Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
Farmacologia e Oncologia Molecolare

CICLO XXIII

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Osteogenic differentiation: a novel role of Slug protein.

Settore Scientifico Disciplinare BIO/10

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Anni 2008/2010

SUMMARY

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The regeneration of bone tissue depends on the concerted actions of a plethora of signals that recruit mesenchymal stem cells for lineage-specific differentiation. The signals are conveyed in hormones, growth factors and transcription factors. These molecules are crucial for the osteoblast commitment, differentiation, functions and, consequently, ensure the proper bone modelling and remodelling. Among these factors, Wnt proteins have a critical role in bone development and homeostasis. Accumulated evidences have shown that lymphocyte enhancer binding factor 1/T cell factor (Lef1/Tcf) transcription factors, the nuclear effectors of the Wnt/β-catenin signaling pathway, influence osteoblast proliferation, function, and regeneration. Nevertheless, most downstream bone-specific target genes of this pathway are only partially known. Among these, Slug has been recently implicated in osteosarcoma progression as a Wnt-responsive molecule strongly correlated with a loss of tumor suppressors such as E-cadherin. Slug, also named Snail2, belongs to the Snail family of genes encoding zinc-finger transcription factors. It is expressed at different stages of development in different tissues, mediates epithelial–mesenchymal transition and directs cell motility during embriogenesis. Slug is also expressed in most normal adult human tissues, but little is known about its potential functions.

In order to identify new potential osteoblast-specific proteins, in this study we analysed the expression, regulation and role of Slug in human normal primary osteoblasts (hOBs) and in their mesenchymal precursors (hMSCs), in relation to the expression of Wnt/β-catenin signalling mediators and genes which are required in the control of osteochondroprogenitors differentiation. The experiments were performed on hOBs and hMSCs, obtained from bone marrow iliac crest, bone marrow tibial plateau and Wharton's jelly umbilical cord. Using several molecular analysis including siRNA strategy and Chromatin Immunoprecipitation (ChIP) assay, we demonstrated that:

- Slug is expressed in hOBs as well as their mesenchymal precursors;
- In hOBs, Slug is regulated by β-catenin and Lef1 that, together with Tcf-1, Tcf-4 and Runx2 are recruited to the Slug gene promoter *in vivo*;
- In hOBs, Slug is positively correlated with osteoblastic markers, such as Runx2, osteopontin, osteocalcin, collagen type I, CXCL12, Wnt/β-catenin signalling and mineral deposition. At the same time, it negatively correlated with Sox9, a factor indispensable for chondrogenic development;
- In hMSCs, Slug acts as a negative regulator of Sox9 and Sox5 expression and a positive regulator of Sox6 and STAT1 genes. Regarding Runx2, the role of Slug seems influenced by cell type;
- Slug interacts *in vivo* with Runx2 and Sox9 promoters in hOBs and hMSCs.

Our results support the hypothesis that Slug functions as a novel regulator of osteoblast activity, even if with a different role in mature committed osteoblasts and in their undifferentiated progenitors. Furthermore, these findings suggest Slug as a new potential therapeutic target for bone tissue repair and regeneration.

Key words:
Slug, Human osteoblasts, Osteogenic differentiation, Human mesenchymal stem cells, Wnt signalling, Osteoblast regulators.

INTRODUCTION

Chapter 1

The Snail/Slug family members: characterization and functions.

Snail/Slug family members encode transcription factors of zinc-finger type which play a key role in various differentiation processes in vertebrate and invertebrate organisms (Nieto., 2002; Hemavathy *et al.*, 2000). At first described in *Drosophila melanogaster* (Grau *et al.*, 1994; Nusslein-Volhard *et al.*, 1984), subsequently, Snail/Slug transcription factors have been found in many species including humans, other vertebrates, non-vertebrates chordate (protochordates), insects, nematodes, annelids and mollusks (Nieto., 2002). To date, about 50 members of this family have been isolated, showing significant differences between them even if a phylogenetic analysis indicates having a common ancestral precursor. Several studies suggested that numerous genes coding for Snail/Slug factors of all Metazoa derived from a common ancestor, *snail*, which, after gene duplication, gave rise to the *Snail* and *Scratch* genes, which one inter-related but independent (Ohno., 1999; Manzanares *et al.*, 2001; Barrallo-Gimeno *et al.*, 2005). In particular, in this thesis I will delve into the role of transcription factors encoded by *Snail* genes. These proteins have overall an homology, in terms of the sequence, which varies between 50% and 70% (Metzstein *et al.*, 1999). In particular, three Snail/Slug proteins have been identified in vertebrates: Snail (Snail1), Slug (Snail2 or Snai2), and Smuc (Snail3 or Snai3) (Nieto., 2002; Barrallo-Gimeno *et al.*, 2005; Katoh and Katoh., 2005).
They all share a similar organization, being composed of a highly conserved carboxy-terminal region, which contains zinc fingers, and a much more divergent amino-terminal region. While many of the zinc fingers are employed in nucleic acid binding, an increasing

numbers of reports have demonstrated that zinc fingers in some proteins are also utilized for protein-protein interaction (Mackay and Crossley., 1998). The fingers are structurally composed of two β-strands followed by an α-helix, the amino-terminal part of which binds to the major groove of the DNA. The two conserved cysteines and histidines (C_2H_2) coordinate the zinc ion. The number of zinc fingers in the family varies from four to six. Probably a minimum of four fingers are required for functioning (Fuse *et al.*, 1994; Grimes *et al.*, 1996; Nakayama *et al.*, 1998). Both random selection and transfection experiments with different promoters have shown that the consensus binding site for Snail-related genes contains a core of six bases, 5'-CANNTG-3'. This motif is identical to the so-called E-box, the consensus of the core binding site of basic helix-loop-helix (bHLH) transcription factors. Under some circumstances, the Snail family proteins compete directly with bHLH proteins for the same binding sequences (Fuse *et al.*, 1994; Nakayama *et al.*, 1998; Kataoka *et al.*, 2000). Moreover, Snail/Slug family members show different affinity toward the E-box flanking sequences. This will decide whether they can regulate similar or different target genes when multiple members are expressed in the same cell (Hemavathy *et al.*, 2000). Binding of Slug/Snail proteins to their target promoters leads to repression of gene activity (Hemavathy *et al.*, 2000). The repressor activity depends not only on the finger region, but also on at least two different motifs that are found in the amino-terminal region. In *Drosophila,* Snail (dSnail) contains two conserved motifs that allow interaction with the corepressor dCtBP (*Drosophila* C-terminal Binding Protein) (Nibu *et al.*, 1998). These motifs allow the binding of dCtBP with dSnail *in vitro* and are essential for transcriptional repression *in vivo*. dSnail's specific role in mesodermal invagination is mostly dependent on the amino-terminal transcription regulatory domain with the two dCtBP interaction motifs, and not on the carboxy-terminal DNA-binding domain (Hemavathy *et al.*, 2004); but there is evidence that dSnail would also be able to repress target genes in the absence of dCtBP.

Vertebrate members have an amino-terminal SNAG (Snail/Gfi-1) domain that has been found in three diverged types of families: vertebrate Snail/Slug proteins, Gfi proteins and IA-1 proteins (Tateno *et al.*, 2001). The SNAG domain was originally characterized in the

growth factor independence-1 (Gfi-1) oncoprotein, where it forms part of the first 20 amino acids that suffice for transcriptional repression (Grimes *et al.*, 1996). For the vertebrate Snail members, the maximal repressive effect is dependent on both the zinc finger DNA-binding domain and the amino-terminal SNAG domain. The SNAG domain is of particular interest because it is indispensable to the recruitment of histone deacetylase (HDACs) for the formation of a multimolecular complex that drives remodeling and compaction of chromatin mediating gene silencing (Tripathi *et al.*, 2005).

From data reported in the literature, it appears that many of the Snail/Slug protein functions have been preserved during evolution from invertebrates to vertebrates, although the knowledge of the different roles that they mediate in the embryonic and adult life of many organisms is still limited. At the cellular level, Snail/Slug factors regulate cell differentiation, cell adhesion, cell movement, cell cycle regulation, and apoptosis (Hemavanthy *et al.*, 2000). The main experimental evidences regarding the functions that these proteins play, are described below.

Snail /Slug and embryonic development

The first evidence about the functions of the Snail family members have been found in *Drosophila*, where these proteins are involved in embryonic development (Ashraf *et al.*, 1999). Both Snail RNA and protein expression have been detected precisely in the ventral region of the blastoderm stage embryos where mesoderm arises (Alberga *et al.*, 1991; Kosman *et al.*, 1991; Leptin., 1991). The expression persists during gastrulation in the invaginated mesoderm and disappears during late gastrulation. Expression reappears in the neuroectoderm and persists as neuroblasts delaminate from the neuroectoderm. Later, during mid-embryogenesis, expression can be detected in precursor cells of some imaginal tissues, including the wing discs. At mesoderm specification stage, Snail acts as a repressor to restrict lateral genes from being expressed in the ventral region to establish mesodermal cell fate (Kosman *et al.*, 1991; Leptin, 1991; Ip *et al.*, 1992). The expression is also

required to control ventral cell invagination to form the mesoderm layer. Indeed, in *Snail* mutants, it was found that no mesoderm derivative is formed and that the ventral cell invagination does not occur (Boulay *et al.*, 1987; Grau *et al.*, 1984; Nusslein-Volhard *et al.*, 1984). Although the Snail-related proteins Escargot and Worniu have redundant functions during asymmetric division of neuroblasts later in development (Ashraf *et al.*, 1999; Cai *et al.*, 2001), these proteins cannot substitute for Snail's normal role in mesoderm invagination (Hemavathy *et al.*, 2004). The elucidation of the significant role of *Snail* genes family in *Drosophila* embryogenesis, prompted the search for similar functions of their homologs in other organisms. Several authors have analyzed the involvement of Snail/Slug proteins in the embryonic development of amphibians.

Numerous studies in *Xenopus* have shown an interest of the Snail/Slug family members in gastrulation, and especially in the development of neural crest (Carl *et al.*, 1999).

Neural crest cells are proliferative, migratory, tissue-invasive stem cells that originate in the ectoderm of vertebrate embryos (LaBonne and Bronner-Fraser., 1998a). The precursors of these cells arise at the lateral edges of the neural plate during mid-gastrulation. Following neural tube closure, these cells undergo an epithelial to mesenchymal transition (EMT), a process that involves the modification of the transcriptional activity, cell adhesion, cytoskeleton assembly, and cell-matrix interaction of prospective neural crest cells (Savagner., 2001). Cells then delaminate from the dorsal aspect of the neural tube, and migrate extensively to populate distant sites throughout the embryo. The neural crest contributes to a large and diverse set of derivatives that include most of the neurons and glia of the peripheral nervous system, melanocytes and elements of the craniofacial skeleton, such as cells of upper and lower jaw bone, dermis, and adipose tissue of epidermis, and connective tissue cells (Bronner-Fraser., 1995) (see Figure 1).

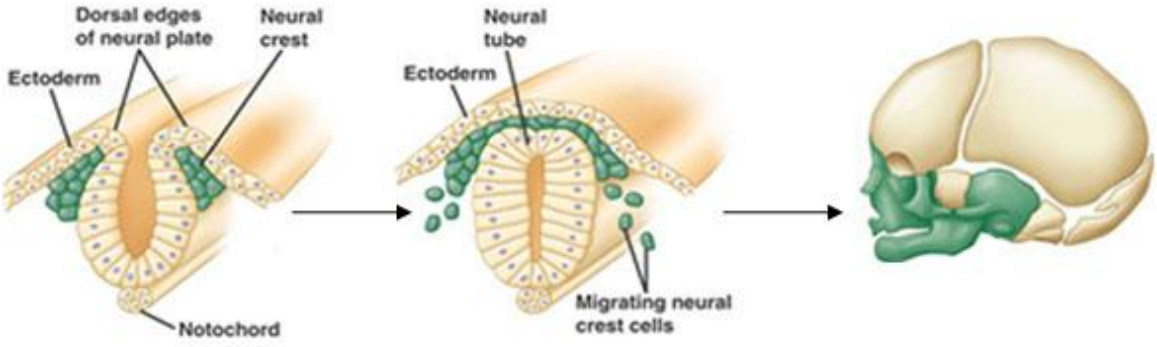


Figure 1. *Successive stages of neural crest formation*. The neural crest consists of bilateral bands of cells near the margins of the embryonic folds that form the neural tube. After neural tube closure, the cells originally located at the crests of the neural folds, known as neural crest cells, come to lie on top of the neural tube, from where they migrate to different locations, giving rise to some of the anatomical structures unique to vertebrates, including some of the bones and cartilage of the skull. (font: http://bio1152.nicerweb.com/Locked/media/ch34/craniate.html).

The induction and regulation of this process are extremely complex and, to date, only partially understood. The involvement of different signaling pathways mediated by Wnt, fibroblast growth factor (FGF), bone morphogenic proteins (BMPs) has been amply demonstrated by several studies in *Xenopus*, zebrafish and mice (Ikeya *et al.*, 1997; Dorsky *et al.*, 1997; LaBonne and Bronner-Fraser., 1998b; Marchant *et al.*, 1998; Vallin *et al.*, 2001). In addition, a lot of transcriptional regulatory proteins have been implicated in one or more aspects of neural crest formation. Among these, some Snail/Slug family members have been identified as targets of the signaling pathways mentioned above. In *Xenopus* both Snail (xSnail) and Slug (xSlug), can be detected in neural crest–forming regions of the neural plate border by late gastrula stages, and both factors are expressed at the lateral edges of the open neural plate in midbrain, hindbrain and spinal cord regions. *Snail*, is also transiently expressed in the transverse neural fold, from which neural crest does not arise (Essex *et al.*, 1993; Mayor *et al.*, 1995). *xSnail* expression is the earliest marker for the neural crest. At the early gastrula stage, the coordinated action of Xiro1 as a positive regulator and xSnail as a repressor restricts the expression of Delta1 at the border of the neural crest territory, which will later on lead to proper formation of the neural folds (Glavic *et al.*, 2004). *xSnail* is upstream of *xSlug* in the specification of neural crest cells,

as it is able to trigger the expression of a series of early and late neural crest markers, such as products of the *Zic5*, *FoxD3*, *Twist*, *Ets1* and *Sox10* genes (Aybar *et al.*, 2003; Honore *et al.*, 2003). *xSlug* overexpression alone is insufficient to direct neural crest formation in ectodermal explants, but it can do so in cooperation with a Wnt or FGF signal. Similarly, *xSlug* overexpression causes an expansion of the neural crest domain and an increase in melanocyte formation in whole embryos, where all inductive signals are present (LaBonne and Bronner-Fraser., 1998a). In avian embryos, both *Slug* and *Snail* overexpression have also been shown to result in excess neural crest production, although in these experiments the effects were confined to cranial regions (del Barrio and Nieto., 2002). Treatment of chick (Nieto *et al.*, 1994) or *Xenopus* (Carl *et al.*, 1999) embryos with antisense oligonucleotides to *Slug* mRNA results in an inhibition of cranial neural crest cell migration. By testing the effects of expressing fusions with transcriptional repressor or activator domains, it was shown that *Xenopus* Slug protein normally acts as a repressor (LaBonne and Bronner-Fraser., 2000). In an analogous manner to *Drosophila* Snail, Slug may down-regulate genes whose expression needs to be excluded from neural crest and have an indirect role in the upregulation of genes in the neural crest. Studies performed using a fusion protein acting as a hormone-inducible dominant negative inhibitor of Slug and Snail protein function, demonstrate that these factors are required for the expression of *Twist*, which encodes a specific marker of neural crest (LaBonne and Bronner-Fraser., 2000).

Interestingly, whereas Slug and/or Snail clearly play essential roles in neural crest formation in both *Xenopus* and avian embryos, a recent double knockout of their orthologs in the mouse epiblast displays no defects in neural crest development, at least in cranial regions before E9.5 (Murray and Gridley., 2006). These mutant mice do have defects in left–right asymmetry, however, further highlighting the essential role of Snail family proteins in patterning and regulatory events beyond cell survival and EMT. Neural crest specific Snail1 deletion on a *Slug* $^{-/-}$ background does lead to later craniofacial and palate defects (Murray and Gridley., 2006; Murray *et al.*, 2007). Nevertheless, the surprising finding that neural crest cells form and migrate normally in the double mutant mice

suggests that murine neural crest cells express one or more factors that can carry out the essential functions that Snail family proteins mediate in frog and chick.

Multiple levels of regulation, including transcriptional, post-transcriptional and post-translational modification, are employed to ensure tight control of the activity of these key regulatory proteins.

The characterization of regulatory regions of *Slug* gene in *Xenopus laevis* and in *Xenopus tropicalis* has allowed the identification of preserved sequences, which seem to be necessary and sufficient for the *Slug* expression in neural crest cells. These sequences contain, binding sites for the Lef/Tcf transcription factors, which are effectors of the Wnt signaling pathway. This supports a direct role for this signaling pathway in *Slug* induction in *Xenopus* (Vallin *et al.*, 2001). Further confirmation of the importance of Wnt signaling in *Slug* regulation comes from the identification of binding sites for Lef/Tcf transcription factors also in chicken *Slug* (*cSlug*) promoter (Liem *et al.*, 1995; Sakai *et al.*, 2005). In addition, the presence of Smad1 binding sites in the proximal sequence of *cSlug*, suggests that BMP signaling is a further important regulator for *Slug* expression in neural crest formation (Sakai *et al.*, 2005). Moreover, it has been demonstrated that, in avian neural crest cells, Sox9 together with Slug directly activates the *Slug* promoter through a direct binding to E-box site, promoting crest formation and EMT (Sakai *et al.*, 2006). The *xSlug* promoter also contains binding sites for YY1 (Ying Yang). The YY1 protein is expressed in the ectoderm at about the time of *Slug* induction and knockdown of YY1 with morpholino oligos specifically represses Slug but not Snail or AP2. Disrupting the YY1 cis-regulatory element also affected *Slug* promoter-mediated transcription (Morgan *et al.*, 2004). Finally, recent work in *Xenopus* has shown that the stability of Slug and Snail is dynamically regulated during neural crest development via the ubiquitin-proteasome system, and that the F-box protein Ppa is essential for directing their degradation (Vernon *et al.*, 2006). Point mutations that abrogate Ppa targeting were found to stabilize the Slug protein and allow it to elicit premature migration. Work in mammalian cell culture has suggested that GSK-mediated phosphorlyation directs β-Trcp mediated degradation of

Snail (Yook *et al.*, 2006; Yook *et al.*, 2005; Zhou *et al.*, 2004); however, this mechanism does not appear to function in the neural crest, at least in *Xenopus* (Vernon *et al.*, 2006). Indeed, Slug and many other Snail factors lack the β-Trcp destruction box present in mammalian Snail, suggesting that this is unlikely to be an evolutionarily conserved means of targeting Snail proteins for degradation. Snail is also regulated at the level of nuclear translocation (Zhou *et al.*, 2004; Dominguez *et al.*, 2003) and it will be important to determine the role that this mechanism plays in neural crest development.

Snail /Slug and EMT

The study of neural crest cells is particularly interesting for understanding the mechanisms of migration, differentiation and cell survival. During embryonic development, in fact, this population undergoes a process that occurs in many physiological and pathological events: the ephitelial-mesenchymal transition (EMT) (Kang and Svoboda., 2005). This process involves the acquisition of the proper mesenchymal phenotype by the epithelial cells of different embryonic and adult tissues. The normal epithelial tissue is characterized, in fact, by the presence of cell-cell interactions crucial for the development of tissues during embryogenesis, and for the maintenance of homeostasis and architecture of epithelial structures. Through epithelial-mesenchymal transition, cells acquire the ability to migrate into tissues, acting on the regulation of specific genes. The EMT process involves, therefore, the activation of genes encoding proteolytic enzymes for extracellular matrix degradation the inactivation of genes coding for epithelial markers and adhesion molecules, including E-cadherin and α- and γ-catenin (Yang *et al.*, 2004). During the EMT process, epithelial cells lose the expression of E-cadherin and other components of epithelial cell junctions, adopt a mesenchymal cell phenotype, and acquire motility as well as invasive properties (Acloque *et al.*, 2009; Kalluri *et al.*, 2009). The lack of adhesion proteins expression by epithelial cells, mean the loss of apical-basal polarity, which is characterized, also, by an extensive cytoskeleton remodeling and a redistribution of organelles (Boyer *et al.*, 2000; Shook and Keller., 2003; Kang and Massagué., 2004). The

cells that undergo epithelial-mesenchymal transition acquire, therefore, the expression of mesenchymal characteristic markers, such as, fibronectin, vimentin and N-cadherin (Yang *et al.*, 2004). Several studies also showed that as this is a reversible process, mesenchymal cells may, in some cases, restore the differentiated epithelial phenotype, through the so-called mesenchymal-epithelial transformation (MET) (Thiery., 2003; Kang and Svoboda., 2005). In particular, this event has been demonstrated in the development of somites, kidney and neural tube (Griffith *et al.*, 1992).

EMT is triggered by several extracellular induction signals such as extracellular matrix (ECM) components and growth factors. Furthermore, EMT is mediated by several signaling pathways, including epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor β (TGFβ), bone morphogenic proteins (BMPs) and Wnt signaling pathway. The involvement of these signaling pathways in modulating the expression of Snail/Slug family factors has been widely demonstrated in several animal models, and has led to attribute to these proteins a major role in the EMT activation (Barrallo-Gimeno *et al.*, 2005; De Craene *et al.*, 2005) (see Figure 2).

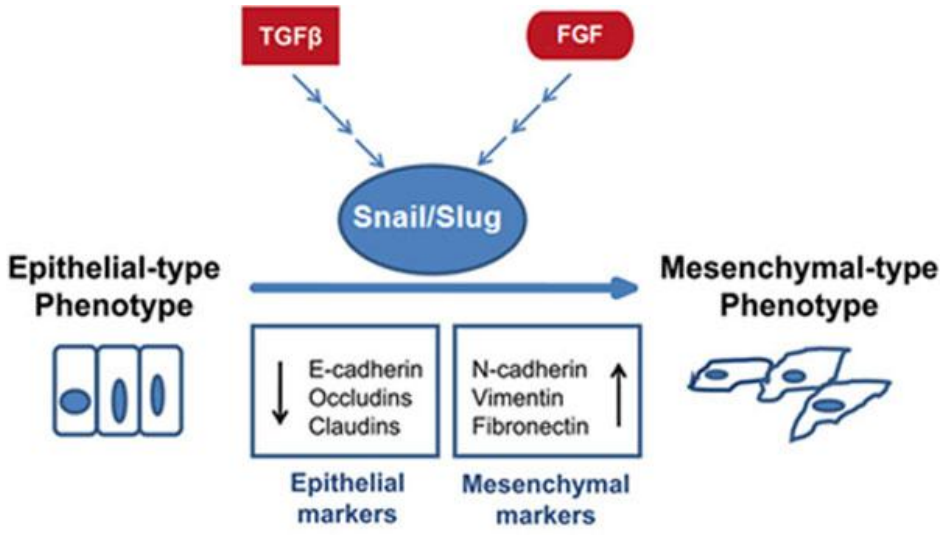


Figure 2. *Role of Snail/Slug transcription factors in epithelial-mesenchymal transition (EMT).* The diagram shows general signaling pathways regulating Snail/Slug and some of the molecules known to be affected by Snail/Slug activity. (modified from: Alves C.C., Carneiro F., Hoefler H., *et al.* (2009). Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers. *Front Biosci.*, 14:3035-3050).

For example Snail is expressed during the early stages in chick development and its induction is promoted by FGF-2 and FGF-8 (Isaac *et al.*, 2000; Montero *et al.*, 2001). Slug, instead, is required for the initial steps of the EMT, which occur during endocardial cushion formation in chicken heart (Romano and Runyan., 1999). Slug appears to be an essential target of TGFβ2 signalling during the initial steps of EMT in the heart, as Slug is sufficient to rescue the inhibitory effect of an anti-TGFβ2 antibody on EMT (Romano., 2000). Slug also plays an important role in the palate fusion, which require a combination of EMT and apoptosis. In fact, in some pathological conditions, Slug acts as anti-apoptotic factor causing a pathological cleft palate and the consequent differentiation of surviving cells in keratinized stratified epithelium (Martinez-Alvarez *et al.*, 2004). In humans, TGFβ factors represent the main candidates for EMT induction that occurs during the development of lens cataracts. It has been shown that TGFβ1 can induce Slug expression, which negatively regulates the expression of E-cadherin in epithelial cells of the lens, thus promoting the migration (Choi *et al.*, 2007).

Studies performed in adult keratinocytes have highlighted that the presence of Slug is also required during cutaneous wound reepithelialization after skin injury. The phenotypic plasticity observed at margins of healing wounds during the reepithelialization process resembles certain aspects of the epithelial–mesenchymal transition (EMT) that occurs during embryonic development (Arnoux *et al.*, 2008; Savagner *et al.*, 2005). It has been observed that adult mouse and human keratinocytes taken from the wound margins, show high Slug expression levels. In mice, *Slug* knockout experiments leads to a reduced cell migration from skin explants (Coulombe., 2003; Chandler *et al.*, 2007; Hudson *et al.*, 2009), while overexpression of this protein reduces cell adhesion in human keratinocytes (Savagner *et al.*, 2005) and this is related to a reduced presence of adhesion molecules such as E-cadherin and integrins, which result in decreased interaction with the extracellular matrix (Turner *et al.*, 2006).

Snail /Slug and cancer

The transformation from epithelial to mesenchymal cell requires the acquisition of an invasive cell phenotype, able to break free from cell-cell interactions and to migrate extensively. This means that this event is not only involved in embryonic development, but also in neoplastic transformation. In particular, cells acquire this feature in the later stages of tumor progression, when they reach the ability to penetrate the basal lamina and give rise to metastases in other areas of the body. Aberrant expression of Slug protein has been found in many types of cancer: esophageal cancer, gastric and colon cancers, liver, pancreas and lung cancers, ovarian and breast cancer (Barrallo-Gimeno *et al.*, 2005; Shih *et al.*, 2005). The involvement of Slug in neoplastic transformation is primarily related to its ability to induce epithelial-mesenchymal transition, with particular reference to the regulation of adhesion and cell migration. Several experimental evidences have shown that this factor can regulate the expression of cell adhesion molecules, particularly E-cadherin. It is known that Snail/Slug factors possess binding sites within the promoter region of the gene encoding E-cadherin (Cano *et al.*, 2000; Perez-Moreno *et al.*, 2001). For instance, in esophageal squamous cell carcinomas (SCC), *Slug* overexpression is correlated with reduced E-cadherin expression and with depth of tumor invasion, lymph node metastasis, stage and lymphatic invasion (Jethwa *et al.*, 2008). *Slug* and *E-cadherin* expression levels are also inversely related in melanoma cells, breast cancer, hepatocellular carcinoma, gastric and colon cancers (Poser *et al.*, 2001; Jiao *et al.*, 2002; Sugimaschi *et al.*, 2003; Hajra *et al.,* 2002). In the latter has been demonstrated that *Slug* expression is regulated by the proto-oncogene c-Myb and the down-regulation of Slug and/or c-Myb led to increased E-cadherin, desmoplakin, and occluding expression, and decreased vimentin expression and reduced invasion capability (Tanno *et al.*, 2010). Furthermore, Larriba *et al.*, demonstrated that Slug inhibits *vitamin D receptor (VDR)* gene promoter activity and decreases VDR mRNA and protein levels by binding to the three E-boxes present in the *VDR* promoter, in colon adenocarcinoma cell lines (Larriba *et al.*, 2009). Similar finding was reported by Mittal and co-workers. They demonstrated that Slug binds *in vivo* to the

VDR gene promoter in human breast cell nucleus and inhibits *VDR* gene expression by chromatin remodeling, thus leading to an alteration of mammary epithelial cells proliferation, differentiation and survival (Mittal *et al.*, 2008). In breast cancer, also, it has been observed a correlation between the expression of estrogen receptor α (ERα) and the loss of E-cadherin induced by Snail/Slug proteins. In this tissue ERα and its ligand, estradiol, play an important role in the regulation of cell differentiation and proliferation. ERα, however, is a positive prognostic marker of breast cancer progression. This can be partly explained by the fact that ER activates transcription of MTA3 (metastasis-associated protein 3) in an indirect way. This involved the formation of the transcriptional repression complex Mi-2/NuRD, together with other several factors, including ATPase and histone deacetylases for chromatin remodeling (Wade *et al.*, 1999; Xue *et al.*, 1998; Dhasarathy *et al.*, 2007). Most of the genes modulated by this mechanism are not yet known, but some experimental evidences indicate their involvement in the development of invasive tumors and metastases (Toh *et al.*, 1997). Slug is one of the targets of the negative modulation performed by Mi-2/NuRD transcriptional complex (Dhasarathy *et al.*, 2007). Therefore, the presence of ER indirectly maintains the expression of E-cadherin. In contrast, in the absence of estrogen receptor, MTA3 activity is inhibited, and the loss of Slug repression induces, consequentely, the loss of E-cadherin expression. ER-negative breast cancer cells are, thus, more predisposed to the acquisition of an invasive phenotype (Fujita *et al.*, 2003). Regarding invasive ductal carcinoma of breast, recent studies substantiate the role of active Wnt/β-catenin pathway in this process. In the presence of Wnt signals, β-catenin partners with Lef/Tcf transcription factors to activate target genes, such as *Slug*, which in turn, promote EMT inhibiting *E-cadherin* expression (Prasad *et al.*, 2009). Further confirmation of the correlation between Slug and Wnt signaling derives from studies performed by Medici and co-workers in DLD1 colon carcinoma, MDCKII, and A375 melanoma cells. In these experimental models, TGFβ1 causes up-regulation in Slug and Snail expression levels, which reduce E-cadherin expression. Both Slug and Snail, also, cause a β-catenin-Tcf4-induced up-regulation of TGFβ3, which in turn, is responsible for increases in Lef1 expression. These β-catenin-Lef1 transcription complex then cause

increased expression of target genes (vimentin, fibronectin etc.) that drive formation of the mesenchymal phenotype (Medici *et al.*, 2008). Furthermore, Slug has been recently implicated in osteosarcoma progression as a Wnt-responsive molecule strongly correlated with a loss of tumor suppressors such as E-cadherin (Nieto., 2002; Conacci-Sorrell *et al.*, 2003; Guo *et al.,* 2007).

In addition to E-cadherin, other Slug target genes have been identified, which support the role of this transcription factor in cell motility regulation. Slug negatively modulates the expression of various molecules involved in the formation of tight junctions, adherent junctions and desmosomes. These include: claudine, occludin, integrins, cytokeratins, desmoplakins, mucins, aggrecan, matrix metalloproteinases, collagen type II (Martinez-Estrada *et al.*, 2006; Wang *et al.*, 2007; Seki *et al.*, 2003).

Snail /Slug and apoptosis

Several evidences indicate the existence of a correlation between the expression of Snail/Slug proteins and apoptosis regulation, highlighting the central role of these transcription factors in neoplastic transformation. It is known that apoptosis is an essential process during embryonic development as well as in the adult, because it is able to remodel or remove any structures, controls cell number of a specific organ and eliminates damaged or non-functional cells. Apoptosis is a finely regulated event that, in vertebrates and invertebrates, involves a large number of molecules and intracellular signaling pathways. Alterations in apoptosis regulation are involved in tumors development, as they promote cell survival in combination of adverse environmental conditions and severe DNA damages.

Several Snail/Slug family members are known regulators of this process. First evidence were observed in *Caenorhabditis elegans*, where CES-1 (a protein homologous to *Drosophila* Scratch), was able to prevent the physiological death of a particular neuronal cell type (Metzstein and Horvitz., 1999). CES-1 has the ability to ensure survival of mouse pro-lymphocytes B, cultured without growth factors. Moreover, Slug showed anti-

apoptotic activity in mice (Inukai *et al.*, 1999). Even in fetal rat hepatocytes Snail has been observed to promote cell survival, despite apoptosis induction mediated by TGFβ (Sanchez *et al.*, 1996). A further example of Slug involvement in preventing apoptosis was observed during neural crest development. Experiments performed in *Xenopus* embryos led to demonstrate a correlation between Slug expression and the acquisition of resistance to apoptosis. In this experimental model, Slug seems to be directly involved in the regulation of apoptosis molecules cascade: indeed, it is able to activate the expression of *Bax* and *Bcl-2*, known anti-apoptotic factors, and to repress the transcription of genes coding for various caspases, apoptosis effector proteins.

Apoptosis is also involved in limb embryonic development in chicken. This process is regulated by the action of fibroblast growth factor (FGF) and bone morphogenic (BMPs), which cooperate in inducing Snail/Slug expression, in order to hamper apoptosis (Ros *et al.*, 1997; Montero *et al.*, 2001).

The involvement of Snail/Slug transcription factors in cell survival has also been found in the onset of leukemia. The fusion protein E2A-HLF (E2A-hepatic leukemic factor), which has transforming activity in several cell types, is able to lead an aberrant increase of Slug expression in precursor B lymphocytes. Slug confers protection from apoptosis to these cells, allowing them to acquire additional mutations that free them from control proliferation, thus promoting the development of leukemia (Inukai *et al.*, 1999). The ability of Snail/Slug proteins to promote migration and cell survival was also found in hematopoietic stem cells. In this cell population Slug expression is induced by the interaction between the stem cell growth factor, SCF, and c-kit receptor (a specific marker of stem cells, constitutively activated in many types of cancer). The biological relevance of Slug in these processes has been described: mice knocked out for *Slug* have a complex phenotype, including gonadal, haematopoietic and pigmentation defects, which are similar to the defects of *SCF* and *c-kit* deficient mice. The pigmentation defects suggest a role for Slug in the development of melanocytes from the neural crest. It was demonstrated that SCF/c-Kit signaling specifically induces the expression of *Slug* gene (Perez-Losada *et al.*,

2002). As to its role in haematopoiesis, Slug expression can be detected in diverse subsets of haematopoietic progenitors, and irradiated *Slug* deficient mice have significantly more apoptotic bone marrow progenitor cells than wild-type controls. This indicates that Slug can act as a radioprotection agent, and normally functions to promote the survival of bone marrow progenitors that have undergone DNA damage (Inoue *et al.*, 2002). In addition, in hematopoietic progenitors, Slug has shown an antagonistic activity against p53 and PUMA, two factors involved in the apoptosis induction (Wu *et al.*, 2005).

Analysis of the role of Snail/Slug proteins in cell proliferation control have provided controversial results. In *Drosophila* gastrulation, changes in cell morphology produced by the cytoskeleton reorganization during cell migration, are related to an inhibition Snail-dependent of mitosis and cell division (Grosshans and Wieschaus., 2000). In *Drosophila*, also other Snail/Slug transcription factors are involved in mitosis suppression, inhibiting the transcription of genes involved in this process. These observations are consistent with the reduced replication rate of epithelial cells expressing Snail (Nieto., 2002). The inhibition of proliferation by Snail/Slug family members occurs in cells induced to make the epithelial-mesenchymal transition (Peinado *et al.*, 2003), including keratinocytes (Turner *et al.*, 2006). Despite a lot of evidences confirm the ability of these proteins to promote tumor progression, it was observed that Snail inhibits cell cycle and proliferation, promoting the acquisition of an invasive phenotype rather that cell growth (Vega *et al.*, 2004). On the contrary, during hair follicles formation in mice, the expression of Snail has revealed a proportional reduction of E-cadherin expression and an increased in proliferative activity. A direct correlation between Slug expression and cell proliferation was also found during the development of renal fibrosis (Thiery., 2003).

Chapter 2

Human Slug gene.

Human *Slug* gene is mapped to chromosome 8q11. Analysis of the coding region, which has a size of about 4 kb, revealed that the gene is composed of three exons and two introns. A single transcription start site has been identified and no evidence for alternative splicing have been detected. The human Slug transcript is 2.2 kb in size, and it codes for a putative protein of 268 amino acids and about 30 kDa, with five zinc fingers in its carboxy-terminal portion (Cohen *et al.*, 1998). The amino-terminal portion of the human Slug protein contains regions shared with mouse (Savagner *et al.*, 1997), chicken (Nieto *et al.*, 1994), and *Xenopus* (Mayor *et al.*, 1995) Slug with 89, 87 and 77% identity, respectively, but bears significantly less homology to Snail proteins with 23% identity to the mouse (Smith *et al.*, 1992). When the Slug zinc finger region in human is compared with that of mouse, chicken, and *Xenopus*, their identity is 100, 99, and 98%, respectively. The mouse Snail zinc finger region is only 68% identical to that of human or mouse Slug. The structural differences in this region between Slug and Snail suggest that they possess distinct affinities and specificities in DNA binding as the basis for their functional differences. The identity over the entire protein sequence between human and each of the mouse, chicken, and *Xenopus* Slug protein is 95, 93, and 88%, respectively (Cohen *et al.*, 1998).

As mentioned before, Slug respressor activity, is localized in the amino-terminal domain, mainly at the level of the first 32 amino acids (see Figure 1). These represent the “core repressor domain” and their deletion, leads to loss of protein function. Slug has a domain with seven amino acid residues at its “core repression domain” known as SNAG domain (Snail/Gfi-1). Through SNAG domain, Slug may recruit other co-repressor proteins,

interacting with them directly or through adapter proteins, including Sin3A (Bailey *et al.*, 2007) and HDAC1 and 2 (Tripathi *et al.*, 2005). Following deletion analysis, it has been shown that the 30 amino acid residues of the central amino-terminal domain can activate transcription, although this ability is hidden by the presence of the "core repression domain". The possibility that the Snail/Slug proteins, as well as other transcription factors, are able, in certain situations, to act as activators of transcription, has been considered by some authors, because various members of that family (including *Drosophila* Snail and human Slug) possess a domain able to regulate positively the transcription process, although to date, examples in favor of this hypothesis have not yet been reported in the literature (Han and Manley., 1993; Hemavathy *et al.*, 2000; Hemavathy *et al.*, 1997). On the contrary, the last 30 amino-terminal residues are an "helper" domain for the transcriptional repression (Hemavathy *et al.*, 2000).

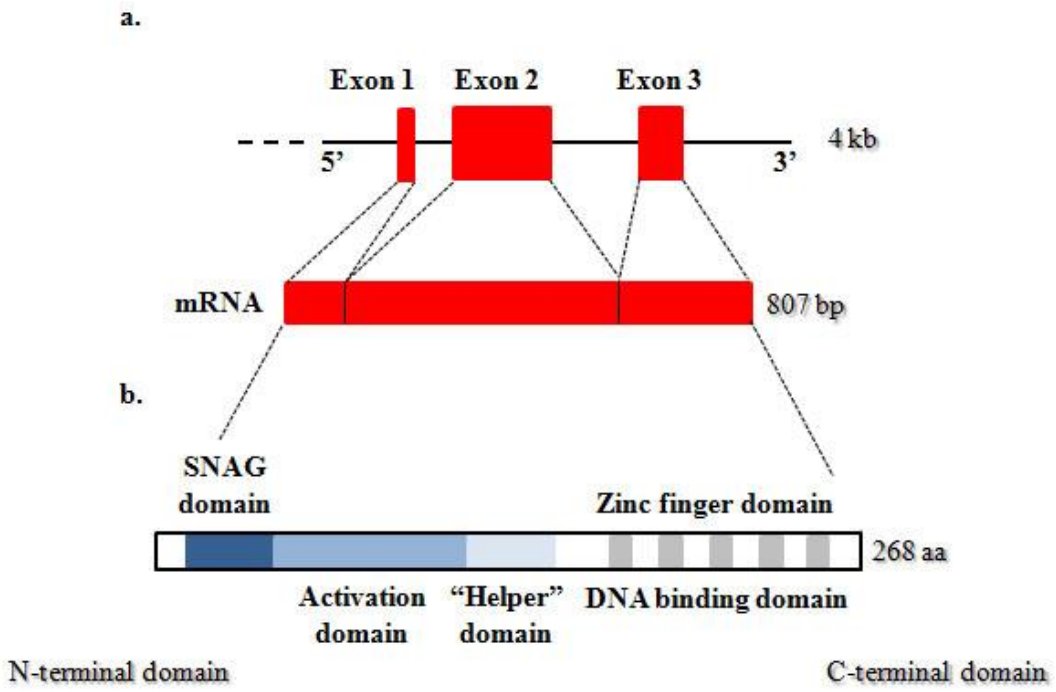


Figure 1. a. *hSlug gene organization. hSlug* gene is composed of three exons and two introns. The coding sequence produces a transcript of 807 bp, which codes for a protein of about 30 Kda. b. *Schematic representation of functional domains in hSlug protein.* Slug protein has five zinc fingers in its carboxy-terminal portion, which are involved in DNA binding, and an amino-terminal domain responsible for Slug repressor activity.

mRNA coding for hSlug has been found in placenta and embryonic stem cells and in most adult tissues, such as spleen, thymus, testis, prostate, ovary, intestine, colon, heart, liver, skeletal muscle, kidney and pancreas and, at lower levels in brain and lung. Its expression is particularly high in the prostate and ovary. Otherwise, the hSlug mRNA is almost undetectable in peripheral blood leukocytes (Cohen *et al.*, 1998; Hemavathy *et al.*, 2000; Katoh *et al.*, 2005).

Although the regulation of *Snail/Slug* expression and their role have been investigated extensively in many animal species, as shown in the previous chapter, mechanisms governing *Snail/Slug* expression in humans are largely unknown. Indeed, most of the data relating to the *Snail/Slug* characterization and functions in humans derived from analysis conducted especially on cancer cells. Studies performed in human breast carcinoma cell lines demonstrated that ligand-activated ERα suppresses *Slug* transcription through direct interaction with the *Slug* promoter. More specifically, ligand-activated ERα forms, together with HDAC1 and N-CoR, a transcriptional inhibitory complex which binds to the *Slug* promoter in a region containing three half-site estrogen response elements (EREs), localized at -467, +182 and +241 respectively. Therefore Slug is an ERα-responsive gene. Human breast cancers which lack ligand-activated ERα may than overexpress Slug, which may down-regulate E-cadherin and lead to EMT (Ye *et al.*, 2008). According to Elloul *et al.* (Elloul *et al.*, 2006), it has been suggested that Snail is regulated at the post-transcriptional level in ovarian carcinoma, whereas Slug is probably regulated transcriptionally. The authors found that the up-regulation of both Snail and Slug by 17β-estradiol (E2) was predominantly mediated through activation of their promoter activities. The ERs elicit gene transcription by interacting with the classical ERE or non-ERE elements that bind heterologous transcription factors, including activator protein 1 sites (Webb *et al.*, 1995), Sp1 sites (Porter *et al.*, 1997) and cAMP-response elements (Sabbah *et al.*, 1999). Of particular note, putative ERE sites can be found in the *Snail* promoter region (Moggs *et al.*, 2005), but not in the *Slug* promoter. By sequence homology search, they found several potential Sp-1, activator protein 1, and cAMP-response element sites within the 5'-flanking regions of *Snail* and *Slug* (Park *et al.,* 2008). In addition, ER might

also be recruited to the 3'enhancer downstream of the coding region, constituting a combinatorial network for transcription regulation (Eeckhoute *et al.*, 2006).

Slug transactivation was observed also in human uterine carcinosarcomasas (UCSs), where Slug up-regulation caused repression of E-cadherin, with subtle changes in cell morphology. In this cellular context, activation of the *Slug* promoter is mediated by β-catenin, through the proximal region (-361 to -257 bp). Although most β-catenin targeted genes require Tcf/Lef factors for their activation (Roose and Clevers., 1999; Tetsu and McCormick., 1999) the observed activation did not require binding sites in the promoter, as evidenced by the failure of deletion or dominant-negative Tcf4, in contrast to *Xenopus* and mouse *Slug* promoters activated by β-catenin/Tcf complexes through the binding sites (Vallin *et al.*, 2001; Conacci-Sorrell *et al.*, 2003). A direct involvement of Wnt signaling pathway in *Slug* regulation was demonstrated also in colorectal carcinogenesis (Jin *et al.*, 2008). There, Tcf4/β-catenin complex binds on a Tcf consensus binding sequence (TACAAAGC), identified within one kilobase upstream of the human *Slug* promoter (Sakai *et al.*, 2005), transactivating *Slug* expression. This leads to suppression of *E-cadherin* transcription and induces EMT. Interaction of Tcf4 with MAD2B (a key mitotic checkpoint protein) inhibits the DNA binding ability of Tcf4, which attenuates transactivation of *Slug*, thus realizing the suppression of E-cadherin and leading to mesenchymal-epithelial transdifferentiation (MET) (Hong *et al.*, 2009).

While several evidence have been described the role of Snail/Slug transcription factors in cancer, more limited indications are available about the physiological role of these proteins, as well as their involvement in the development of non-neoplastic diseases.

Studies performed in human lens epithelial cells obtained from patients with anterior polar cataracts, revealed an up-regulation of Slug levels. Analysis of the human *Slug* promoter indicated that TGFβ1 induced the expression of *Slug* through an Sp1 binding site localized between positions -179 and -141. The increased level of Slug in response of TGFβ1 results in the repression of E-cadherin production through binding to the E-box element of its promoter and subsequently, in the EMT induction of lens ephitelial cells and in formation of anterior polar cataracts (Choi *et al.*, 2007). Ikuta and Kawajiri demonstrated that *Slug*

expression is regulated by the aryl hydrocarbon receptor (AhR) in human keratinocyte. AhR is a ligand-activated transcription factor involved in xenobiotic metabolism, in teratogenesis (Mimura *et al.*, 1997) and thymic atrophy (Fernandez-Salguero *et al.*, 1996). It binds to the xenobiotic responsive elements (XREs) located in the 5'-flanking region of the target genes, controlling their expression levels (Fujisawa-Sehara *et al.*, 1986). In particular, five XREs are located in 3.4 kb upstream of the initiation codon of the human *Slug* gene (Stegmann *et al.*, 1999), but only XRE5 is involved in the binding with AhR/ligand complex, as shown by ChIP assay in HaCaT cells. This observation indicates that AhR participates in *Slug* induction, which regulates cellular physiology such as cell adhesion and tumor cell invasion (Ikuta and Kawajiri., 2006).

Mutations in the coding region for *hSlug* were detected in patients with Waardenburg syndrome, a congenital disorder that involves many genes. The disease has an heterogeneous phenotype, and is characterized by a rare form of hereditary deafness, coupled with abnormalities in pigmentation (Sanchez-Martin *et al.*, 2002). The Waardenburg syndrome involves defects of various cell lines derived from the neural crest, including melanocytes. Defects in the development of pigmented cells thereby cause an abnormal distribution of melanin, as demonstrated by patches of white pigmentation on the skin and white hair. Alterations in the development of this structure are indeed implicated in the pathogenesis of piebaldism (or hypochromia), a rare disorder inherited in an autosomal dominant pattern, which leads to a heterogeneous distribution of pigmentation (Spritz *et al.*, 1997). Large deletions in *hSlug* gene have been reported in patients with this pathology (Murakami *et al.*, 2004).

Furthermore, some studies have identified *hSlug* mutations in patients with neural tube defects. They have an incomplete closure of the neural tube, which causes the development of serious and highly debilitating diseases, such as spina bifida (Stegmann *et al.*, 1999).

As previously mentioned, Slug plays a critical role during neural crest formation, that under the control of this transcription factor, can give rise to different cell types including neurons, glia, facial chondrocytes, osteoblasts, and melanocytes (Nieto., 2002; Basch and

Bronner-Fraser., 2006; Le Douarin *et al.*, 1994). In addition, craniofacial abnormalities have been observed in association with cerebral malformations and cutaneous lesions in some neurocutaneous syndromes, emphasizing an important inductive role of the neural tube in the development of non-neural tissues mediated through neural crest and differentiating genes such as *Slug* and *Sox10* (Sarnat and Flores-Sarnat., 2005; Sakai *et al.*, 2006). Overall, these observations encourage investigation on Slug expression and functions in adult cells, including osteoblasts. This study is further supported by recent evidences that demonstrated the role of Snail1 during osteoblast differentiation and on adult bones. Indeed, its deregulated expression in the developing bone leads to achondroplasia in transgenic mice, the most common form of dwarfism in humans (de Frutos *et al*, 2007). Achondroplasias are associated with activating mutations in FGFR3, which signal in a ligand-independent manner to impair chondrocyte proliferation and differentiation (Ornitz and Marie, 2002). Snail1 is the transcriptional effector of FGFR3 signaling during bone development and disease, and its activity can be inversely correlated with the length of the long bones (de Frutos *et al*, 2007). As in other tissues, Snail1 expression is very tightly regulated in the bone, and thus, its aberrant activation in the adult had any impact on bone homeostasis. de Fructos and co-workers highlighted the fundamental role of Snail1 in controlling bone mass by acting as a repressor of both *Runx2* and *vitamin D receptor (VDR)* transcription during osteoblast differentiation. They observed that, sustained activation of *Snail1* in transgenic mice provoked deficient osteoblast differentiation, which, together with the loss of vitamin D signaling in the bone, impaired osteoclastogenesis (de Fructos *et al.*, 2009). Therefore, these indications shows that the impact of Snail1 activity on the osteoblast population regulates the course of bone cells differentiation and ensures normal bone remodeling.

Chapter 3

Bone tissue and osteogenesis.

Bone is a specialised connective tissue hardened by mineralization with calcium phosphate in the form of hydroxyapatite. Bone has well recognised important functions, such as structural functions that provide mobility, support for and protection of the body. It also has an important function as a reservoir for calcium and phosphorus. Morphologically, bone is characterized either as cancellous (spongy, trabecular) or as a cortical (compact). Functionally, cancellous bone is more closely associated with metabolic capabilities than cortical bone, whereas cortical bone generally provides greater mechanical strength (Downey and Siegel., 2006).

Regarding cellular composition, bone tissue consists of specialized cells including osteoblasts, osteocytes, bone lining cells, and osteoclasts, and the extracellular matrix containing an organic and an inorganic component. Osteoblasts, which mature in ostecytes, are responsible for depositing the proteinaceous and calcified matrix and secreting the growth factors necessary for osteogenesis. Osteoclasts, derived from the monocyte-macrophage lineage, participate in the critical function of bone remodelling. The extracellular matrix is composed of collagenous proteins (predominantly collagen type I), non-collagenous proteins (osteocalcin, osteopontin and bone sialoprotein) and mineralized matrix (hydroxyapatite) (Kwan *et al.*, 2008).

Bone Formation and Remodeling

Several lines of evidence from classical embryology have established that two different embryonic lineages, neural crest and mesoderm, form early skeleton (Aubin and Liu.,

1996; Erlebacher *et al.*, 1995). The branchial arch derivates of the craniofacial skeleton originate from neural crest, whereas the axial skeleton, ribs, appendicular skeletons, and the skull base arise from mesoderm. Among the skeletal tissues formed by the mesoderm, the axial skeleton originates from the sclerotome, and the appendicular skeleton arise from the lateral plate mesoderm.

During skeletogenesis, bone is formed in two different manners, intramembranous ossification and endochondral ossification, regardless of the embryonic lineage (see Figure 1).

Intramembranous ossification is characterized by invasion of capillaries into the mesenchymal zone and the emergence and differentiation of mesenchymal cells into osteoblasts. These osteoblasts costitutively deposit bone matrix, leading to the formation of bone spicules. These spicules grow and develop, eventually fusing with other spicules to form trabeculae. As the trabeculae increase in size and number they become interconnected, forming woven bone (a disorganized weak structure with a high proportion of osteocytes), which eventually is replaced by more organized, stronger lamellar bone. This type of ossification is involved in the development of flat bones in the cranium, various facial bones, parts of the mandible and clavicle and the addition of new bone to the shafts of most other bones (El Tamer and Reis., 2009).

Endochondral ossification forms most of the bones including the axial and appendicular skeletons. It has simplistically been referred to as replacement of cartilage by bone, but this process is very complex in both its molecular and cellular transitions (Caplan and Boyan., 1994). The beginning of the cartilage differentiation process is signalled by cellular condensation of the mesenchyme prior to cartilage matrix secretion. The mesenchymal cells differentiate into chondroblasts, which proliferate and produce a matrix that forms both the shape and position of the eventual bone. The embryonic model for long bones consists of hyaline cartilage, which undergoes appositional growth resulting in a dumbbell-like shape. The shaft of the cartilaginous mass becomes the diaphysis, with the epiphyses located at both ends, and completely surrounded by the perichondrium (Sandberg., 1991). In the central aspect of the forming bone, long linear columns of chondrocytes

progressively hypertrophy, resorb the surrounding cartilage, and leave behind trabeculae of cartilage matrix, which then becomes mineralized. The chondrocytes degenerate, leaving behind interconnected spaces (Marks and Hermey., 1996; Sandberg., 1991).

Along the circumference of the developing endochondral bone, the perichondrium develops osteogenic potential and lays down a thin layer of bone around the shaft, known as the periosteum. Primitive mesenchymal cells and blood vessels then invade the spaces within the shaft of the bone that are left after the chondrocytes degenerate. This mesenchyme differentiates into osteoblasts and bone marrow cells (Marks and Hermey., 1996; Sandberg., 1991). Irregular woven bone is then formed as the osteoblasts layer along the surface of the calcified cartilage remnants. The ends of the original cartilaginous model are now separated through this process known as primary ossification.

Secondary ossification also is occurring within the epiphysis, while a thin layer of hyaline cartilage is retained along the articular surface. The border between the diaphysis and epiphysis of developing long bones is the epiphyseal or growth plate. This is the area of continued longitudinal bone growth until physical maturity, when cartilage is replace by bone, bringing together the diaphysis and the epiphysis (Sandberg., 1991).

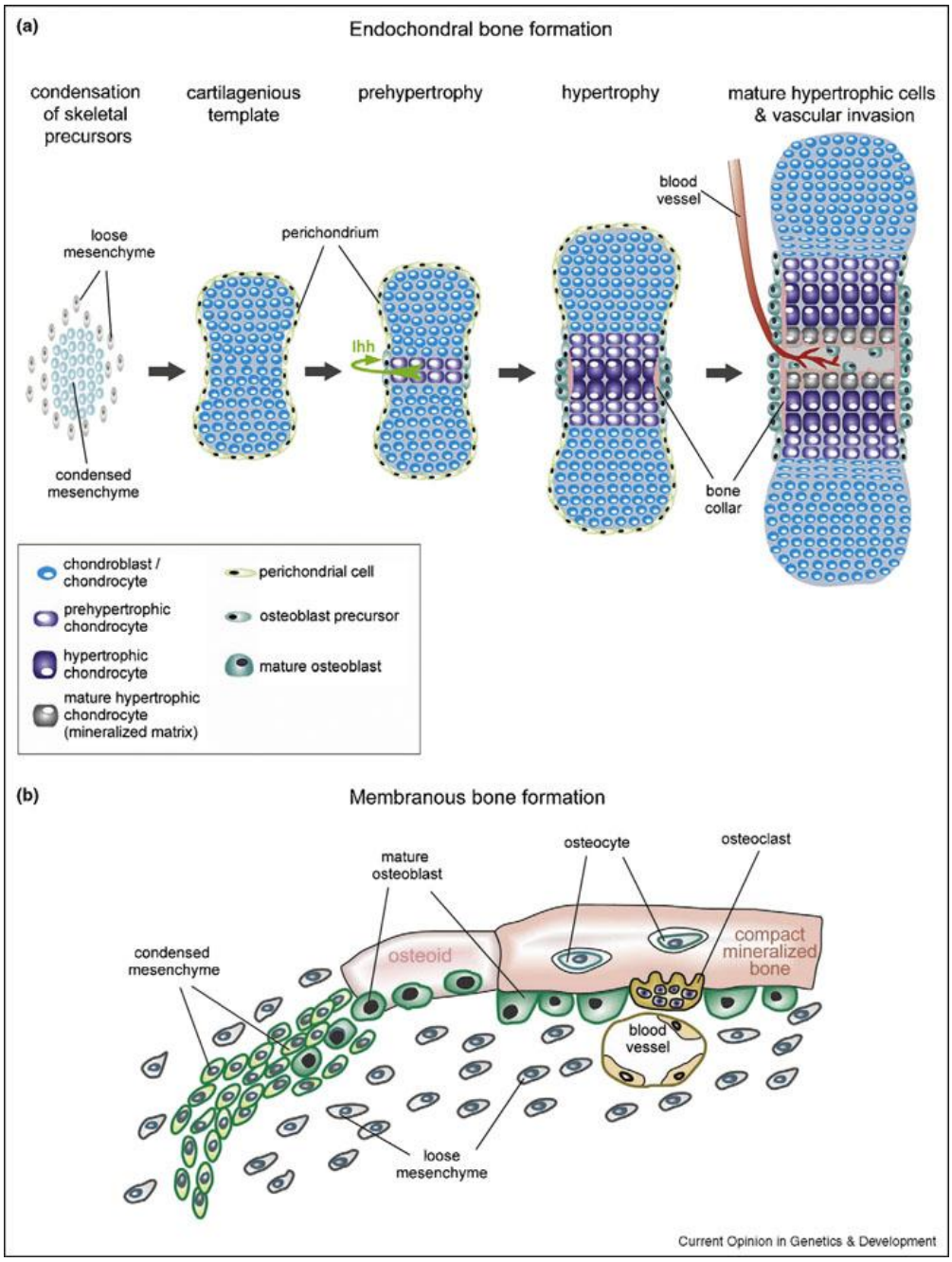


Figure 1. *Schematic representation of the two modes of bone formation.* (a) Endochondral bone formation as it takes place in long bones, starting with the condensation of skeletal precursors, proceeding to the formation of a cartilaginous template. Next prehypertrophic cells differentiate in the center of the cartilaginous template. At the subsequent steps hypertrophic chondrocytes appear, which continue to differentiate into mature hypertrophic chondrocytes producing a mineralized matrix and eventually are removed by chondroclasts leaving behind the mineralized matrix. Concurrently, blood vessels invade and together with those osteoblasts will enter and start to make trabecular bone utilize the mineralized matrix. (b) Membranous bone formation as it occurs in the flat bones of the skull: osteoblast progenitors differentiate directly from condensed mesenchyme and eventually differentiate into osteoid producing mature osteoblasts. Osteoblasts that get entrapped into the compact bone, reside in lacuna and differentiate into osteocytes. (Font: Hartmann C. (2009). Transcriptional networks controlling skeletal development. *Current Opinion in Genetics & Development.*, 19:437–443).

Bone tissue is not static, and healthy bones require continuous remodeling in order to maintain bone mass, to repair microdamage of the skeleton, to prevent accumulation of too much old bone, and for mineral homeostasis. Remodeling is a lifelong coordinated and dominant process in the adult skeleton, whereby cortical and trabecular bone is rebuilt, a process initiated by osteoclastic bone resorption and followed by osteoblastic bone formation at the same site where the resorption process occurs, known as bone multi-cellular units (BMU) (Lerner., 2006).

Bone remodelling has been described as a "bone remodelling cycle" consisting of three phases: initiation, transition, and termination of remodelling (Matsuo and Irie., 2008).

The initiation phase starts with osteoblastic activation of osteoclast differentiation, fusion, and activation. This process involves upregulation of the expression of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) by osteoblastic and other cells (Boyce and Xing., 2007). By cell-to-cell contact, RANKL and M-CSF will activate their cognate receptors, RANK and c-Fms, respectively, on osteoclast progenitor cells. This will lead to an expansion of the osteoclast progenitor pool, increased survival of these cells, and the initiation of a differentiation program that terminates in fusion of the mononucleated progenitor cells and the development of latent multinucleated osteoclasts. Finally, these osteoclasts become activated to bone-resorbing osteoclasts and create resorption lacunae (Lerner., 2006). Osteoclastic bone resorption lasts about three weeks in human bone.

During transition phase, bone-resorbing osteoclasts stimulate differentiation of osteoblast precursors, activating bone formation in bone resorption lacunae. While bone formation is being activated, osteoclastic bone resorption stops and osteoclasts undergo apoptosis in a Bim/caspase-3-dependent manner (Wakeyama *et al.*, 2007) or through estrogen-induced Fas ligand (Nakamura *et al.*, 2007). High extracellular calcium released from bone during resorption induces osteoclast apoptosis (Lorget *et al.*, 2000; Nielsen *et al.*, 2007).

The termination phase includes new bone formation, mineralization and entry into quiescence. This phase proceeds slowly and lasts much longer (about three months) than resorption. Osteoblasts become quiescent presumably with the help of sclerostin, a factor

produced by osteocytes (van Bezooijen *et al.*, 2004). During bone formation in the termination phase, osteoclast differentiation is suppressed, most likely through osteoprotegerin (OPG) produced by osteoblasts (Matsuo and Irie., 2008).

The balance of these three phases, the rate of bone remodelling and the number of remodelling sites are altered in a variety of pathologic conditions affecting the skeleton, leading to loss of bone, as in osteoporosis, hyperparathyroidism, and rheumatoid arthritis, or, more rarely, to gain of bone as in osteopetrosis (Lerner., 2006; Boyce and Xing., 2008).

Bone tissue regeneration

Many pathological conditions (e.g. osteoporosis, osteoarthritis, and cancer metastases to the bone) as well as high-impact bone fractures present with a problem of tissue regeneration and/or replacement due to the extent of the injury and/or the lack of proliferating and tissue-regenerating cells in bone. Regarding this aspect, the currently used therapeutical strategies are based mainly on autografts, allografts, demineralised bone matrix and bone substitutes (Salgado *et al.*, 2006). Autografts and allografts are the most popular methodologies in the field, presenting, however, several disadvantages.

Autografts require a second surgical procedure that can lead to infection and chronic pain in the harvest site. Furthermore, it can also cause donor site morbidity, and the amount of biological material available for grafting is not high, limiting in this sense, the type of injuries in which it can be used (Heary *et al.*, 2002; Perry., 1999).

Allografts are especially attractive when a larger segment of bone needs to be replaced. It introduces however, the possibility of immune rejection and of pathogen transmission from donor to host, and, although infrequent, infections could occur in the recipient's body due to the transplantation (Salgado *et al.*, 2004).

Taking into account these evidences, the need to improve upon the failings with which current interventions remain beset is very important. As such, a shift in focus towards the development of cell-based bone tissue engineering strategies is transpiring, as their potential to provide novel solutions to the persistent inadequacies of current skeletal

reconstructive modalities is being increasingly realized. Recent investigations, probing multiple progenitor cell populations, have produced a compelling body of evidence to support the clinical viability of cell-based bone tissue engineering.

First and foremost, multipotent cells must be able to differentiate toward osteogenic lineage in an efficient manner, thanks to the concerted actions of a plethora of signals (e.g. hormones, growth factors, mechanical forces, transcription factors) important for osteoblastogenesis. However, this alone is insufficient to make a cell source a viable candidate for use clinically. In order for a particular cell population to be successfully utilized in clinical bone tissue engineering applications, it must not only possess the capacity to regenerate mature, functionally competent, bone tissue de novo. Furthermore, the cells must maintain an adequate proliferative capacity post-harvest, be accessible in sufficient numbers, and be able to be obtained without imparting undo donor morbidity. Undoubtedly, human mesenchymal stem cells (MSCs) have shown a clear capacity to fulfil these criteria (Panetta *et al.*, 2009).

"In vitro" experimental models to study osteogenesis process: mesenchymal stem cells (MSCs) and osteoblasts (OBs)

- Mesenchymal stem cells (MSCs)

The use of living cells as therapeutic agents for the maintenance, regeneration, or replacement of malfunctioning tissues has been proposed in last decades (Kirouac and Zandstra., 2008). Stem cells are the basis for cell therapy. They are functionally undifferentiated cells that retain the ability to differentiate in one or more mature cell types under appropriate conditions, and to self-renew, representing a potentially inexhaustible cell source (Alberts *et al.*, 2002). There are two main sources of stem cells: embryonic and non-embrionic stem cells.

Embryonic stem cells (ESCs) are harvested from the inner cell mass of the blastocyst and are acclaimed for their unlimited capacity for self-renewal (Allison *et al.*, 2002; Preston *et*

al., 2003). They were primarily isolated during early 1980s from mouse embryos (Evans and Kaufman., 1981; Martin., 1981), then later from human embryos (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Human ESCs are pluripotent, as they can give rise to essentially all cell types in the body (Buckwalter and Mankin., 1998; Hentthorn, 2002). *In vitro* and *in vivo* experiments have demonstrated the ability of ESCs for osteogenic differentiation (Whang and Lieberman., 2003). In spite of this broad differentiation capability and potential to be used in regenerative medicine, the predisposition of these cells for teratoma formation and the political and ethical debate currently surrounding their use pose substantial challenges for forward progress on these cells (Lauffenburger and Schaffer., 1999; Montjovent *et al.*, 2004).

Non-embrionic stem cells or mesenchymal stem cells (MSCs) are a group of clonogenic cells present in different adult human tissues. These cells are able to self-renew and possess a high proliferative capacity (Minguell *et al.*, 2001; Uccelli *et al.*, 2008). Many studies suggest that MSCs can give rise to different mesenchymal cell type such as osteoblasts (Pittenger *et al.*, 1999; Donald *et al.*, 1996; Jaiswal *et al.*, 1997; Kadiyala *et al.*, 1997; Nilsson *et al.*, 1999), chondrocytes (Kadiyala *et al.*, 1997; Pittenger *et al.*, 1999; Johnstone *et al.*, 1998; Mackay *et al.*, 1998), adipocytes (Young *et al.*, 1998; Pittenger *et al.*, 1999; Endres *et al.*, 2003), tenocytes (Awad *et al.*, 1999) and myocytes (Pereira *et al.*, 1995; Horwitz *et al.*, 1999; Jiang *et al.*, 2002; Bhabavati *et al.*, 2004; Smith *et al.*, 2004; Beyer and da Silva, 2006; Sethe *et al.*, 2006). MSCs can also differentiate into cells of ectodermal origin, such as neurons (Woodbury *et al.*, 2000), and of endodermal origin, such as hepatocytes (Petersen *et al.*, 1999) (see Figure 2).

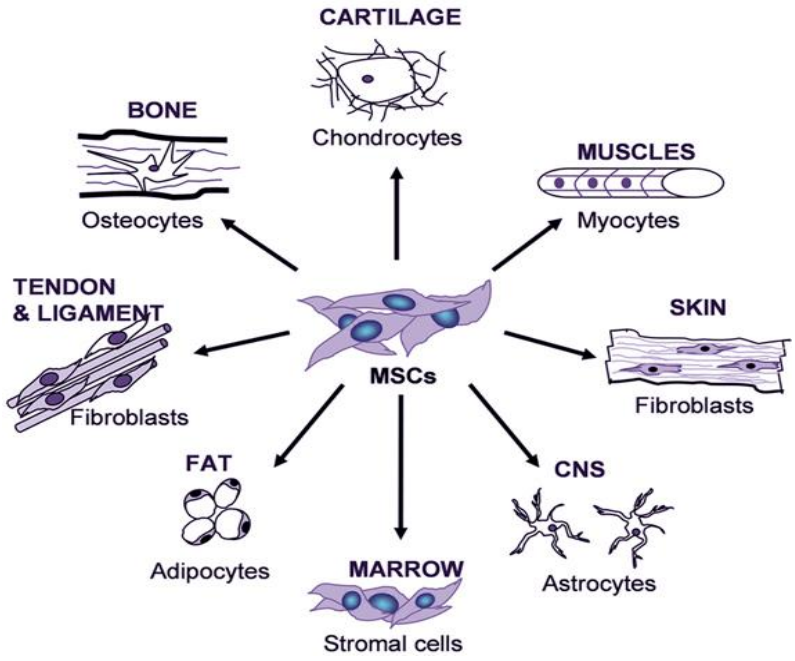


Figure 2. *Multi-lineage potential of mesenchymal stem cells (MSCs).* MSCs can differentiate into multiple lineages as osteocytes, chondrocytes, fibroblasts, adipocytes, astrocytes, myocytes and bone marrow stromal cells. (font: Grassel S and Ahmed N. (2007). Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation. *Front Biosci.*, 12:4946-4956).

Several ideas have been put forward to explain stem cell lineage determination. One current line of research is focused on the stem cells microenvironment or niche. A niche consists of signalling molecules, intercellular communication and the interaction between stem cells and their neighbouring extracellular matrix. This three-dimensional microenvironment is thought to influence/control genes and properties that define "stemness" of the stem cell, i.e. self-renewal or development to committed cells (Watt and Hogan., 2000). An interesting theory put forward is that stem cells might be terminal differentiation cells with the potential to display diverse cell types, depending on the host niche. Adult stem cells that are implanted into a totally different niche (different germ layer) can potentially differentiate into cell types similar to those found in the new environment. For example, human neural stem cells were found to produce muscle cells when they were implanted into skeletal muscle (Galli *et al.*, 2000). Bone marrow cells

were found to differentiate into neural cells when they were transplanted into a neural tissue (Zhao *et al.*, 2002; Mezek *et al.*, 2000). In addition, trans-differentiation of liver cells to islet cells was achieved (Alam and Sollinger., 2000). These findings showed the possibility of "strong" niche influence leading to adult stem cells plasticity (the ability to dedifferentiate into cells from other lineages) (Bajada *et al.*, 2008).

Historically, research involving the cells currently referred to as MSCs dates back to the 60s and 70s, when Dr. Alexander J. Friedenstein and colleagues started researching fibroblastic cells from bone marrow (BM) of rodents and rabbits (Phinney., 2002). At first, these cells were not called MSCs, not even were termed stem cells, but were considered to be fibroblastic precursors derived from an entity with unknown anatomical location in BM termed the colony-forming unit-fibroblast (CFU-F). Experiments involving transplantation of BM cells into ectopic sites including the renal capsule resulted in ectopic bone formation, and brought up the notion that BM housed osteogenic precursors (Phinney., 2002). Later, the fibroblastic colonies derived from BM cells were found to be able to differentiate into cells with characteristics of osteoblasts, chondrocytes, and adipocytes (Phinney., 2002). By that time, Dr. T. M. Dexter and colleagues developed a cultured system to study hematopoiesis *in vitro* (Dexter et al., 1977). In this system, cells bearing hematopoietic stem cell (HSC) characteristics were found to be non-adherent to the culture vessels, and to be dependent on the establishment of a layer of adherent cells that were viewed as representative of the BM stromal environment. The notion that CFU-F was derived from the stromal compartment of BM became established, and the term bone marrow stromal cell used in reference to these culture-adherent BM cells (Lanotte *et al.*, 1981).

Source of MSCs

Adult MSCs have been identified in the vast majority of tissues and organs. They have been isolated from muscles (Deasy *et al.*, 2001), peripheral blood (Kuznetsov *et al.*, 2001; Roufosse *et al.*, 2004), adipose tissue (Lee *et al.*, 2004), tendon (Salingcarnboriboon *et al.*, 2003), synovial membrane (De Bari *et al.*, 2001), hair follicles and scalp subcutaneous

tissue (Shih *et al.*, 2005), periodontal ligament (Trubiani *et al.*, 2005), fetal bone marrow, blood, lung, liver and spleen (In't Anker *et al.*, 2003), as well as pre-natal tissues such as cord blood (Erices *et al.*, 2000) and placenta (Fukuchi *et al.*, 2004; In't Anker *et al.*, 2004). As a result, significant efforts have been directed at identifying postnatal sources for multipotent cells. Multipotent cells have been identified in bone marrow, adipose tissue, placenta, umbilical cord, human amniotic fluid, dental pulp and skeletal muscle among others (Freeman, 1997; Clarkson, 2001; Mitka, 2001; Kadner *et al.*, 2002; Kaviani *et al.*, 2002, 2003; Rosser and Dunnett, 2003; Savitz *et al.*, 2004).

Bone marrow-derived mesenchymal stem cells (BM-MSCs) were the first adult stem cells identified (Friedenstein., 1976). BM-MSCs represent the most characterized type of adult stem cells (Bianco *et al.*, 2001; Owen and Friedenstein., 1988) and remain the most commonly used cell source for bone regeneration and repair in the studies using different animal models (El Tamer and Reis., 2009).

It has since been shown that in the bone marrow, there exists approximately one stem cell for every 100000 bone marrow cells (Polak and Bishop., 2006). Stem cells population derived from the bone marrow are usually a heterogeneous mix of different subpopulations, including bone marrow-derived hematopoietic stem cells (BM-HSCs), mesenchymal stem cells (BM-MSCs) (Friedenstein., 1976) and endothelial progenitor cells (Hristov and Weber., 2003).

Isolation and characterization of MSCs

Currently, the culture of MSCs from human BM most invariably starts with the centrifugation of BM aspirates on a density gradient formed by Ficoll-Paque or Percoll to separate the nucleated cells from the red blood cells (Phinney et al., 1999; Lennon and Caplan., 2006). In rodents, BM is obtained by flushing the BM out of the long bones, and the tissue obtained is usually disaggregated by flushing it in and out a syringe with needle (da Silva Meirelles and Nardi., 2003; Lennon and Caplan., 2006). MSCs obtained from solid tissues can be obtained by digestion using collagenase type I with or without the use of other proteolytic enzymes (da Silva Meirelles *et al.*, 2006; Krampera *et al.*, 2007). The

cells are then counted and placed in plastic culture vessels with culture-treated surface in culture medium. MSCs can be expanded *in vitro* to hundreds of millions of cells from a 10-to-20-mL BM aspirate (Di Girolamo *et al.*, 1999; Sekiya *et al.*, 2002). The cell yield after expansion varies with the age and condition of the donor and with the harvesting technique. Therefore, differences in isolation methods, culture conditions, and media additives greatly affect cell yield and possibly also the phenotype of the expanded cell product (Sekiya *et al.*, 2002; Caterson *et al.*, 2002; Sotiropoulou *et al.*, 2006). For these reasons, efforts have been made within the European Group for Blood and Marrow Transplantation (EBMT) MSC expansion consortium for the standardization of MSC isolation and expansion procedures. This organization, including European centers interested in the biology and clinical application of MSCs, has defined common protocols in order to facilitate comparisons between cell products generated at different sites and to run large-scale clinical studies (Bernardo *et al.*, 2009). In this regard, MSCs are currently expanded *in vitro*, either under experimental or clinical grade conditions, in the presence of 10% fetal calf serum (FCS) (Caterson *et al.*, 2002; Sotiropoulou *et al.*, 2006), and serum batches are routinely prescreened to guarantee both the optimal growth of MSCs and the biosafety of the cellular product. Despite this, the use of FCS has raised some concerns when utilized in clinical-grade preparations, because it might theoretically be responsible for the transmission of prions and still unidentified zoonoses or cause immune reactions in the host, especially if repeated infusions are needed, with consequent rejection of the transplanted cells (Horwitz *et al.*, 2002). In view of these considerations, serum-free media, appropriate for extensive expansion and devoid of the risks connected with the use of animal products, are under investigation. The possibility of using autologous or allogeneic human serum for *in vitro* expansion of MSCs has been tested, and autologous serum has proved to be superior to both FCS and allogeneic serum in terms of proliferative capacity of the expanded MSCs (Shahdadfar *et al.*, 2005).

MSCs characterization consists of the analysis of their adherent properties, proliferation, differentiation potential and surface molecule profile. In detail, *ex vivo* expanded MSCs

have been phenotipically characterized on the basis of the expression of some markers, including CD105 (SH2 or endoglin), CD73 (SH3 or SH4), CD90 (Thy-1), CD166, CD44, and CD29. In addition, culture-expanded cells lack the expression of some hematopoietic and endothelial markers, such as CD14, CD31, CD34, and CD45 (Caplan., 1994; Pittenger *et al.*, 1999). Little is known about the characteristics of the primary precursor cells *in vivo,* since it has not yet been possible to isolate the most primitive mesenchymal cell from bulk cultures. One of the hurdles has been the inability to prospectively isolate MSCs because of their low frequency and the lack of specific markers. Recently, the identification and prospective isolation of the most primitive mesenchymal progenitors, both in murine and human adult BM, have been reported, based on the expression of some markers (Anjos-Afonso and Bonnet., 2007; Gang *et al.*, 2007; Simmons and Torok-Storb., 1991; Quirici *et al.*, 2002; Buhring *et al.*, 2007; Battula *et al.*, 2009; Gentry *et al.*, 2007). One group has reported the identification, isolation, and characterization of a novel multipotent cell population in murine BM, based on the expression of the stage-specific embryonic antigen-1 (SSEA-1). This primitive subset, found both directly in the BM and in mesenchymal cell cultures, can give rise to SSEA-1+ MSCs and is proposed to be placed at the apex of the hierarchical organization of the mesenchymal compartment (Anjos-Afonso and Bonnet., 2007). In human cells, surface markers such as SSEA-4, STRO-1, and the low-affinity nerve growth factor receptor (CD271) (Gang *et al.*, 2007; Simmons and Torok-Storb., 1991; Quirici *et al.*, 2002; Buhring *et al.*, 2007), which enrich for MSCs, have been employed with the aim to prospectively isolate MSCs. Moreover, Battula and colleagues have recently isolated by flow cytometry MSCs from human BM using antibodies directed against the surface antigens CD271, mesenchymal stem cell antigen-1 (MSCA-1), and CD56, and identified novel MSC subsets with distinct phenotypic and functional properties (Battula *et al.*, 2009). Platelet-derived growth factor receptor-beta (PDGF-RB; CD140b) has also been identified as a selective marker for the isolation of clonogenic MSCs (Buhring *et al.*, 2007), and other reports have demonstrated a 9.5-fold enrichment of MSCs in human BM cells with prominent aldehyde dehydrogenase activity (Gentry *et al.*, 2007). The relevance and usefulness of these markers for the prospective isolation and consequent

expansion of MSCs from BM and/or other sources is being evaluated and will possibly allow a more precise definition of the cell products employed both in the experimental and clinical setting.

However, due to the lack of specific markers that define the multipotent MSC, coupled with the lost of certain markers during MSCs *in vitro* expansion, these cells are usually defined in functional terms based on *in vitro* and *in vivo* functional assay. The International Society for Cellular Therapy has provided the following minimum criteria for defining multipotent human MSCs (Dominici *et al.*, 2006):

- Plastic-adherent under standard culture conditions;
- Positive for expression of CD105, CD73, and CD90, and absent for expression of hematopoietic cell surface markers CD34, CD45, CD11a, CD19, and HLA-DR;
- Under specific stimulus, cells should differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*.

In addition, "the gold standard" assay for MSCs stemness is based on the ability of the cells to form ectopic bone and bone marrow microenvironment supporting hematopoiesis upon implantation in an open system (subcutaneous implantation) in immune deficient severe combined immunodeficiency disease mice. This assay has also been employed to demonstrate the ability of the multipotential MSC cells to exhibit self-renewal and maintenance of stemness capacity during serial implantations (Piersanti *et al.*, 2006).

Wharton's Jelly mesenchymal stem cells (WJMSCs)

As mentioned above, different adult human tissue have been considered as MSCs sources, including bone marrow, trabecular bone, adipose tissue, peripheral blood, synovium, skeletal muscle, dental pulp and periodontal ligament (Dennis *et al.*, 1999; Kern *et al.* 2006; Peng *et al.*, 2008). Although bone marrow still represent the main and most investigated source of adult MSCs, the isolation and use of these cells still present some drawbacks. For instance, the number of MSCs found in bone marrow, decrease

progressively starting at age 17 and the harvesting techniques are invasive, often causing severe infections, bleeding and chronic pain for donors (Bajada *et al.*, 2008). Looking for alternative MSCs sources, fetal tissue (Hu *et al.*, 2003; Guillot *et al.*, 2008) and exta-embryonic tissues (Yen *et al.*, 2005; De Coppi *et al.*, 2007; Lee *et al.,* 2004; Parekkadan *et al.*, 2007; Sarugaser et al., 2005; Can and Karahuseyinoglu., 2007), have recently been considered.

In particular, in this thesis I will focus the attention on umbilical cord stroma.

Umbilical cord, due to the unique morphological properties, represents an interesting alternative source for MSCs, especially if compared with umbilical cord blood (Secco *et al.*, 2008; Troyer and Weiss., 2008). The human umbilical cord weighs approximately 40 g, its length reaches to approximately 60–65 cm, and it has a mean diameter of 1.5 cm at term (Raio *et al.*, 1999; Di Naro *et al.*, 2001). The inner tissue architecture is composed of a set of two arteries and one vein and a surrounding matrix of mucous connective tissue called Wharton's Jelly, initially described by Thomas Wharton in 1956 (see Figure 3).

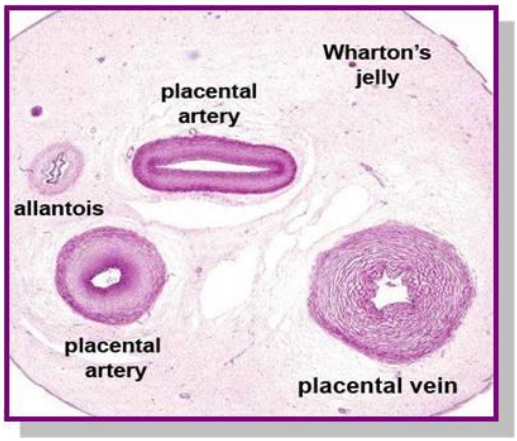


Figure 3. *Umbilical cord in cross-section.*
(modified from: http://embryology.med.unsw.edu.au/Science/ANAT2341lab04_4.htm)

This jelly acts as a protective tissue for vessels and contains into its stromal compartment, cells with specific mesenchymal characteristics, called Wharton's Jelly Mesenchymal Stem Cells (WJMSCs) (Wang *et al.*, 2004). Therefore, Wharton's Jelly can be an ideal, unique, easily accessible and uncontroversial source for early MSCs, due to the simple

collection procedure once umbilical cord is routinely discharged at parturition (Penolazzi *et al.*, 2010).

Wharton's Jelly is constituted of specialized fibroblast-like cells and occasional mast cells embedded in high amounts of extracellular matrix (ECM) components, mainly collagen, hyaluronic acid and several sulphated proteoglycans (Sobolewski *et al.*, 1997; Franc *et al.*, 1998). The accumulation of peptide growth factors, such as insulin-like growth factor (IGF) (Edmondson *et al.*, 2003), fibroblast growth factor (FGF) (Yu *et al.*, 2003), and transforming growth factor β (TGF-β) (Shalitin *et al.*, 2003), followed their release and activation, may strongly promote the biosynthesis of these ECM components. This amorphous ground substance would provide a strong mechanical resistance, elasticity and high degree of hydration to prevent the umbilical cord vessels from occlusion, such as that induced by bending, torsion or scretch evoked by uterine contraction or fetal movement (Pennati 2001). Within Wharton's Jelly, WJMSCs have been isolated from three relatively indistinct regions: the perivascular zone, the intervascular zone, and the subamnion (Troyer and Weiss., 2008).

WJMSCs have some genetic and surface markers that are also common in MSCs. Among these, CD105, CD73, and CD90, which are known to characterize MSCs (Dominici *et al.*, 2006), were consistently found to be positive in WJMSCs. They do not express hematopoietic stem cell markers such as CD45, CD34, and human leukocyte antigen (HLA)-DR, which are also lacking in MSCs (Dominici *et al.*, 2006).

WJMSCs principally display a fibroblast-like appearance, are plastic-adherent and multipotent, so they can be differentiated into bone, cartilage, fat and neural cells (Karahuseyinoglu *et al.*, 2007; Wang *et al.*, 2004; Lund *et al.*, 2007). Many reports have demonstrated that WJMSCs have faster proliferation and greater *ex vivo* expansion capabilities than BM-MSCs. First, the isolation frequency of colony forming units (CFU-F) from bone marrow is estimated to be in the range of 1-10 CFU-F per 10^6 mononuclear cells (MNCs), while in WJMSCs is reported to have a higher frequency of CFU-F (Karahuseyinoglu *et al.*, 2007; Sarugaser *et al.*, 2005). Second, coupled with the greater CFU-F frequency, the doubling time of WJMSCs is shorter than BM-MSCs

(Karahuseyinoglu *et al.*, 2007; Baksh *et al.*, 2007; Lund *et al.*, 2007). This may be due to the expression of telomerase by WJMSCs (Mitchell *et al.*, 2003).

Telomerase activity (TA) in proliferating cells is an active area of research since, unlike embryonic stem cells and tumorigenic cells, somatic cells display low levels of TA (Greider., 1998). Few studies dealing with the TA of human WJMSCs revealed that TA in those cells is higher than in somatic cells of the body. Mitchell *et al.* (Mitchell *et al.*, 2003) showed that TA in isolated porcine WJMSCs was about 10% of that expressed by carcinoma cell lines, whereas Weiss *et al.* (Weiss *et al.*, 2006) found that telomerase reverse transcriptase gene expression is elevated in cultured human WJMSCs. Karahuseyinoglu *et al.* (Karahuseyinoglu *et al.*, 2007) demonstrated a stable but higher than normal TA in those cells during early passages, which then decreased and reached a level below control HeLa cell lines. Since the transplanted WJMSCs did not develop any tumorigenic formation (Conconi *et al.*, 2006), it is possible to conclude that WJMSCs have a certain limit of TA that provide cells with the ability to proliferate up to 30–60 divisions but never to a level of tumorigenic state.

WJMSCs have, also, stromal support properties. For example, extra-embryonic mesenchyme, that is primitive Wharton's Jelly, surrounds the migrating embryonic blood island cells during their migration to the aorta-gonad mesonephros from the yolk sac region prior to day E10.5 (Sadler., 2004). WJMSCs retain this property as demonstrated by their role in *ex vivo* hematopoietic expansion (Lu *et al.*, 2006) and *in vivo* engraftment of HSCs. In fact, Friedman and co-workers (Friedman *et al.*, 2007) shown that in a NOD/SCID mouse model, human WJMSCs, when co-injected with human umbilical cord blood cells (UCB), can accelerate human hematopoietic stem cell recovery when limited numbers of UCB cells or CD34 cells are injected.

Regarding immune properties, many results indicate that WJMSCs are immunosuppressive and have reduced immunogenicity (Troyer and Weiss., 2008). Indeed, WJMSCs exert immunosuppression by inhibiting T-cell responses to polyclonal stimuli (Di Nicola *et al.*, 2002; Glennie *et al.*, 2005) and suppress lymphocyte proliferation *in vitro* (Bartholomew *et al.*, 2002). In addition, WJMSCs express HLA-G, do not express co-stimulatory molecules,

such as CD80, CD86 or CD40 and express cytokines which may modulate immune function, like interleukin (IL)-6 and vascular endothelial growth factor (VEGF) (Weiss *et al.*, 2006).

Differentiation potency of culture-expanded MSCs

One of the criteria to define MSCs is their ability to differentiate into the adipogenic, chondrogenic and osteogenic lineages (Dominici *et al.*, 2006).

For adipogenic differentiation, MSCs are treated with dexamethasone, insulin, isobutyl metyl xanthine, and indomethcin (added to medium containing FBS), and the differentiation is revealed by the detection of lipid vacuoles with oil red O staining (da Silva Meirelles *et al.*, 2006; Mackay *et al.*, 2006). The chondrogenic differentiation is performed using a high cell-density pellet or micromass culture treated with transforming growth factor-β (TGF-β), ascorbic acid, and β-glycerophosphate; this results in production of cartilage-specific highly sulphated proteoglycans and type II collagen (Johnstone *et al.*, 1998). Classically, osteogenic differentiation of human MSCs requires incubation in FBS-containing medium supplemented with ascorbic acid, β-glycerophosphate, and dexametasone, resulting in an increase in alkaline phosphatase activity and calcium deposition. However, other supplements, such as 1,25-dihydroxyvitamin D_3 and members of the bone morphogenic protein (BMP) family are also routinely used for osteoinduction (Tuan *et al.*, 2003). Indeed, BMP-2 together with the aforementioned agents, successfully mediates the osteogenic differentiation of human WJMSCs and BM-MSCs inducing the expression of osteoblast-specific markers such as alkaline phosphatise (ALP), Runx2, osteocalcin (OC), collagen type I (COL I) and osteopontin (OPN) (Hou *et al.*, 2009). In addition, BMP-2 alone appears to increase bone nodule formation and the calcium content of osteogenic cultures *in vitro*, while concomitant application of BMP-2 and basic fibroblast growth factor increases MSCs osteogenesis both *in vivo* and in *vitro* (Hanada *et al.*, 1997).

Increasing evidence, supports that MSC populations are heterogeneous with coexisting subsets having varying potency; this applies to bone marrow as well as to MSCs from

other tissues. As an example, Karystinou and co-workers, recently reported that human synovium-derived clonal MSCs were capable of osteogenic and chondrogenic differentiation though with varying potency, but only 30% of the clonal populations tested were able to differentiate into adipocytes (Karystinou *et al.*, 2009).

Osteogenic differentiation potency of MSCs

The osteogenic potential of culture-expanded MSCs (Friedenstein *et al.*, 1976; Ashton *et al.*, 1985) has been studied extensively *in vitro* and *in vivo*. The first *in vivo* experiments with MSCs were performed using diffusion chambers loaded with culture-expanded cells (Ashton *et al.*, 1980). Later, the adoption of bioscaffolds such as hydroxyapatite (HA) implanted in immunocompromised mice proved useful to help studying MSC osteogenic differentiation *in vivo* (Ohgushi and Okumura., 1990). It was possible to obtain donor MSC-derived bone by subcutaneous implantation of HA scaffolds seeded with human MSCs (De Bari *et al.*, 2008; Muraglia *et al.*, 1998; De Bari and Dell'accio., 2008; Bluteau *et al.*, 2008; Dell'accio *et al.*, 2008). Using HA-based bioscaffolds, it became than possible to repair segmental bone defects *in vivo* by using autologous MSCs, in loaded conditions, both in large animals (Kon *et al.*, 2000) as well as in humans in proof-of-concept studies (Quarto *et al.*, 2001).

The use of MSCs in bone tissue engineering remains challenging for issues such as the plethora of tissues sources and culture conditions, with resulting biological variability. For instance, human periosteum contains cells that upon enzymatic release and culture expansion display MSCs phenotype and capacity at the single-cell level to differentiate into multiple skeletal lineages including bone (De Bari *et al.*, 2006). Notably, in a proof-of-concept study, De Bari and co-workers quantified the bone-forming potency of matched human MSCs from synovium and periosteum and analyzed the sources of variability in osteogenic outcome. They identified the tissue of origin of MSCs as the main source of variability, since MSCs from periosteum had significantly greater osteogenic potency than MSCs from synovium. The different origin of MSCs may influence also the beginning and the achievement of osteogenesis. Indeed, Baksh and co-workers demonstrated that

WJMSCs undergo osteogenesis at a faster rate than BM-MSCs, based on the increase in ALP positive cells, and after 5 weeks of culture under osteogenic conditions, WJMSCs generated a greater extend of mineralization than BM-MSCs, including bone nodule generation (Baksh *et al.*, 2007). A second source of variability is related to the individual donor, within each tissue. In particular, donor's age in relation to osteogenic differentiation capacity of MSCs, has been well documented in both human and animal models (Zhang *et al.*, 2008; D'Ippolito *et al.*, 1999). For instance, the BM-MSCs/progenitor cell number and differentiation ability decrease in an age-dependent manner (Bajada *et al.*, 2008; Hamrick *et al.*, 2006).

- Osteoblasts (OBs)

Osteoblasts, bone producing cells, originate from multipotent mesenchymal stem cells and are responsible for the secretion of the organic extracellular matrix of bone (ECM), both during development and later during the remodeling of mature bone (Karsenty and Wagner., 2002). These cells also express genes that are necessary and sufficient to induce mineralization of this ECM and deposit osteoid on the pre-existing mineralized matrix only (Huang *et al.*, 2007).

Despite that, osteoblasts are required for bone resorption: indirectly, because they produce some factors, including RANKL (Theoleyre *et al.*, 2004) and interleukin (IL)-6 (Ishimi *et al.*, 1990), necessary to induce osteoclast differentiation; directly, because they release metalloproteinases, enzymes involved in the extracellular matrix digestion (Filanti *et al.*, 2000).

To date, some specific osteoblast differentiation markers have been defined, which allow to discriminate between osteoblasts and other cells (Gundberg., 2000; Titorencu *et al.*, 2007):

- Runx2 (Runt-related transcription factor 2) or Cbfa1 (Core binding factor 1): is the master gene regulator of osteoblast differentiation;

- Collagen type I (Col1a1): is a significant component of bone matrix, which is involved in the mineralization process;
- Osteopontin (OPN): is an extracellular structural phosphoprotein which is rich in sialic acid;
- Osteocalcin (OC) or Bone gamma-carboxyglutamic acid-containing protein (BGLAP): is a noncollagenous protein, vitamin K-dependent, which binds bone mineral calcium and hydroxyapatite;
- Osteonectin: is a glycoprotein secreted by osteoblasts during bone formation, initiating mineralization and promoting mineral crystal formation. It is also shows affinity for collagen type I in addition to bone mineral calcium.
- Bone Sialoprotein (BSP): is a component of bone extracellular matrix;
- Alkaline phosphatise (ALP): is implicated in mineralization mechanisms.

Recently, also collagen type XV (COL15A1), previously described as being expressed in the basement membrane in other cell types, was identified as a matrix protein released by osteoblasts. It was demonstrated that, the expression of collagen XV significantly increases during the osteogenic differentiation *in vitro*. On the contrary, the presence of free ionised extracellular calcium in the medium, significantly down-modulates its expression (Lisignoli *et al.*, 2009).

Besides the estrogen receptor ERα is a key regulator of bone and its mRNA is actively regulated throughout development of the osteoblastic phenotype. The activation of this receptor has a direct effect on the expression of genes coding for enzymes, bone matrix proteins, hormone receptors, transcription factors, cytokines and growth factors of osteoblasts and osteoclasts (Zallone., 2006).

With regard to the precise effect of estrogen on osteoblastic differentiation and proliferation of mature osteoclasts, the data reported in the literature are partly conflicting and in some cases seem to vary depending on the experimental model adopted.

It is possible to outline osteoblastogenesis in three major stages: proliferation, matrix maturation, and mineralization, which are characterized by sequentially expressed distinctive osteoblast markers. Proliferation is characterized by increased expression of genes related to cell cycle; at the end of this phase and during the matrix deposition, it is possible to detect mRNA for collagen I, osteonectin and ALP. Indeed, ALP is an early marker of osteoblast differentiation. The production of OPN, OC and BSP occurs between the end of matrix deposition and the beginning of mineralization; these proteins are therefore classified as late markers of differentiation (Olsen *et al.*, 2000; Zur Nieden *et al.*, 2003). This is associated with the deposition of calcium and phosphate salts, which can be detected by specific assays.

The implementation of osteoblast differentiation process is controlled by several factors, some of which have been characterized and are currently successfully used to induce specific differentiation of mesenchymal progenitor cells. As mentioned before, the main agents reported in the literature capable of inducing osteogenesis are: ascorbic acid, vitamin D_3, β-glycerophosphate and dexamethasone (Zur Nieden *et al.*, 2003; Kawaguchi., 2006).

Runx2 (which will be discussed more fully in the following paragraphs) is certainly the most studied transcription factor and was indicated as the main regulator of the "osteogenic commitment"; indeed, it is always expressed in the three phases, even if its role is decisive, especially during stem cell-preosteoblast and preosteoblast-osteoblast transition (Komori., 2008).

Chapter 4

Osteogenic differentiation: molecular signaling pathways.

Progenitor cells will likely form the cornerstone of future skeletal tissue engineering modalities. Yet, gaining an understanding of the intra and intercellular processes which transpire on a molecular level to guide the osteogenic differentiation of skeletal progenitors will be pivotal in optimizing their potential to enhance osseous healing. Multiple signaling pathways, including those involving TGFβ/BMP, Wnt, FGF, Hedgehog, Notch and Ephrin, are now known to be intimately involved in skeletal development, homeostatic bone turnover, and skeletal regeneration after injury (see Figure 1).

More importantly, many have been found to in part regulate the osteogenic differentiation of MSCs. Though not an exhaustive list, these factors represent excellent targets for MSCs manipulation on a molecular level with the goal of augmenting the osteogenic potential of MSCs implemented in skeletal reconstruction, as well as enhancing the endogenous regenerative capacity of native tissues.

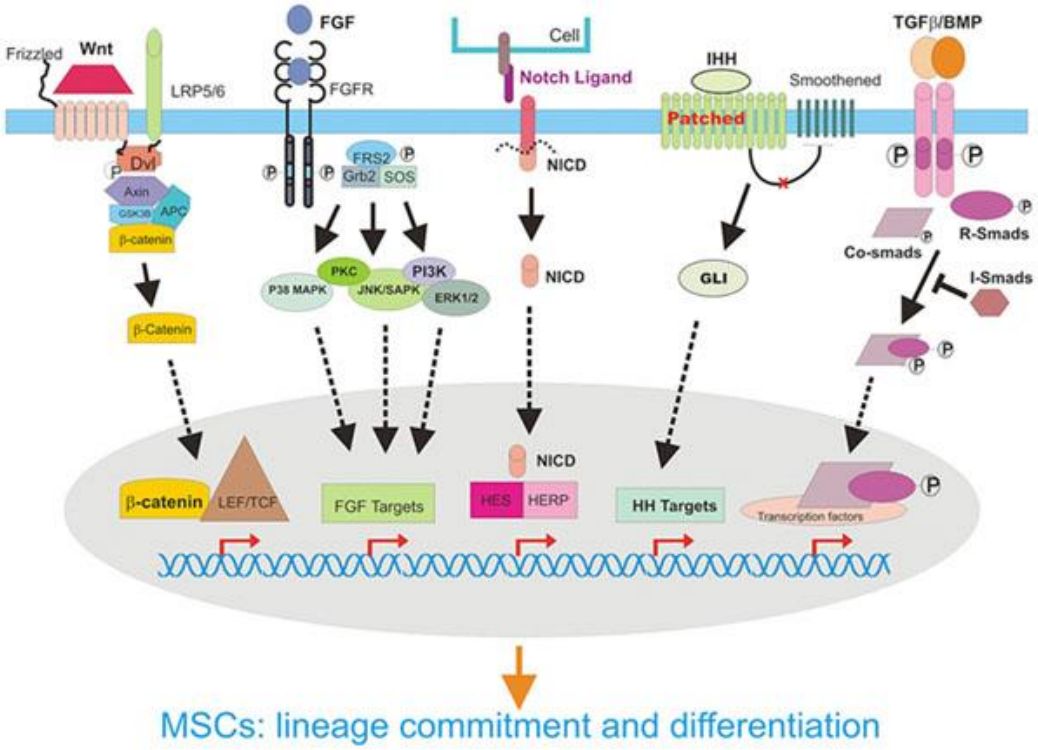


Figure 1. *Schematic representation of the major signalling pathways that play an important role in regulating skeletal development*. These include Wnt/β-catenin, TGFβ/BMP, Notch, Ihh, and FGF signalling pathways. (font: Deng Z.L., Sharff K.A., Tang N., *et al.* (2008). Regulation of osteogenic differentiation during skeletal development. *Front Biosci.*, 13:2001-2021).

TGFβ/BMP signaling: TGFβ superfamily consists of over 30 secreted dimeric polypeptide factors that play critical roles in regulating a diverse set of cellular functions including proliferation, differentiation and embryonic development (Luu *et al.*, 2007; Shi and Massague., 2003; Attisano and Wrana., 2002). TGFβ family consists of three isoforms TGFβ1, TGFβ2, TGFβ3, which are highly conserved in mammals. BMPs belongs to the TGFβ1 superfamily. They are a group of phylogenetically conserved signalling molecules, and were initially identified by their capacity to induce endochondral bone formation (Canalis., 2003; Cao and Chen., 2005). Many of these proteins, mostly notably BMP-2,-4,-6,-7,-9 initiate their signalling cascade by binding to the dimeric complex of two transmembrane serine-threonine kinase receptors, termed Type I and Type II (Hogan., 1996; Cheng *et al.*, 2003). The activated receptors, transmit signals through Smad-

dependent and Smad-indipendent pathways, including ERK, JNK, and p38 MAP kinase (MAPK) pathways (Derynck et al., 2001). There are three classes of Smads: 1) receptor-regulated Smads (BR-Smads) that can be BMP activated, or TGFβ activated (TR-Smads); 2) common partner BMP and TGFβ mediator Smads (Co-Smads); 3) inhibitrory Smads (I-Smads). The BR-Smads/Co-Smads complexes then traslocate into the nucleus and regulate transcription of target genes by interacting with various transcription factors and transcriptional co-activators or co-repressors. The BR-Smads activates expression of Distal-less homeobox 5 (Dlx5), that induces expression of Runx2 and Osterix in osteoprogenitors cells (Miyama *et al.*, 1999; Lee *et al.*, 2003). Although the Smads are critical mediators in the TGFβ signalling pathway, BMP-2 can activate ERK, JNK and p38 in osteoblastic cells and provide evidences that these MAP kinases have dinstinct roles in regulating alkaline phosphatise and osteocalcin expression (Guicheux et al., 2003; Lai and Cheng., 2002).

Hedgehog (Hh) signalling: The Hedgehog family of secreted proteins consists of three mammalian orthologs, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). Ihh is produced by prehypertrophic chondrocytes and appears to act directly on perichondrially located osteoblast progenitors to specify the osteoblast precursors (St-Jacques *et al.*, 1999; Long *et al.*, 2004). The failure of activation of Runx2, indicates that hedgehog (Hh) signalling acts to initiate an osteogenic program (Razzaque *et al.*, 2005). Ihh, is essential for skeletal development as demonstrated by knockout of *Ihh* in transgenic mice. *Ihh*-knockout mice demonstrate reduced chondrocyte proliferation, inappropriate chondrocyte maturation and an absence of osteoblast differentiation in endochondral bones, suggesting the importance of Ihh in endochondral bone development (St-Jacques *et al.*, 1999).

FGF signalling: The fibroblast growth factors (FGFs) are a family of secreted polypeptides that acts through four related tyrosine kinase receptors (Fgfr1-Fgfr4) to regulate a plethora of developmental processes, and they are critical for the control of endochondral and intramembranous ossification (Ornitz and Marie., 2002). Both FGF ligands and FGF receptors have a demonstrated role in osteogenic differentiation (Chen

and Deng., 2005; Ornitz., 2005; Jackson et al., 2006). For example, FGF-2 has been shown to induce alkaline phosphatase activity in rat bone marrow precursor cells, and FGF-2,-4 and -8 have been shown to induce Runx2 (Woei *et al.*, 2007). Furthermore, FGF-9 can induce osteocalcin expression, and FGF-2, -9 and -18 are important in subsequent matrix mineralization. Regarding FGF receptors, FGFR1 plays a dominant role during osteogenic differentiation, and FGFR2 appears to function during both osteogenic proliferation and differentiation. Although FGFR3 has been shown to function primarily during endochondral ossification to control chondrocyte proliferation, recent evidence suggests it may also play a critical role in osteogenesis. FGFR4 is expressed in rudimentary membranous bone and localized to the osteoblasts, suggesting that it may also be an important regulator of osteogenesis and play a critical role in intramembranous ossification (Cool *et al.*, 2002).

Notch signalling: The *Notch* gene encodes a single pass transmembrane receptor that is activated by a membrane bound ligand. In mammals, four Notch receptors (Notch 1-4) and five ligands (Delta-like1, Delta-like3, Delta-like4, Jagged 1 and Jagged 2) have been identified (Ehebauer *et al.*, 2006; Chiba., 2006; Hurlbut *et al.*, 2007). Notch 1 and Notch 2 are expressed by osteoblasts, while Notch 3 and Notch 4 have been identified in subgroups of the osteogenic lineage (Chau *et al.*, 2009). Notch signalling regulates osteogenesis, although the mechanisms involved in this pathway are poorly understood (Tezuka *et al.*, 2002; Sciaudone *et al.*, 2003; Schnabel *et al.*, 2002). Notch/TGFβ cross talk between the Notch1 and TGFβ ligand BMP-2 promotes osteogenic differentiation (Nobta *et al.*, 2005), although a recent study demonstrated that Notch1 overexpression inhibits osteogenesis by repressing Wnt/β-catenin but not BMP signalling (Deregowski *et al.*, 2006).

Ephrin signaling: Ephrins have the capacity for bidirectional signalling. That is, when a cell expressing an ephrin receptor contacts a cell expressing an ephrin ligand, signals are transduced into both the ephrin receptor-expressing cell (forward signalling) and the ephrin ligand-expressing cell (reverse signalling). There are two classes of ephrins, the B class (ephrin B1 to B3) are ligands for EphB tyrosine kinase receptors (B1 to B6),

whereas class A ephrins (A1 to A5) are ligands for GPI-anchored EphA receptors (A1 to A10) (Mundy and Elefteriou., 2006). In bone biology, ephrinB and EphB receptors control patterning of the developing skeleton (Compagni *et al.*, 2003). Zhao *et al.* suggested that ephrin signalling is critical to the two-way communication between osteoclasts and osteoblasts (Zhao *et al.*, 2006). This bidirectional signalling is mediated by transmembrane ephrinB2 ligand in osteoclasts and EphB4, a tyrosine kinase receptor, in osteoblasts. EphB4 expression is constitutive and the forward signalling through EphB4 induces osteogenic regulatory factors, such as Dlx5, Osterix, and Runx2, in calvarial osteoblasts, suggesting that EphB4 ia at the top of the regulatory cascade during osteoblast differentiation (Huang *et al.*, 2007).

Wnt signalling: The Wnt family consists of a large number of secreted glycoproteins (Cadigan and Nusse., 1997; Woderz and Nusse., 1998; Logan and Nusse., 2004). The Wnt pathway plays an important role in embryonic development, tissue induction and axis polarity (Cadigan and Nusse., 1997; Croce and McClay., 2006; Luo *et al.*, 2007). As described in detail below, Wnt plays an important role in skeletal development and osteoblast differentiation (Fischer *et al.*, 2002; Wang and Wynshaw-Boris., 2004; Gregory *et al.*, 2005).

Wnt signaling: a critical pathway for bone differentiation

Discovered in 1980s, the Wnt (Wingless and int-1) signaling cascade is involved in embryonic development and homeostasis, through regulation of cell proliferation, differentiation, and apoptosis (Rubinfeld *et al.*, 1993; Su *et al.*, 1993). Besides, it has been well established that Wnt signaling plays an integral role in many physiologic and pathologic processes, such as various types of cancer (Polakis., 2000; Reya and Clevers., 2005), Alzheimer's disease (De Ferrari., 2000), bipolar disease (Gould and Manji., 2002) and bone disorders (Wagner *et al.*, 2011).

Wnt genes encode a family of approximate 20 cysteine-rich secreted glycoproteins, which interact with cell surface receptors and trigger a cascade of intracellular events. Cell

signaling cascades provoked by Wnt proteins have been highly conserved across species, including *Caenorhabditis elegans, Drosophila melanogaster*, Zebrafish, *Xenopus laevis*, chicken, mouse, and human (Behrens., 2000). There are three distinct pathways through which Wnt signaling can be transduced: the Wnt/β-catenin or canonical pathway, a planar cell polarity pathway and a Ca^{2+}/Protein kinase A pathway (see Figure 2). The best studied of the pathways is the so-called canonical Wnt payhway. Activation of the canonical pathway is initiated when Wnt proteins, such as Wnt 1, Wnt 3a, or Wnt 8, bind to the Frizzled (Frz) receptors, seven-span transmembrane receptor proteins, and co-receptor LRP5/6 (low-density lipoprotein-receptor-related protein 5/6), activating the associated kinases, which in turn phosporylates the intracellular protein disheveled (Dvl). Activation of Dvl inhibits glycogen synthase kinase 3 β (GSK3β), resulting in subsequent stabilization of β-catenin (Chen and Alman., 2009). Stabilized β-catenin then translocates to nucleus and forms a transcriptional complex with lymphoid-enhancer binding factor (Lef)/T-cell specific transcription factors (Tcfs) to regulate target gene expression (Westendorf *et al.*, 2004). Examples of the β catenin/Tcf target genes include *c-Myc*, *cyclin D1* and *PPARδ* (Clevers., 2006; He *et al.*, 1999; Tetsu and McCormick., 1999).

In the absence of Wnt ligands, a complex consisting of GSK3β, Axin and adenomatous polyposis coli (APC), phosphorylates the N-terminal of β-catenin and initiates its proteosomal degradation (Kubota *et al.*, 2009; Piters *et al.*, 2008).

The non-canonical Wnt pathways, which occur independently of β-catenin, include the calcium dependent and cell polarity pathways. Well characterized non-canonical Wnts include Wnt 5a and Wnt 11 (Rao and Kuhl., 2010). The calcium-dependent Wnt pathway plays important roles in embryonic development, cell migration and motility, and possibly tumor suppression (Piters et al., 2008; Torres et al., 1996). In the calcium-dependent Wnt pathway, the Wnt ligands bind to Frz receptor and trigger intracellular calcium release by heterotrimeric G protein stimulation. The released intracellular calcium activates protein kinase C (PKC) or calcineurin (CaCN), which then turns on transcription factors Elk-1 and NFAT (Kuhl., 2004). The cell polarity pathway functions through Frz receptors, activating Rho and Rac, which lead to c-jun NH2 terminal kinase (JNK) activation (Davis., 2000).

This pathway may modify the actin cytoskeleton, while also plays a role in cell proliferation, differentiation, and apoptosis (Chen and Alman., 2009; Habas et al., 2003).

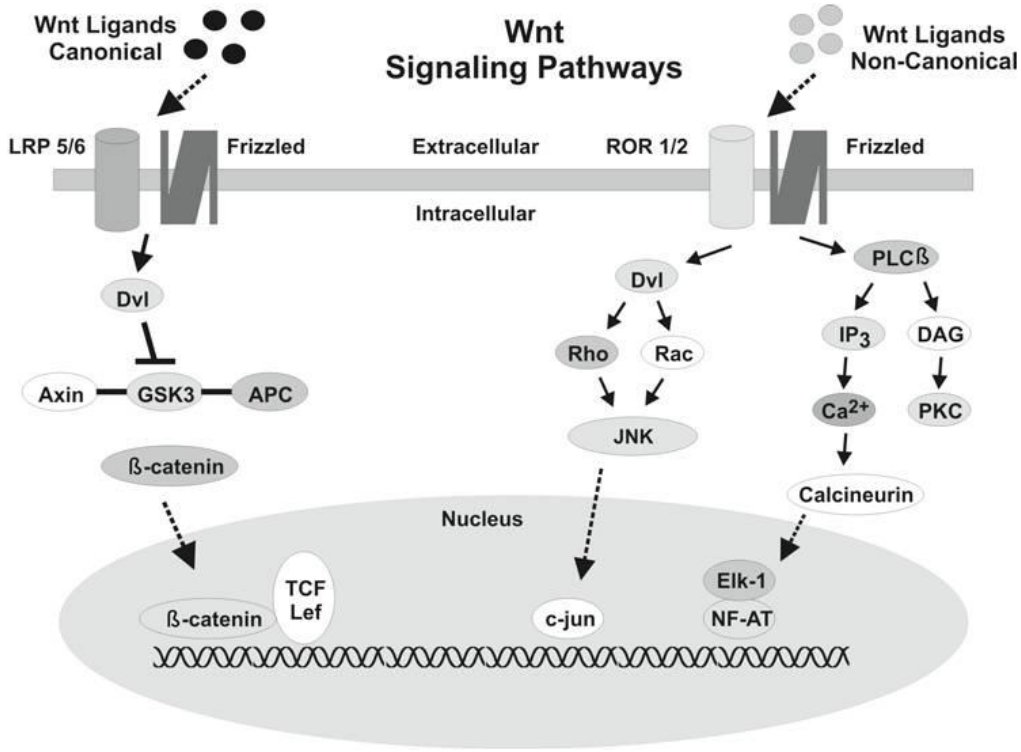


Figure 2. *Schematic representation of Wnt signaling pathways*. Wnt ligands can activate either the canonical pathway (through β-catenin) or the non-canonical pathway (through Ca++ signaling or Rac/Rho pathway) to achieve distinct biological functions. (font: Wagner E.R., Zhu G., Zhang B.Q., *et al.* (2011). The therapeutic potential of the Wnt signaling pathway in bone disorders. *Curr Mol Pharmacol*., 4(1):14-25).

Wnt signaling is tightly controlled by several groups of negative regulators that interfere acting either outside and within the cell. Extracellular inhibitors of the Wnt proteins include Wnt Inhibitory Factors (WIFs), Dickkopfs (Dkks), secreted frizzled-related proteins (SFRPs), Kremen1 and 2 (Krm 1/2), and Sclerostin (Sost). These regulatory molecules act by either binding the Frz (SFRPs) or LRP 5/6 (Sclerostin, Dkks, Krm) receptors to prevent Wnt association, or by directly binding the Wnt proteins (WIFs) (Piters *et al.,* 2008). Dkk 1 and Dkk 2 are secreted proteins that bind to the LRPs, leading to a cross-linking and internalization of receptor (Clevers., 2006). Intracellular inhibitors

include the GSK3β-Axin-APC complex and Chibby (Cby). Cby competes with Tcf/Lef-1 to block β-catenin signaling (Takemaru *et al.*, 2003). This nuclear control involves modifying the Tcf and Lef transcription factors in the Wnt signaling cascade (Cavallo *et al.*, 1998; Semenov *et al.*, 2005). The activity of Wnts is not only regulated by the components in signaling pathways, but also regulated by extracellular factors, specifically heparin sulfate proteoglycans. By binding to Wnt ligands at different affinity, heparin sulfates fine-tune the access of Wnts to Frz receptors and regulate various processes, like proliferation and differentiation of MSCs as well as progenitors in certain lineage such as osteoprogenitors (Manton *et al.*, 2007a; Cool and Nurcombe., 2006; Dombrowski *et al.*, 2009). The antagonist regulation of the Wnt cascade is critical in a variety of disease processes, and therefore, has tremendous potential in therapeutic regimens.

Wnt signaling in MSCs

Wnt signaling plays a vital role in the regulation of proliferation and differentiation of MSCs. Etheridge *et al.* demonstrated that MSCs express a number of Wnt ligands, including Wnt2, Wnt4, Wnt5a, Wnt11 and Wnt16 and several Wnt receptors, including FZD2, 3, 4, 5 and 6 as well as various co-receptor and Wnt inhibitors (Etheridge *et al.*, 2004). Studies have suggested canonical Wnt signaling keeps stem cells in a self-renewing and undifferentiated state. Exogenous application of Wnt3a to cell cultures expands the multipotential population of MSCs as well as human adipose-derived stem cells as a result of both increased self-renewal and decreased apoptosis. (Boland *et al.*, 2004; Cho *et al.*, 2006; Ling *et al.*, 2009). Moreover, the overexpression of *LRP5,* has been reported to increase proliferation of MSCs (Baksh *et al.*, 2007). Conversely, Wnt5a, by activating the non-canonical pathway, is able to suppress β-catenin/Tcf signaling, leading to a decline of both the level of cyclin D1 and the proliferation rate of MSCs (Baksh *et al.*, 2007; Baksh and Tuan, 2007). However, canonical Wnt signaling has also been reported to inhibit human MSCs proliferation in an autocrine or paracrine fashion (Qiu *et al.*, 2007). Supportively, Dkk1, is required for the arrested human MSCs to re-enter into cell cycle and subsequent proliferation through antagonizing canonical Wnt signaling (Gregory *et al.*,

2003). Interestingly, one study has revealed that canonical Wnt signaling stimulates human MSCs proliferation at low dose while inhibits it at high dose (De Boer *et al.*, 2004b). This dual effect of Wnt signaling suggests the intensity of Wnt signals can lead to different or even opposite biological functions. Furthermore, these controversial findings about the role of canonical Wnt signaling in MSCs self-renewal may also come from the difference in culture conditions and manipulation of the cells.

Recently, Quarto *et al.* (Quarto *et al.*, 2010) reported that Wnt3a has differential effects when using different *in vitro* models and an *in vivo* model of bone regeneration; the effects were dependent on the dose as well as the differentiation state of the recipient cell. When added to undifferentiated MSCs, Wnt3a inhibited osteogenic differentiation. By contrast, when added to calvarial osteoblasts, Wnt3a at high doses had an inhibitory effect in cells from juvenile mice but induced bone production in cells from adult animals, as assessed by alkaline phosphatase activity and Alizarin red mineralization assay. The defect repair was influenced once again both by Wnt3a dose and by the age of the animal, mimicking the *in vitro* results. These findings are in accordance with previous investigations (Kahler and Westendorf., 2003; Kahler *et al.*, 2006; Eijken et al., 2008; Kahler *et al.*, 2008), showing that the effect of canonical Wnt signaling on osteogenesis is influenced by the differentiation stage of target cells. Overall, the canonical Wnt signaling appears to stimulate the differentiation of MSCs committed to osteogenic lineage, while it inhibits the terminal differentiation of mature osteoblasts.

Wnt signaling in bone

The canonical and non-canonical Wnt signaling pathways play an important role in bone metabolism and osteogenesis, and contribute to osteogenic pathologies, as shown in both animal studies and genetic disorders in humans. The first indication that Wnt signaling play a critical role in bone formation came from human studies performed in the Wnt co-receptor LRP5.

Loss-of-function mutations in *LRP5* was found to associate with osteoporosis-pseudoglioma syndrome (OPPG), an autosomal recessive disorder, characterized by

blindess, low bone mineral density and skeletal fragility (Gong *et al.*, 2002). Similar results have been seen in *LRP5* knockout mice, where, a significant reduction in bone matrix deposition was observed, although expression of Runx2 and osteocalcin was not affected, suggesting that *LRP5* deletion affects osteoblast function not osteoblast differentiation (Kato *et al.*, 2002). In contrast, mutations in the N terminus of human LRP5 (e.g. G171V) that reduce affinity of LRP5 for Dkk1, was found to associate with high bone mass (Ai *et al.*, 2005; Boyden *et al.*, 2002). Also mice that overexpress the G171VLRP5 mutant in osteoblast have enhanced osteoblast activity, reduced osteoblast apoptosis, and a high bone mass phonotype reminiscent of that observed in human with this mutation (Babij *et al.*, 2003).

The role of LRP6 is analogous to that of LRP5 in initiating the Wnt signaling pathway. A mutation in the *LRP6* gene in humans leads to osteoporosis and other metabolic abnormalities (Mani *et al.*, 2007). In mice, a spontaneous missense mutation in the *LRP6* gene leads to defects in somitogenesis and reduced bone mass in adults (Kokubu *et al.*, 2004). Null mutations in the *LRP6* gene lead to prenatal lethality secondary to truncated distal limb and axial skeletal structures (Williams and Insdogna., 2009; Pinson *et al.*, 2000).

Although a few attentions have been paid to the Wnt ligands, there appears to be some associations of the extracellular Wnt agonists with metabolic abnormalities. Some Wnts including Wnt7b, Wnt10b, and Wnt5a are shown to function in bone homeostasis mice. Wnt7b is expressed in osteoblasts, and its expression is elevated along with osteoblast differentiation (Hu *et al.*, 2005; Li *et al.*, 2005). Removal of *Wnt7b* in skeletal progenitor cells using *Dermo1-Cre* mice results in defects in chondrogenesis and osteoblastogenesis (Tu *et al.*, 2007). Wnt10b promotes osteoblast differentiation by induction of the osteoblastogenic transcription factors Runx2, Dlx5 and Osterix and suppression of the adipogenic transcription factors CCAAT/enhancer-binding protein-a (C/EBPa) and peroxisome proliferator-activated receptor-c (PPARc) (Bennett *et al.*, 2005; Kang *et al.*, 2007). Overexpression of *Wnt10b* using the *osteocalcin* promoter in mice increases bone mass by stimulating osteoblastogenesis (Bennett *et al.*, 2007). *Wnt10b*$^{-/-}$ mice have

decreased trabecular bone with reduced bone formation rate (Bennett *et al.*, 2005; Bennett *et al.*, 2007). *Wnt5a*$^{+/-}$ mice have low bone mass with enhanced adipogenesis and reduced osteoblastogenesis in bone marrow mesenchymal progenitors (Takada *et al.*, 2007).

As an important mediator of the canonical Wnt signaling pathway, β-catenin is crucial in bone formation and regeneration (Krishnan *et al.*, 2006). Deletion of β-catenin in mesenchymal precursors of chondrocytes and osteoblasts, using *Dermo1-Cre* mice and *Prx1-Cre* mice, blocks osteoblast differentiation and causes ectopic formation of chondrocytes during both intramembranous and endochondral ossification. In the absence of β-catenin, the expression of collagen I, osterix, and osteocalcin is greatly diminished, suggesting that β-catenin is required for osteoblast differentiation in early stages (Hu *et al.*, 2005; Day *et al.*, 2005; Hill *et al.*, 2005). In mature osteoblasts, β-catenin plays another important function. Inactivation of *β-catenin* in osteoblasts using *a1 (I) collage-Cre* mice leads to low bone mass caused by increased bone resorption through decreased expression of *osteoprotegerin (Opg)* (Glass *et al.*, 2005). In addition, loss of *β-catenin* using *osteocalcin-Cre* mice severe osteopenia with increases in osteoclasts (Holmen *et al.*, 2005). *In vitro*, osteoblasts lacking *β-catenin* exhibited elevated expression of *Rankl* and diminished expression of *Opg*. These findings suggest that β-catenin in mature osteoblasts regulates osteoclastogenesis and osteoclast function.

Some extracellular proteins which function as antagonist of Wnt signaling, such as sclerostin, Dkks, SFRPs and axin are involved in the development of skeletal tissues. Sclerostin (Sost) is a protein secreted by osteocytes that binds to and blocks Wnt binding to co-receptors LRP5/6, leading to inhibiting osteogenic differentiation (ten Dijke *et al.*, 2008). During bone remodeling, osteocytes regulate bone formation by expressing sclerostin to complete a negative feedback cycle of Wnt signaling in mature osteoblasts (van Bezooijen *et al.*, 2005). Furthermore, mechanical stimulation of long bones reduces the expression of sclerostin and enhances bone formation, while mechanical unloading increases the sclerostin antagonistic effects and diminishes bone strength (Lin *et al.*, 2009; Moustafa *et al.*, 2009). In addition, sclerostin represents one of the best documented examples that offer a link between Wnt antagonists and specific pathologies. The

autosomal recessive diseases Sclerostosis and Van Buchen disease form secondary to homozygous mutations of *Sost* encoding the secreted protein sclerostin (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). These disorders are associated with a progressive increase in bone mass, hyperostotic skeleton and increased bone mineral density (Li *et al.*, 2008). Sclerostin is directly involved in the regulation of osteoblastic differentiation through the antagonism of the Wnt signaling pathway (Li *et al.*, 2008). Furthermore, specific polymorphisms of the *Sost* promoter have been implicated in osteoporosis (Huang et al., 2009).

Another group of extracellular regulators are the Dkk proteins, which play an important role in regulating bone matrix mineralization through the Wnt pathway. *Dkk1*$^{-/-}$ mice die because of failure of head induction (Mukhopadhyay *et al.*, 2001). *Dkk1*$^{-/-}$ mice are viable and exhibit an high bone mass (HBM) caused by an increase in bone formation without any changes in bone resorption (Morvan *et al.*, 2006). Mice overexpressing *Dkk1* in osteoblasts develop osteopenia because of reduced osteoblast number and bone formation (Li *et al.*, 2006). Unexpectedly, *Dkk2*$^{-/-}$ mice are osteopenic with increased osteoid and impaired mineralization. They display enhanced osteoclastogenesis with the upregulation of *Rankl* expression (Li *et al.*, 2005), indicating that Dkk2 affects both bone formation and bone resorption.

Although the antagonists to LRP 5/6 co-receptors are well established in their osteogenic regulatory roles, the Frizzled receptor antagonists play a key function in osteogenesis and osteogenic pathologies. Osteogenic abnormalities are associated with SFRPs, as increased incidence of hip osteoarthritis has been associated with alterations of the *Sfrp* gene (Loughlin *et al.*, 2004). *Sfrp1* knockout mice exhibit increased trabecular bone volume. The deletion of *Sfrp1* inhibits osteoblast lineage cell apoptosis *in vivo* and increases osteoclastogenesis *in vitro* (Bodine *et al.*, 2004). Furthermore, deficiencies in both sclerostin and Sfrp1 result in increased bone mass (Hartmann., 2006; Li *et al.*, 2005).

Also the Axin-GSK3β-APC complex has been shown to play a role in osteogenesis. *Axin* mutations lead to both osteogenic and chondrogenic abnormalities as familial tooth agenesis has been linked to *Axin2* mutations (Lammi *et al.*, 2004). Through the activation

of the β-catenin signaling pathway, *Axin2* knockout mice present with increased osteoblast proliferation, craniosynostosis, increased chondrocyte differentiation, reduced limb length, and increased bone mass phenotypes (Dao *et al.*, 2010; Yan *et al.*, 2009; Yu *et al.*, 2005). GSK3β also regulates osteogenesis, as GSK3β inhibitors, such as lithium, are able to increase bone density and enhance osteoblast differentiation (Clement-Lacroix *et al.*, 2005; Kulkami *et al.*, 2006). Mice heterozygous for *Gsk3*β alleles exhibit increased bone mass (Kugimiya *et al.*, 2007).

Cross-talk between Wnt signaling and other pathways

The differentiation of osteoblasts from the mesenchymal progenitors is a complex process, which remains to a great extent an unknown cascade of complex biological events, particularly at the interactions between various intracellular and extracellular molecular signaling pathways. Many signaling pathways are involved in osteoblast differentiation, such as the Wnt/β-catenin pathway, BMP/Smad pathway, Hedgehog pathway, and Notch pathway. Due to BMP's utmost importance in stimulating bone formation, the crosstalk between Wnt and BMP pathway during chondrogenic or osteogenic differentiation has received increasing attentions.

During osteogenic differentiation of multipotent cell lines, β-catenin and BMP-2 synergize to promote osteoblastic differentiation such induction of ALP activity (Mbalaviele *et al.*, 2005). In addition, BMP-2 enhances β-catenin-induced transcriptional activation of the *osteoprotegerin (OPG)* promoter, while the *OPG* gene promoter functionally interacts with β-catenin/Tcf-1 in cooperation with Smad1/4, and these complexes then cooperate to regulate graded expression of *OPG* (Sato *et al.*, 2009). Chen *et al.* recently demonstrated that in cultured osteoblasts that had been treated with BMP-2, several Wnt ligands (e.g., Wnt-7a, Wnt-10b) and their receptors (e.g., Fz-1, LRP-10) were upregulated at their mRNA level, together with an increased β-catenin-mediated Tcf-dependent transcription (Chen *et al.*, 2007b). Intramuscular implantation of BMP-2 in mice caused a highly expressed β-catenin and resulted in ectopic endochondral ossification. In another study, Fischer *et al.* demonstrated that BMP-2 treatment upregulates expression of *Wnt- 3a* and *β-*

catenin in C3H10T1/2 cells, whereas overexpression of *Wnt-3a* in this cell lines not only increases total and nuclear levels of both β-catenin and Lef-1, but also leads to enhanced BMP-2-mediated chondrogenesis, an early stage during endochondral ossification and fracture healing. Interestingly, in response to BMP-2, both nuclear levels of Smad-4 as well as the interaction between Smad-4 and β-catenin are enhanced upon BMP-2 treatment, thus providing a direct mechanism for a crosstalk between Wnt and BMP-2 pathway (Fischer *et al.*, 2002a).

Hedgehog (Hh) signal is another pathway that is crucial to embryonic skeletogenesis (Day and Yang., 2008). It has been shown that Wnt signaling controls Hh signal transduction through its regulation of Gli2 and Gli3, the key mediators to transduce Hh signals (Ogden *et al.*, 2004), and provides a synergistic effect on surface ectoderm/neural tube and notochord signaling in somite cell specification (Borycki *et al.*, 2000).

Notch pathway is also involved in embryonic skeletogenesis. Among Notch ligands genes, *JAG-1* gene is predicted as an evolutionarily conserved target of the canonical Wnt signaling pathway based on the conservation of double Tcf/Lef-binding sites within the 5' promoter region of mammalian *JAG-1* orthologues (Katoh., 2006). Additionally, when expressed in osteoblast cell line (MC3T3), the notch intracellular domain (NICD) impaired differentiation and blocked expression of Tcf/Lef-1 target promoter, thus resulting in inhibition of the canonical Wnt pathway (Sciaudone et al., 2003).

Transcriptional factors involved in bone differentiation

Osteoblast commitment, differentiation, and functional activity are all governed by several regulatory signalling pathway, as mentioned above, and specific transcription factors that promote expression of phenotypic genes and establishment of the osteoblast phenotype (see Figure 3). Regulatory factors that influence various aspects of osteoblast biology can be divided into five major categories (Javed et al., 2010). These include (1) cis-acting DNA binding transcription factors such as Runx2, Osterix, Sox9, Lef/Tcf transcription factors, C/EBPs, ATF4, NFATc, AP-1, Dlx5, Smads and Twist (Javed *et al.*, 2010; Javed

et al., 2008; Yang *et al.*, 2004) ; (2) signal transducers and non-DNA binding coregulatory proteins, such as Satb2, Cbfβ, Stat1 and TLE (Kundu *et al.*, 2002; Dobreva *et al.*, 2006; Javed *et al.*, 2000; Kim *et al.*, 2003); (3) posttranslational regulatory proteins, such as Shn3, WWP1, Smurf1, CHIP and Schnurri-3 (Jones *et al.*, 2006, Li *et al.*, 2008); (4) chromatin-modifying enzymes, such as p300 acetyltransferase and histone deacetylase (Vega *et al.*, 2004; Lian *et al.*, 2004); (5) chemokines such as such as MCP-4/CCL13, PARC/CCL18, Mig/CXCL9, SDF-1/CXCL12, and MCP-1/CCL2 (Iwamoto *et al.*, 2008). Only some of the osteoblastic regulatory factors just mentioned, will be taken into account in this thesis.

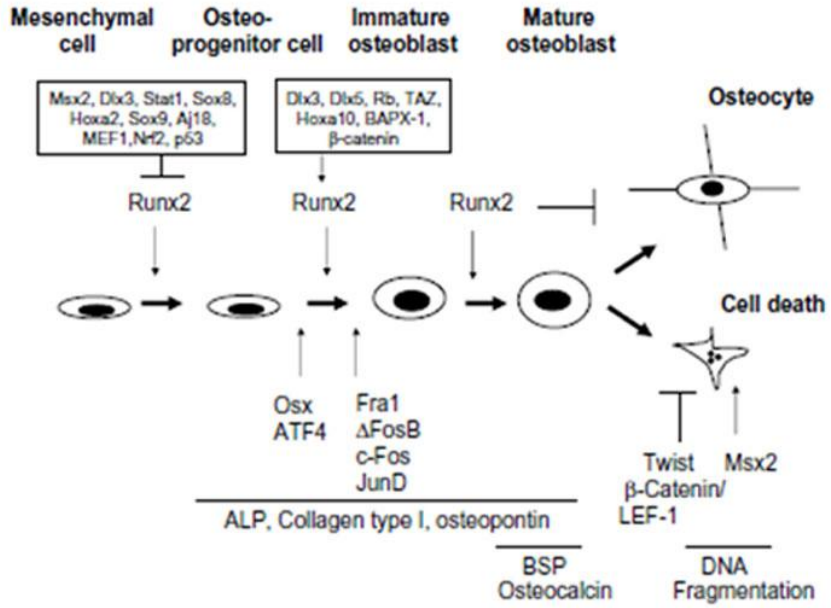


Figure 3. *Control of osteoblast differentiation by transcription factors*. Osteoblast differentiation starts with the commitment of osteoprogenitor cells from mesenchymal cells, their differentiation into immature and more mature functional osteoblasts expressing osteoblast phenotypic genes, and ends by becoming osteocytes within the bone matrix, or cell death for a fraction of osteoblasts. The indicated transcription factors control osteoblastogenesis in a time and space coordinated way. Transcription factors themselves are tightly regulated by the indicated multiple proteins acting positively or negatively on their expression or transcriptional activity to modulate osteoblast gene expression. (font: Marie P.J. (2008). Transcription factor controlling osteoblastogenesis. *Archives of Biochemistry and Biophysics* 473:98–105).

Runx2

Runx2 (CBFA1, AML3), an essential factor for bone and hypertrophic cartilage formation, is a member of the Runt family of transcription factors that is expressed by mesenchymal cells at the onset of skeletal development and is present in osteoblasts throughout their differentiation. Although Runx2 is the most abundant factor in mature osteoblasts, Runx2 and Runx3 are also present in osteoblast lineage cells (Lian *et al.*, 2004). Runx2 was found to control bone formation during both skeletal development and post-natal life (Karsenty and Wagner., 2002). Molecular studies and genetic manipulation of Runx2 *in vivo* indicated that the expression of Runx2 is both necessary and sufficient for mesenchymal cell differentiation towards the osteoblast lineage (Karsenty and Wagner., 2002; Komori., 2006). Runx2 invalidation inhibits the differentiation of mesenchymal cells into osteoblasts (Ducy *et al.*, 1997; Komori *et al.*, 1997). Consistently, *Runx2* haploinsufficiency in humans results in cleidocranial dysplasia, a disease characterized by defective bone formation (Lee *et al.*, 1997; Otto *et al.*, 1997).

As a member of the Runx family of transcription factors, Runx2 operates in bone lineage cells by binding to the Runx consensus sequence (PuACCPuCA), first named the osteoblast specific element (OSE2). The Runx regulatory element can be found in the promoter of all major osteoblast genes controlling their expression, including *type I collagen alpha 1 chain*, *osteopontin*, *bone sialoprotein* and *osteocalcin*, resulting in the establishment of an osteoblast phenotype. In addition to control osteoblast differentiation, Runx2 was found to negatively control osteoblast proliferation by acting on the cell cycle (Pratap *et al.*, 2003). Recent studies indicate that Runx2 interacts with several regulatory proteins within the nuclear architecture, resulting in activation or repression of genes which control the program of osteoblast proliferation and differentiation (Stein *et al.*, 2004). Despite its important role in osteoblast commitment, Runx2 is not essential for the maintenance of the expression of the major bone matrix protein genes in mature osteoblasts (Maruyama *et al.*, 2007). Mice overexpressing Runx2 exhibit osteopenia, as a result of reduced number of mature osteoblasts, indicating that Runx2 negatively controls osteoblast terminal differentiation and maintain osteoblastic cells in an immature stage

(Liu *et al.*, 2001). Thus, Runx2 can act differently at multiple levels to control osteoblast differentiation and bone formation. As expected from the important role of Runx2 in osteoblastogenesis, both expression and activity of Runx2 are tightly controlled by transcription factors, protein–DNA or protein–protein interactions. Notably, Runx2 is controlled by Hoax2, a member of the Hox homeodomain family of transcription factors that regulate skeletal patterning, inhibiting *Runx2* expression and thereby bone formation (Dobreva *et al.*, 2006). SATB2, a nuclear matrix protein, represses *Hoxa2* expression and thereby activate Runx2-dependent osteoblast differentiation (Dobreva *et al.*, 2006). Other complex mechanisms can negatively regulate Runx2 expression. The transcription factors Stat1 and Sox9 interact with Runx2 and blunt its transcriptional activity (Kim *et al.*, 2003; Zhou *et al.*, 2006). In particular, Stat1 exerts its inhibitory effect mainly, if not exclusively, in the cytoplasm. In addition, it has been demonstrated that the loss of inhibition of Runx2 activity by Stat1 results in increased bone mass in the adult *Stat1*$^{-/-}$ mice without affecting bone formation during the developmental period, suggesting that Stat1 is selectively involved in the Runx2 regulation in bone remodeling at the postnatal stage (Kim *et al.*, 2003). Runx2 activity is also positively controlled by transcriptional activators such as Rb, TAZ, HOXA10 or BAPX-1 (Tribioli and Lufkin., 1999; Hassan *et al.*, 2007; Luan *et al.*, 2007; Hong *et al.*, 2005). It can be phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway by binding of type I collagen to alpha2beta1 integrins on the osteoblast surface (Xiao *et al.*, 2000). CBFb1 is the most important co-activator of Runx2 which is required for Runx2-dependent bone formation (Miller *et al.*, 2002; Kanatani *et al.*, 2006). Runx2 directly interacts with estrogen receptor α (ERα) in an estrogen-dependent manner, and the interaction enhances Runx2 activity (McCarthy *et al.*, 2003); but Runx2 seems to suppress *Esr1/ERα* promoter activity in osteoblasts (Lambertini *et al.*, 2007). Another important regulator of Runx2 activity is its interaction with signal transducers of transforming growth factor beta superfamily receptors, Smads. Runx2 functions synergistically with Smad1 and Smad5 to regulate bone-specific genes (Lee *et al.*, 2000; Zhang *et al.*, 2000). The importance of the Runx2-Smad complex in driving osteoblastogenesis has been demonstrated both *in vitro* and *in vivo* (Phimphilai *et al.*,

2006; Javed *et al.*, 2008). In addition to these important mechanisms controlling Runx2, other transcriptional coactivators of Runx2, such as C/EBPb, C/EBPd and Menin have been identified (Westendorf., 2006). Interestingly, recent studies showed that Runx2 interacts with factors with histone acetyl transferase activity such as p300, allowing a tight control of transcriptional activity by a balance between acetylation and deacetylation of the chromatin (Westendorf., 2006). Finally, Runx2 regulation also occurs at the level of protein stability. Smurf1 and Schnurri-3 interacts with the Runx2 protein promoting its degradation (Zhao *et al.*, 2004; Jones *et al.*, 2006).

Lef/Tcf transcription factors

Human and mouse Lef1/Tcf family members are encoded by four genes: *Tcf1 (Tcf7), Lef1 (Tcf1a), Tcf3 (Tcf7L1), and Tcf4 (Tcf7L2).* As mentioned above, Lef1/Tcf transcription factors are nuclear high mobility group (HMG) proteins that mediates gene transcription in response to canonical Wnt/β-catenin signalling pathway (Westendorf *et al.*, 2004; Barker., 2008; Leucht *et al.*, 2008). These factors are involved in many processes occurring both in embryonic and adult tissues. In particular, experiments performed in mutant mice have allowed to demonstrate the involvement of these factors in the regulation of skeletal development and bone homeostasis. *Tcf1* null mice exhibit a low bone mass with an increased in *Osteoprotegerin* expression and in osteoclast number, while osteoblast number and function are not altered. These findings are similar to those in osteoblast-specific *β-catenin*-deficient mice (Glass *et al.*, 2005). *Lef1*-deficient mice are smaller than wild type mice and die shortly after birth (van Genderen et al., 1994). A mouse model expressing a mutant form of *Lef1* lacking the HMG domain (Lef1-βgal) displays several skeletal abnormalities, mostly associated with skeletal patterning (Galceran *et al.*, 2004). In addition, an age- and gender-dependent role for Lef1 in regulating bone mass phenotype and turnover *in vivo*, has recently been described (Noh *et al.*, 2009).

Controlling the development and homeostasis of bone tissue, the Wnt signaling pathway, and in particular their effectors Lef1/Tcf proteins, directly interact with other proteins involved in the regulation of osteoblastogenesis. It was demonstrated that osteoblast-like

cell lines treated with differentiation-inducing factor-1 (DIF-1), show a significant reduction of *alkaline phosphatase (ALP)* reporter gene activity through a specific Lef/Tcf binding site, suggesting that Wnt/β-catenin signaling affects *ALP* expression (Matsuzaki *et al.*, 2006). Furthermore Lef1 is reported to up-regulate the expression of *Col11a1* (Kahler *et al.*, 2008) and *Runx2* (Gaur *et al.*, 2005). On the contrary, Kahler and Westendorf found that Lef1 binding to Runx2 through the Lef1 HMG domain, represses Runx2-induced transcription of the *osteocalcin* promoter. (Kahler and Westendorf, 2003). Lef1, suppressing the activity of *Runx2*, prevents the expression of this gene in the early stages of osteoblast maturation. In addition, it has been showed that osteoblasts with reduced Lef1 expression mineralize at an accelerated rate while osteoblasts over expressing Lef1 do not mineralize (Kahler *et al.*, 2006).

Sox9

Sox9 is a member of the *Sox* gene family characterized by high–mobility-group-box DNA binding motif related to that of the sex determining factor SRY. Normally, Sox9 is expressed predominantly in mesesnchymal progenitor cells and in proliferating chondrocytes, but not in hypertrophic chondrocytes and osteoblasts (Zhao *et al.*, 1997). The malfunction of Sox9 results in campomelic dysplasia (CMD1), which is a semilethal osteochondrodysplasia, characterized by skeletal anomalies that include bending of the long bones and XY sex reversal (Sock *et al.*, 2003; Giordano *et al.*, 2001). Sox9 is a potent transcriptional activator for chondrocyte-specific genes such as *Collagen2α1 (Col2α1)* (Lefebvre *et al.,* 1997), *Col1α1* (Bridgewater *et al.*, 1998), or *aggrecan* (Sekiya *et al.*, 2000) genes by binding to their enhancer sequences. Nevertheless, it requires the activity of two other Sox family members, L-Sox5, the large Sox5 isoform and Sox6 in order to transform cells into early chondrocytes the so-called chondroblasts (Hartmann., 2009). Sox5 and Sox6 are present after cell condensation (Smits *et al.*, 2001; Akiyama *et al.,* 2002), are highly related, coexpressed with and regulated by Sox9, but belong to a different Sox family subgroup (Lefebvre and Smits., 2005). In addition to its well established role as transcriptional activator for chondrogenesis, Sox9 also acts as a

transcriptional repressor for osteoblast differentiation and chondrocyte hypertrophy in part via inhibition of Runx2 transactivation of its target genes. (Zhou *et al.*, 2006). The precise mechanisms involved in this process are unclear; however, some evidences have shown that Sox9 promoted the degradation of Runx2, directing it toward lysosomal degradation (Zhou *et al.*, 2006). Sox9 can repress the expression of *Runx2* in chondrocytes also indirectly, through the transcription factor Nkx3.2/Baxp1 (Yamashita *et al.*, 2009). This indicates that Sox9 regulated Runx2 through a variety of mechanisms both transcriptionally and posttranscriptionally. Moreover, Eames *et al*, demonstrated by retroviral injection experiments in chicken embryos that *Sox9* misexpression repressed Runx2 function and diverted cell fate from bone to cartilage in the craniofacial region (Eames *et al.*, 2004). In a *Sox9* knockin mouse model in which *Sox9* was overexpressed in proliferating chondrocytes during endochondral ossification, osteoblast differentiation was also delayed (Akiyama *et al.*, 2004). Interestingly, mouse genetic studies showed that *Sox9*-expressing precursor cells could eventually differentiate into tendons and osteoblasts (Akiyama *et al.*, 2005). Cheng and Genever, found that Runx2 inhibited the transactivity of Sox9, which is consistent with the findings that Sox9 is expressed in mature osteoblasts (Akiyama *et al.*, 2005) and provides a mechanism whereby the expression level of Sox9 may be maintained but its transactivity repressed by Runx2 during the process of osteogenic differentiation in MSCs (Cheng and Genever., 2010).

CXCL12

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a member of the CXC chemokine family (Shirozu *et al.*, 1995; Zlotnik and Yoshie., 2000), wirh several isoforms that vary in the number of amino acid extensions at the carboxyl (C) terminus (Yu *et al.*, 2006). CXCL12 signals through its G-protein-coupled transmembrane receptor CXC chemokine receptor-4 (CXCR4) (D'Apuzzo *et al.*, 1997; Zlotnik and Yoshie., 2000); and a second receptor, CXCR7, was identified in several types of cells (Hartmann *et al.*, 2008; Levoye *et al.*, 2009). CXCL12 is constitutively expressed in various cell types (Baggiolini *et al.*, 1997), including immature osteoblasts lining the bone endosteum, MSCs and

endothelial cells (Ponomaryov *et al.*, 2000; Lisignoli *et al.*, 2006). Accumulated evidence suggests that constitutive and induced expression of CXCL12 is tightly regulated in different cells. Nevertheless, the molecular mechanisms involved in directing the correct levels of this chemokine remain unclear. Recently, a functional characterization of the *CXCL12* gene promoter has been performed (Garcia-Moruja *et al.*, 2005; Calonge *et al.*, 2010), and potential binding sites for transcriptional regulators including Sp1, AP2, HNF-3, NF-X3, glucocorticoid receptor, NFAT, c-myb, c/EPBβ and bHLH have been identified. CXCL12/CXCR4 signaling plays pleiotropic functions in organogenesis, developmental or pathologic angiogenesis, hematopoietic myeloid and lymphoid cell homing and differentiation, and in tumorigenesis process (Rossi and Zlotnik., 2000; Kim *et al.*, 2007; Chen *et al.*, 2008). The important role of CXCL12/CXCR4 signaling in tumorigenesis was recently confirmed in pancreatic cancer, in which the abrogation of CXCR4 inhibited invasion-related genes and the invasive ability of cancer cells (Wang *et al.*, 2008). Moreover this CXCL12/CXCR4 orientated chemotaxis regulates the homing into and retention of hematopoietic stem cells and MSCs within marrow microenvironments, as well as the metastatic colonization of bone and bone marrow by breast and prostate cancer cells (Lataillade *et al.*, 2000; Majka *et al.*, 2000). It has been highlighted that CXCL12/CXCR4 signaling finely modulates craniofacial development and both their knockdown and over-expression result in cranial neural crest cell migration defects and ectopic craniofacial cartilage formation (Olesnicky Killian *et al.*, 2009). Concerning bone tissue, it has recently been suggested that this signaling is involved in the remodeling process (Ponomaryov *et al.*, 2000; Dominici *et al.*, 2009; Otsuru *et al.*, 2008), has a critical role in the recruitment of MSCs to the fracture site during skeletal repair (Kitaori *et al.*, 2009), and is highly expressed and secreted by regenerating/proliferating osteoblasts after irradiation in mouse models (Dominici et al., 2009). Furthermore, CXCL12 has also been found to have a regulatory role in the osteogenic differentiation of murine MSCs. Zhu *et al*, (Zhu *et al.*, 2007) reported that blocking of the CXCL12 signaling inhibited the progression of C2C12 cells towards osteoblastic cells in response to BMP-2 stimulation. Hosogane and co-workers (Hosogane *et al.,* 2010) demonstrated that the perturbation of

CXCL12/CXCR4 signal in primary MSCs derived from human and mouse bone marrow, significantly reduced *Runx2* and *Osterix* expression, affecting the differentiation of MSCs towards osteoblastic cells in response to BMP-2 stimulation. Finally, Kitaori *et al*, (Kitaori *et al*., 2009) using mouse segmental bone graft models, found that CXCL12 recruits MSCs to bone repair sites during the early phase of bone endochondral repair, suggesting the importance that this chemokine may have to promote successful bone healing.

As can be deduced from the evidences reported above, bone tissue homeostasis depends on the concerted actions of a plethora of signals that control osteoblast commitment, differentiation, and functions. Therefore, a better understanding of molecular mechanisms behind osteogenic differentiation and bone remodeling may help to identify the role of a specific transcription factor in mediating these processes and consequently, may help to discover new molecular targets to use in bone repair regenerative medicine and in the treatment of bone and skeletal diseases.

AIM

In recent years much attention has been paid to strategies enhancing bone regeneration in severe osteogenic abnormalities, such as critical sized defects, non-union fractures, and vertebral interventions, as well as in many pathological conditions (e.g. osteoporosis, osteoarthritis, and cancer metastases to the bone). The regeneration of bone is a multidisciplinary approach consisting of stem and progenitors cells, various types of scaffolds and different signaling pathways involved in the commitment of mesenchymal stem cells towards osteogenic lineage. These signals, that conveys in hormones, growth factors and transcription factors, are crucial for osteoblast differentiation, proliferation, functions and, consequently, ensure the proper bone modelling and remodeling.

Among these factors, Wnt proteins have a critical role in bone development and homeostasis.

Most downstream bone-specific target genes of this pathway are only partially known. Among these, Slug has been recently implicated in osteosarcoma progression as a Wnt-responsive molecule strongly correlated with a loss of tumor suppressors such as E-cadherin (Nieto., 2002; Conacci-Sorrell., 2003). To date, for what concerns bone tissue, Slug is considered exclusively a marker of malignancy, and there are no data about its expression and regulation in human normal osteoblasts.

In order to identify new potential osteoblast-specific proteins, the aim of this thesis is addressed to explore a possible function of Slug in normal bone tissue by:

- analyse Slug expression in human normal osteoblasts (hOBs) and in their mesenchymal precursors (hMSCs), obtained from bone marrow iliac crest, bone marrow tibial plateau and Wharton's jelly umbilical cord. Despite the difficulties of experimental set up, indeed, the use of a model obtained from human primary cells,

represents the chosen system for a detailed study of processes related to bone tissue, as it allows to mimic the physiological conditions that occur *in vivo*;

- analyse Slug gene regulation in hOBs in relation to Wnt/β-catenin signaling mediators and osteoblast regulators such as Runx2 transcription factor;
- evaluate the role of Slug in hMSCs and hOBs in relation to Wnt/β-catenin signaling mediators and genes involved in the control of osteoprogenitors differentiation;
- use experimental targeted strategies, such as siRNA approach or chemical compound (e.g. SB216763) administration, to identify the role of specific transcription factors, by modifying their expression levels.

On the whole this study aims to investigate the role of Slug in the context of bone tissue, in order to identify a new potential therapeutic target for bone tissue repair and regeneration.
In addition, and more generally, the work of this thesis aims to underline the concept that the appropriate manipulation of gene expression might affect cell behaviour leading to significant improvement in the efficiency of tissue engineering and enhancing the therapeutic value of stem cells/osteoprogenitors for the restoration of bone defects.

MATERIALS AND METHODS

Cell cultures

Human cell lines

Osteosarcoma cell line SaOS-2, osteoblast-like cells CAL72 and Hobit, and breast cancer cell lines MCF-7 and MDA-MB-231 were maintained in Eagle's MEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (CELBIO EuroClone, Milan, Italy), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml ascorbate at 37°C in a humidified atmosphere of 5% CO2.

Human osteoblasts (hOBs)

Craniofacial osteoblasts

Human craniofacial osteoblast cells were obtained from bone samples collected during nasal septum surgery. Recruitment of subjects donating osteoblasts was in accordance with approved procedures, and informed consent was obtained from each patient. Bone samples were collected from patients that underwent septoplasty surgery, FESS or both procedures from June 2006 to February 2010 at the ENT Department of the University Hospital of Ferrara. This type of surgery consists in the exeresis of the lower part of the osseous nasal septum (this portion has been used in this study). This sampling is very handily and costless.

Briefly, the bone samples, about 1 cm^2 in size, were cut into small fragments that were washed several times in phosphate-buffered saline (PBS) (136 mM NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4) to remove blood cells and debris with a final wash in culture medium. Fragments were then collected in culture flasks containing 6 ml of 1:1 mixture of DMEM/Ham's F12 (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS) (CELBIO EuroClone, Milan, Italy), 2 mM glutamine, 100 units/ml

penicillin, 100 mg/ml streptomycin, and 50 mg/ml ascorbate at 37°C in a humidified atmosphere of 5% CO2. After about 5-7 days, outgrowth of bone cells from the bone chips commenced, and confluency in 25 cm^2 dishes was usually reached after 3-4 weeks. For the data here presented, only first and second passage cells were used.

Tibial plateau osteoblasts

Human osteoblasts were obtained from trabecular bone located in the inner portion of the tibial plateau of patients undergoing total knee replacement. The study was approved by the Istituto Ortopedico Rizzoli (Bologna, Italy) ethical committee and informed consent was obtained from each patient. Briefly, bone chips were removed from the tibial plateau, collected in a V-glass tube containing 1.5 ml of 1:1 mixture of DMEM/Ham's F12K no calcium (Gibco, Invitrogen Corporation, Paisley, Scotland, UK) and supplemented with 15% FBS, antibiotics (100 U/ml penicillin and 100μg/ml streptomycin), 25 μg/ml ascorbic acid, 4mM glutamine (Sigma-Aldrich, St Louis, MO, USA), and 2 mM calcium chloride (referred to as enzyme medium) according to the methods previously described by Robey and Termine (Robey and Termine., 1985).

Human mesenchymal stem cells (MSCs)

Tibial plateau MSCs

Human tibial plateau mesenchymal stem cells (hTP-MSCs) were isolated from bone marrow tibial plateau aspirates using Ficoll-Hypaque density gradient (d=1.077 g/ml) (Sigma, St Louis, MO, USA). Briefly, nucleated cells were collected at the interface, washed twice, suspended in α-MEM supplemented with 15% FBS and penicillin G (Sigma), counted and plated at a concentration of $2X10^6$ cells/T150 flask. After 48h non-adherent cells were removed and the adherent MSCs expanded *in vitro*.

Iliac crest MSCs: Human bone-marrow mesenchymal stem cells were isolated from bone-marrow aspirates of the iliac crest (hIC-MSCs) and cultured as described for hTP-MSCs.

Wharton's jelly MSCs: Human Wharton's jelly mesenchymal stem cells (hWJ-MSCs) were isolated from Wharton's jelly of human umbilical cords collected from full-term births, after mother's consent and approval of the "Ethical commitee of University of Ferrara and S.Anna Hospital", as previously described (Penolazzi *et al.*, 2010). Harvesting procedures of Wharton's jelly from human umbilical cords were conducted in full accordance with the "Declaration of Helsinki" as adopted by the 18th World Medical Assembly in 1964 successively revised in Edinburgh (2000) and the Good Clinical Practice guidelines.

Cords were processed within 4 hours, and until that moment had been stored at 4°C in sterile saline. Typically, the cord was rinsed several times with sterile PBS before processing and was cut into pieces, 2-4 cm in length. Blood and clots were drained from vessels with PBS, to avoid any contamination. Single pieces were dissected, first separating the epithelium of each section along its length, to expose the underlying Wharton's jelly. Later cord vessels (the two arteries and the vein) were pulled away without opening them. The soft gel tissue was then finely chopped. The same tissue (2-3 mm^2 pieces) were place directly into 25-cm^2 flask culture expansion in DMEM low glucose media (Euroclone S.p.a., Milan, Italy), supplemented with 10% FCS, penicillin 100 mg/ml and streptomycin 10 mg/ml at 37°C in a humidified atmosphere of 5% CO_2. After 5-7 days, the culture medium was removed and changed twice a week.

Human chondrocytes

Human primary chondrocytes were obtained from patients with osteoarthritis undergoing knee arthroplasty. The chondrocytes were isolated from minced tissue by sequential

enzymatic digestion with 0.25% trypsin (Biochrom KG, Seromeds, Berlin, Germany) at 37°C for 15 min and with 300 U/ml collagenase II (Worthington, Lakewood, NJ, USA) at 37°C for 4 h in Hams's F12 (Biochrom KG). Digested material was centrifuged at 1000 rpm for 10 min, and pellets were resuspended and expanded in monolayer cultures in complete medium: Hams's F12 supplemented with 10% fetal calf serum (FCS), 1% pennicillin–streptomycin, 1% L-glutamine and 50 mg/ml L-ascorbic acid (Sigma, St. Louis, MO, USA).

Flow cytometric analysis

hMSCs and hOBs (at passage 2) were fixed in 4% paraformaldehyde. hMSCs were then incubated at 4°C for 30 min with 5µg/ml of the following monoclonal antibodies: anti-human-CD3, -CD34, -CD45, (DAKO Cytomation, Glostrup, Denmark), -CD31 (Chemicon International, temecula, CA), -CD73, -CD90, -CD146 (Becton Dickinson, Mountaine View, CA), -CD105 (produced from the hybridoma cell line, clone SH2, ATCC, Rochville, MD). Instead, hOBs were incubated at 4°C for 30 min with 5µg/ml of monoclonal antibodies anti human -CD45, -STRO-1 (DakoCytomation; Glostrup, Denmark), and –CD90 (Becton Dickinson, Mountaine View, CA). The cells were washed twice and incubated with 2.4 µg/ml of a polyclonal rabbit anti-mouse immunoglobulins/FITC conjugate or with 8 µg/ml of a polyclonal rabbit anti-rat immunoglobulins/FITC conjugate or with 1µg/ml of a polyclonal swine anti-rabbit immunoglobulins/FITC conjugate (DAKO Cytomation) at 4°C for 30 min. After two final washes, the cells were analysed using a FACStar plus Cytometer (Becton Dickinson). For isotype control, FITC-coupled non-specific mouse IgG was substituted for the primary antibody.

Viability analysis (Calcein-AM uptake assay) of MSCs populations

The viability of the cells was analyzed by double staining with propidium iodide (PI) and Calcein-AM according to the manufacturer's instructions and as previously described (Penolazzi *et al.*, 2010). For propidium iodide and calcein analysis the cells were visualized under a fluorescence microscope (Nikon, Optiphot-2, Nikon corporation, Japan) using the filter block for fluorescein. Dead cells were stained in red, while viable ones appeared in green.

Analysis of the osteoblast phenotype

Alkaline phosphatase staining and activity

For alkaline phosphatase staining, the Alkaline phosphatase (ALP) Leukocyte kit (Sigma) was used, as previously described (Penolazzi *et al.*, 2008). To perform the test, prefixed mono-layered cells were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (AMPD). The presence of sites of ALP activity appeared as blue cytoplasmatic staining.

ALP activity was evaluated in hOBs and hMSCs by the hydrolysis of p-nitrophenylphosphate (PNPP), as indicated by Ibbotson *et al.* (Ibbotson *et al.*, 1986). The cells were washed in cold PBS 1X pH 7.4, were mechanically detached and centrifuged at 1200 rpm for 30 min at room temperature. The pellet was then resuspended in 500 µl of Triton X-100 (0.1%) and subjected to rapid freezing and thawing in liquid nitrogen for three times, in order to facilitate the breakdown of cell membranes; the lysate was then recovered after a quick spin at 1200 rpm. Different amounts of lysate (25, 50 and 100 µl) are added to a fixed quantity (100 µl) of substrate represented by 10 mM p-nitrophenylphosphate solution in a diethanolamine buffer 100 nM (pH 10.5, 0.5 mM MgCl2). After 30 min at 37 ° C, the reaction was stopped with 100 µl of 0.2 M NaOH each well; the enzyme activity was expressed as µmol of substrate hydrolyzed for time unit

(minutes) for mg cellular protein. The total quantity of protein was determined by the "Bradford method" (Bradford Reagent for Protein Determination, Sigma) The enzyme activity, expressed as μmol/min/μg of protein, was evaluated 6 days after siRNA/Slug treatment in hOBs and in MSCs before and after osteogenic induction.

Osteogenic induction

Osteogenesis of hMSCs and hOBs was induced 24 h after seeding (in 12 well), by incubating hMSCs in α-MEM medium supplemented with 10% FBS, 100 μM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (all purchased from Sigma) and by incubating hOBs in DMEM high-glucose (Euroclone S.p.a., Milan, Italy), supplemented with 10% FBS, 100 μM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone for 3/4 weeks. In the committed cells, the osteogenic medium was changed every three days. The cells were analysed after 24h (day 0) and at days 7, 14, 21 and 28.

Mineralization assay

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma). The cells were then fixed in 70% ethanol for 15 min at room temperature, washed with deionized water, stained with 40 mM ARS (pH 4.2) for 20 min at room temperature, and washed five times with deionized water to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope. Concerning osteogenic induction of hOBs, matrix mineralization was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer (''Quantity one'' software, Biorad). The surface and the number of all mineralized nodules were quantified in 2 wells per condition at day 14 and 21 of culture. With regard to hMSCs osteogenesis, mineralized matrix positive to Alizarin Red S was quantified analyzing 10

different RGB images, that were acquired under white transmitted light from each well by a linear (gamma=1) CCD camera mounted on an inverted microscope (DS Camera Control Unit DS-L2, Nikon Instruments Europe BV, Amstelveen, Netherlands), using an objective at 10X magnification and maintaining the sampling area fixed. Photometric analysis were conducted on the monochrome red channel of the RGB stack. Images were calibrated by adjusting the brightest gray level to 255 and then analyzed using the public domain ImageJ software (rsbweb.nih.gov/ij). The data were expressed as integrated optical density (O.D.).

Immunocytochemistry

A sample of 10^4 human osteoblasts obtained from tibial plateau (at passage 2) were seeded in 8-well chamber slide and allowed to adhere for 96 h. Human osteoblasts were fixed in 4% PFA for 20 min at room temperature and then hydrated with TBS 1% BSA for 5 min at room temperature. The slides were incubated with monoclonal antibodies anti-human -CD146 (Nocastra, Newcastle, UK), -CD105 (produced from the hybridoma cell line, clone SH2; ATCC, Rockville, MD), -Runx-2, -osteocalcin (OC), anti-human-CXCL12 (all purchased from R&D Systems, Minneapolis, MN), -alkaline phosphatase (ALP), -collagen type 1 (Col1a1) (both obtained from DSHB, Department of Biological Sciences, Iowa city, IA), -bone sialoprotein (BSP), (Fisher Scientific,Pittsburg, PA, USA), and -estrogen receptor alpha (ERα) (Upstate Biotechnology, Lake Placid, NY), for 1 h at room temperature. The slides were washed three times with TBS 1% BSA and then sequentially incubated with multilinker biotinilated secondary antibody and alkaline phosphatase-conjugated streptavidin (Kit BioGenex, San Ramon, CA, USA) at room temperature for 20 min. The slides were developed using fast red as substrate, counterstained with haematoxylin, mounted with glycerol jelly, and evaluated in a brightfield microscope. Negative and isotype matched controls were performed. Positive cells were manually counted by two evaluators blinded to marker evaluated. For each well, we randomly

selected 20 fields at high magnification (X40). Results were expressed as the percentage of positive cells on the total number of cells counted.

For immunocytochemical analysis of human craniofacial osteoblasts and osteogenic - induced hWJMSCs (at days 0, 7, 14, 21 and 28) the cells were fixed in 4% paraformaldheide (PFA) for 20 min at room temperature, washed twice in PBS, treated with 3% H_2O_2 (in PBS) and incubated in 2% normal horse serum (S-2000, Vector labs, CA, USA) for 15 min at room temperature. After the incubation in blocking serum, the slides were incubated with monoclonal antibodies anti-human-Slug and –Runx2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1hour. The slides were washed three times with PBS 1X and then were visualized under a brightfield microscope fluorescence.

Construction of reporter plasmid

Promoter region (+1 to -982 bp) of the human Slug promoter was amplified by PCR from human genomic DNA using Slug F genomic primer (5'-gaagatctTGTCAAAAGTGTGAGAGAAT-3') as sense primer and Slug R genomic primer (5'-cgacgcgtCTTGCCAGCGGGTCTGGC-3') as antisense primer (in lower font are shown the recognition sequences for MluI and BglII restriction enzymes). The PCR product was subcloned upstream of a firefly luciferase (LUC) gene in the promoter-less pGL3-Basic vector (Promega, Madison, WI) using MluI and BglII restriction sites, and the presence of the insert was confirmed by restriction digestion.

Plasmids and transient transfection

The expression vector for full-length Lef-1 (K14-myc-hLEF1) was a gift from Elaine Fuchs and Rebecca C. Lancefield (Howard Hughes Medical Institute, The Rockfeller University, Lab. of Mammalian Cell Biology & Development, New York U.S.A.).

The TCF reporter plasmid TOP FLASH was kindly provided by Rolf Kemler (Max Planck Institute, Heidelberg, Germany). TCF reporter plasmid containing two sets (with the second set in the reverse orientation) of three copies of the TCF binding site upstream of the Thymidine Kinase (TK) minimal promoter and Luciferase open reading frame.

Luciferase reporter gene assays

The cells were cultured in 9.4 cm^2 wells, to a confluence of 60-70% and maintained in D-MEM supplemented with 10% FCS for 24 hours. The cells were transfected with 1 μg of reporter plasmid pGL3-Basic or the reporter plasmid containing the insert (+1 to -982 bp) of the human Slug promoter, or TOPflash plasmid, previously complexed with a 1 μg of Lipofectamine reagent (Invitrogen, Carlsbad, CA), by incubation at room temperature for 20 min. The cells were cotransfected with 0.25 μg of pCMV-Sport-βgal (Invitrogen) to evaluate transfection efficiency and 2.5 μg of expression vectors for Lef1 (K14-myc-hLEF1). Where indicated, the cells were treated with 10 μM SB216763 (purchased from Sigma, and dissolved in DMSO), for 24 h prior to harvest. The cells were lysed 48h after transfection using the reporter lysis buffer (Promega, Madison, WI). As previously described, Luciferase and β-galactosidase activities were determined with luciferase and Beta-Glo assay systems respectively (Promega, Madison, WI). Their activities were normalized with respect to total protein amount (Lambertini *et al.*, 2008).

Small interfering RNA (siRNA) transfection

Three sets of Stealth RNAi duplexes and corresponding Stealth control were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). Stealth RNAi compounds are 25 mer dsRNA containing proprietary chemical modifications that enhance nuclease stability and reduce off-target effects. The following Stealth RNAi sequences were used:

siRNA/Slug1 sense: 5'-CCGUAUCUCUAUGAGAGUUACUCCA-3',

antisense: 5'-UGGAGUAACUCUCAUAGAGAUACGG-3';

siRNA/Slug2 sense: 5'-CCCUGGUUGCUUCAAGGACACAUUA-3',

antisense: 5'-UAAUGUGUCCUUGAAGCAACCAGGG-3';

siRNA/Slug3 sense: 5'-GGCUCAUCUGCAGACCCAUUCUGAU-3',

antisense: 5'-A UCAGAAUGGGUCUGCAGAUGAGCC-3'.

The most effective fragments used for targeting human Slug were siRNA/Slug2.

Twenty-four hours before siRNA transfection, hOBs and hMSCs were seeded in triplicate at density of 16 X 10^3/cm^2 and 12 X 10^3/cm^2, respectively, in DMEM with 10% FCS. Cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's instructions. Transfected cells were incubated for 6 days at 37°C before gene silencing analysis. As a negative control for the siRNA treatment, Medium GC Stealth RNAi Negative Control Duplex (Invitrogen) was used. Knockdown of Slug expression was verified by Real-Time RT-PCR.

Real-time quantitative RT-PCR

Cells from three wells were harvested and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden,Germany) according to the manufacturer's instruction. One microgram of the total RNA was reverse transcribed with the "High Capacity cDNA Reverse Transcription" kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). TaqMan technology, the Assays-On-Demand kit for human Slug, Lef1, Snail1, Snail3, Runx2, BSP, Sox9, Sox5, Sox6, STAT1,OPN, OC, Col1a1, Rankl, c-myc, and CXCL12 were used. The mRNA levels of target genes were corrected for GAPDH mRNA levels (endogenous control). ΔΔCt method was used to value the gene expression, as previously described (Lambertini *et al.*, 2008). Ct was calculated as the difference between the Ct of the target gene and that of GAPDH. Then a calibrator sample (which is the value of ΔCt of the

sample with the lowest levels of the target cDNA) was chosen. Then ΔΔCt was calculated as the difference between the ΔCt of each sample and ΔCt of calibrator. According to a process validated by the custodian of the technology, the expression levels of samples are expressed as $2^{-\Delta\Delta Ct}$, named "fold induction", which estimates the times of increase of each sample compared to the sample chosen as calibrator. All PCR reactions were performed in triplicate for each sample. All experimental data were expressed as the mean ± SEM.

Cell lysates

As previously reported (Penolazzi *et al.*, 2007), the cells were washed twice with ice-cold PBS, containing protease inhibitors (AESBF 104 mM, Aprotina 0.08 mM, Leupeptina 2 mM, Bestatina 4 mM, Pepstatina A 1.5 mM, E-64 1.4 mM; protease inhibitor cocktail, Sigma) and PMSF (Phenylmethanesulfonyl fluoride), harvested and centrifuged for 5 min at 1200 rpm at 4°C. The cell pellet corresponding approximately to 5 x 10^7 cells, was resuspended in 200 µl of a lysing solution (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, water) to which was added protease inhibitors. The cells were kept on ice for about 30 min and then centrifuged for 2 min at 14,000 rpm at 4°C. Then the supernatant was collected and 5 µl were removed for spectrophotometric determination of protein concentration. The rest was stored at -80°C before use.

Western blotting

For western blot analysis, the cells were washed twice with ice-cold PBS and cell lysates were prepared as above mentioned. Then, 10 µg of each sample was electrophoresed on a 12% SDS-polyacrylamide gel. The proteins were then transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, USA). After blocking with PBS-0.05% Tween 20 and 5% dried milk, the membrane was probed with the following antibodies: Slug (L40C6) from Cells Signaling Technology (Danvers, CA, USA), Runx2 (sc-10758), Sox9 (sc-

20095), Tcf-1 (sc-13025), and Tcf-4 (sc-13027), from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Lef1 (L7901) from Sigma Aldrich, IP3K (06-195), and Active β-Catenin (05-665) from Upstate Biotechnology (Lake Placid, NY). After washing with PBS-Tween, the membranes were incubated with peroxidase-conjugated anti-rabbit antibody (1:50000) or anti-mouse (1:2000) (Dako, Glostrup, Denmark) in 5% non-fat milk. Immunocomplexes were detected using Supersignal West Femto Substrate (Pierce, Rockford, IL, USA). Anti-IP 3K was used to confirm equal protein loading.

Osteocalcin assay

Osteocalcin secretion was measured in cell culture supernatants collected from osteoblasts plated in 24-well dishes and cultured with D-MEM, 10% FCS in presence or in absence of 30 nM siRNA/Slug for 6 days. Prior to measurements, the media were collected, centrifuged at 1,300g for 5 min, and tested by using a human osteocalcin enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer's instructions (DRG Diagnostics, Germany). Osteocalcin levels were corrected with total protein content and expressed as nanograms per micrograms of cell protein and each treatment was performed in duplicate.

Secretion of CXCL12 chemokine

Supernatants from both control and silenced-hOBs were collected. CXCL12 production was evaluated by a specific immunoassay standardized in our laboratory using antibody pairs matched for CXCL12 (R&D Systems, Minneapolis, MN, USA). Sensitivity of the assay was 15 pg/ml. Data were expressed as measured pg/mlCXCL12/μg of total proteins ± s.e.m.

Scratch wound assay

hMSCs were seeded at density of $12X10^3/cm^2$, grown to 90% confluency, transfected with siRNA/Slug or scramble RNAs. 72h later the cells were wounded by scratching with a sterile 200 µl pipette tip. 24 and 48 hours after wounding, the cells were incubated with Calcein AM (final 1 µM) at 37°C in the incubator for 30 min. Images were taken at 0, 24 and 48 hours.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described (Brugnoli *et al.*, 2010) using the ChIP kit from Upstate Biotechnology, Inc. (Lake Placid, NY). The cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C, washed in ice-cold PBS, and resuspended in SDS lysis buffer for 10 minutes on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors, and precleared with 80 µl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 5 µg of anti-Lef1 (sc-8591), Tcf-1 (sc-13025), Tcf-4 (sc-13027), Runx-2 (sc-10758) and Slug (sc-10436) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Immunocomplexes were mixed with 80 µl of DNA-coated protein A-agarose followed by incubation for 1 h at 4°C. Beads were collected and sequentially washed five times with 1 ml each of the following buffers: low-salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high-salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris, pH 8.1) and TE buffer. The immunocomplexes were eluted two times by adding a 250-µl aliquot of a freshly prepared solution of 1% SDS and 0.1 M NaHCO3, and the cross-linking reactions were reversed by incubation at 65°C for 4 h. Furthermore, the samples were digested with proteinase K (10 mg/ml) at 42°C for 1 h, DNA was recovered by phenol/chloroform extractions, ethanol was precipitated using 1 µl of 20 mg/ml

glycogen as carrier and resuspended in sterile water. For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). PCR was performed to analyze the presence of DNA precipitated by specific antibodies and by using specific primers (Table 1) to amplify fragment of Slug, Runx2, Sox-9 and CXCL12 gene promoters.

Each PCR reaction was performed with 5 µl of the bound DNA fraction or 2 µl of the input. The PCR was performed as follows: preincubation at 95°C for 5 minutes, 30 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 62°C, and 1 minute at 72°C, with one final incubation at 72°C for 5 minutes. No-antibody control was included in each experiment.

Table1. *PCR primers used for chromatin immunoprecipitation assay (ChIP)*

Gene		Primer sequences	Product size (bp)
Slug	Forward F1:	5'-GAGGTTACCTCTCTTGAAAATACT-3'	178
	Reverse R1:	5'-GGAAGAAAGATCCAATCACA-3'	
	Forward F2:	5'-CCAGGCCAGATCCCAGGAGAGC-3'	164
	Reverse R2:	5'-GCCTCTGGTGTTAATGAGAGCCTA-3'	
	Forward F3:	5'-TGCCCCCCTTCTCTGCCAGAGTT-3'	165
	Reverse R3:	5'-TTCCGCGAAGCCAGGGGCAGCG-3'	
	Forward F4:	5'-ATATAGGCTCTCATTAACAC-3'	218
	Reverse R4:	5'-AGTATTTTCAAGAGAGGTAA-3'	
	Forward F5:	5'-GAAATGGAGTGAAAAGCAAG-3'	175
	Reverse R5:	5'-TTGCAGGAGAGAGGAAAATA-3'	
Runx2	Forward F1:	5'-ATATCCTTCTGGATGCCAGG-3'	167
	Reverse R1:	5'-AAGCACTATTACTGGAGAGGC-3'	
	Forward F2:	5'-GTTTCAGTGAATGCTAATGTAG-3'	290
	Reverse R2:	5'-AAGCGTTCATTTAACATGCAG-3'	
	Forward F3:	5'-CAAGAGCTTTATTTGCATTGAC-3'	282
	Reverse R3:	5'-TTGTCCTCTGTGAGGCCTAT-3'	
Sox9	Forward F1:	5'-GATAGTGTCCTCACTTCGCA-3'	467
	Reverse R1:	5'-TCCACTCTGGCGGAGTCATG-3'	
	Forward F2:	5'-CAGCCACCACCATCCAAGTT-3'	470
	Reverse R2:	5'-GAAGGGCATTGTGTGTACAG-3'	
CXCL12	Forward F1:	5'-TACAGGCGAGGAAACTGAGGCT-3'	243
	Reverse R1:	5'-GGACTGAATGAGAACCAATGAA-3'	
	Forward F2:	5'-ATGCGTAATTACTTATGCTTAGC-3'	292
	Reverse R2:	5'-TCAGGTGGGCAGCTGGACCTA-3'	

Statistical analysis

Statistical analysis was performed using the non-parametric tests (Wilcoxon matched pair test and Friedman ANOVA & Kendall's concordance non-parametric test) to evaluate if there was any tendency to increase or decrease along the time points analyzed and to compare basal versus treated cells. The Kruskal-Wallis ANOVA and Mann-Whitney U test were also used for comparing non-matched group of data. All values were expressed as the mean ± s.e.m. of at least three different experiments. The analyses were performed using CSS Statistica Statistical Software (Statsoft Inc., Tulsa, OK, USA). Values of $p<0.05$ were considered significant.

RESULTS

Chapter 1

Slug expression in human normal osteoblasts and their mesenchymal precursors.

In this chapter it will be considered: 1. the cellular models on which it based the analysis of the expression and regulation of Slug, and 2. Slug expression in relation with other transcription factors involved in OB lineage cells.

Isolation and characterization of human primary osteoblasts (hOBs)

Human primary osteoblast cultures (hOBs) were generated from bone chips removed from nasal septum, and were subjected to the experimental procedure schematized in Figure 1. Briefly, the bone samples, about 1 cm^2 in size, were cut into small fragments and collected in culture 25-cm^2 flasks. The appearance of isolated human osteoblastic cells was examined and cell morphology was found to be consistent with what has been reported in the literature (Robey and Termine., 1985) (see Figure 1). It was observed that immediately following isolation, there was limited cellular migration from the trabecular bone chips at day 1. After about 7 days, outgrowth of bone cells from the bone chips commenced, and confluency in 25-cm^2 flask was usually reached after 3-4 weeks. The cellular morphology was spindle-shape as well as circular for some osteoblastic cells. On examination of the cells at day 21, there was a substantial increase in the number of primary cells that had migrated from the bone chips, increasingly further away from the bone chips.

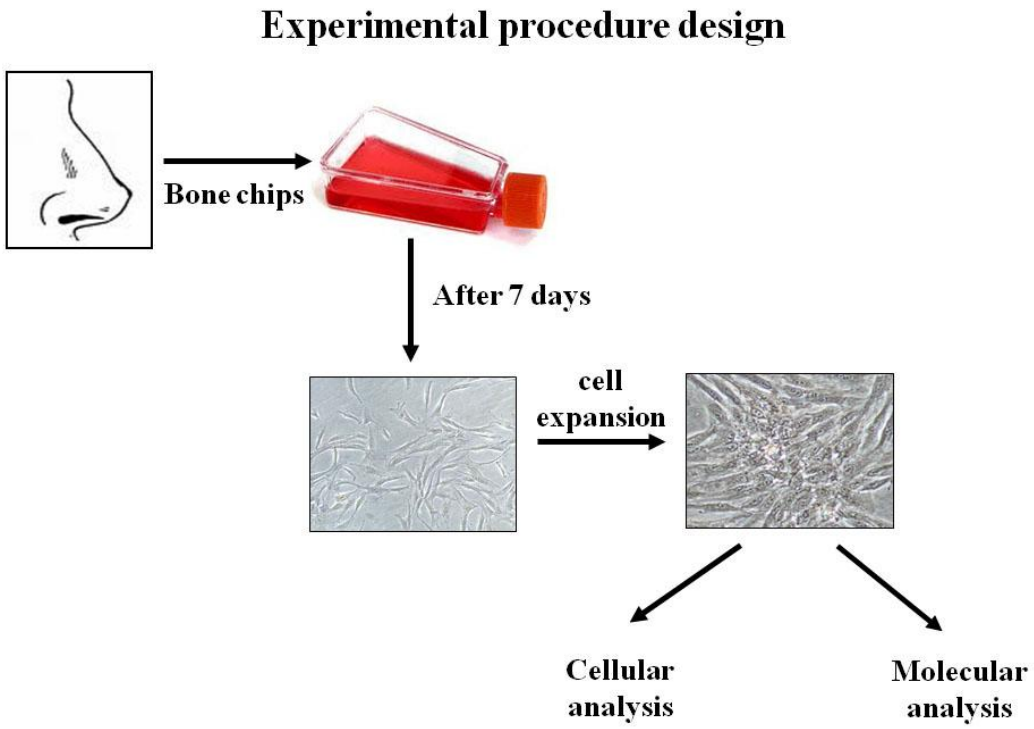


Figure 1. *Isolation and use of human primary osteoblasts (hOBs).* Human primary osteoblasts were obtained from bone chips removed from nasal septum. Bone was cut in small pieces which were rinsed and then cultured in 25-cm^2 flask. About 7 days after isolation, outgrowth of bone cells from bone chips began. As shown in this figure, cells present a spindle-shape morphology. Once hOBs reached confluency, they were used for cellular and molecular analysis.

To characterize hOBs, the immunophenotypical profile of the cells obtained from bone chips was determined by testing three specific surface markers using flow cytometric analysis. The results indicate that most of the cells (98.12%) expressed cell surface marker CD90 (Thy-1) (see Figure 2A) that is detected in the early stage of osteoblast differentiation and declines as osteoblasts differentiate into osteocytes (Chen *et al.*, 1999). The hematopoietic marker CD45 was not detectable on these cells (see Figure 2A), indicating that cells were not contaminated with cells of hematopoietic origin. In addition, flow cytometry showed that the cells did not express STRO1 (a marker that recognizes osteoprogenitor stem cells of the colony-forming unit-fibroblastic) (see Figure 2A), suggesting that they are mostly mature osteoblasts (Byers *et al.*, 1999).

At the same time hOBs phenotypic characterization was analysed by immunocytochemistry staining for Runx2, a typical osteogenic protein. As shown in Figure 2B, the cells were highly positive for this marker and this result was further confirmed by data from gene expression analysis based on RT-PCR (see Figure 2B). In addition, a significant component of the bone extracellular matrix, the bone sialoprotein (BSP), was detected at mRNA level by real time RT-PCR. These data confirm the level of maturation of the cells giving a definite indication of their commitment.

Osteogenic ability of hOBs

Next, the cells were characterized for their osteogenic capacity in terms of alkaline phosphatase (ALP) activity and mineral deposition (see Figure 2C). Alkaline phosphatase is an early osteogenic marker whose activity tends to increase with increasing in osteoblastic differentiation. The presence of blue staining, indicating an intense ALP activity, was detected in the cells analysed (see Figure 2C). In addition, Alizarin Red staining showed an evident extracellular matrix mineralization deposition after 14 days of culture under osteogenic conditions (see Figure 2C).

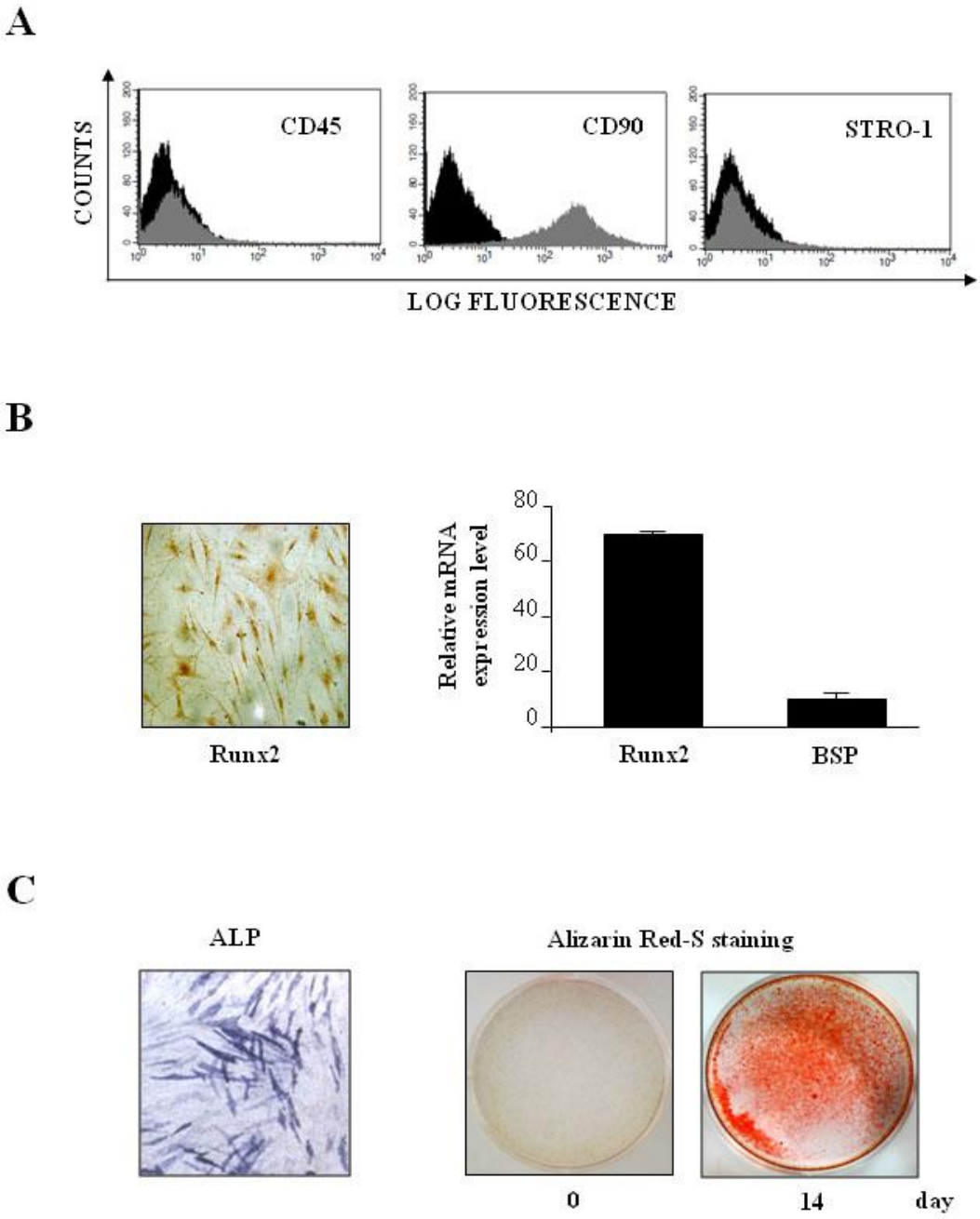


Figure 2. *Phenotypical characterization of human primary osteoblasts (hOBs).* A. The characterization of hOBs has been performed by flow cytometric analysis of CD45, CD90 and STRO-1 phenotypical markers. X-axis, mean fluorescent channel; Y-axis, number of events. B. hOBs samples were subjected to immunocytochemical analysis for Runx2 osteoblastic marker. A representative sample is shown (X10 magnification). The level of Runx2 and BSP expression was examined by quantitative RT-PCR in five hOB samples. The experiments were carried out in triplicate, the expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. ΔΔCt method was used to value the gene expression; SEM was calculated. C. The cells were treated with β-glycerophosphate, ascorbic acid, and dexamethasone. The authentic osteoblast phenotype was confirmed in hOBs by staining for alkaline phosphatase (ALP) activity. Deposition of extracellular matrix was evaluated.Mineral formation was examined by Alizarin Red-S staining after 14 days of osteogenic induction.

Isolation and characterization of human mesenchymal stem cells (hMSCs)

As sources of human MSCs, adult hMSCs from tibial plateau (TP) trabecular bone, iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord, were used. We chose to examine these three populations in order to analyze their different characteristics in terms of isolation, proliferation and differentiation potential.

Due to the simple collection procedure, Wharton's jelly represents, among all three, the easiest accessible source for MSCs. The choice to use solely Wharton's jelly instead of the whole cord was made with the aim of isolating a relatively homogeneous cell population, possibly avoiding any epithelial cell contamination. Contrary to the majority of groups working on WJ-MSCs, we choose to process the Wharton's jelly based essentially on the capacity of MSCs to adhere to a plastic surface, without enzymatic digestion treatment. As previously described in "Materials and Methods" section, umbilical cords were chopped and scraped to obtain only the jelly mucoid connective tissue from each sample. Then, the small pieces (2-3 mm^2) were placed directly into 25-cm^2 flasks in D-MEM Low Glucose medium supplemented of 10% FCS. After 3 to 5 days of culture, the WJ-MSCs form adherent colonies reaching confluence after 10-14 days, while hTP-MSCs and hIC-MSCs usually reach the confluence after 2-3 weeks from seeding. However, the cells isolated from the three sources, display both a monocyte-like and a spindle-shape morphology (see Figure 3 and 4).

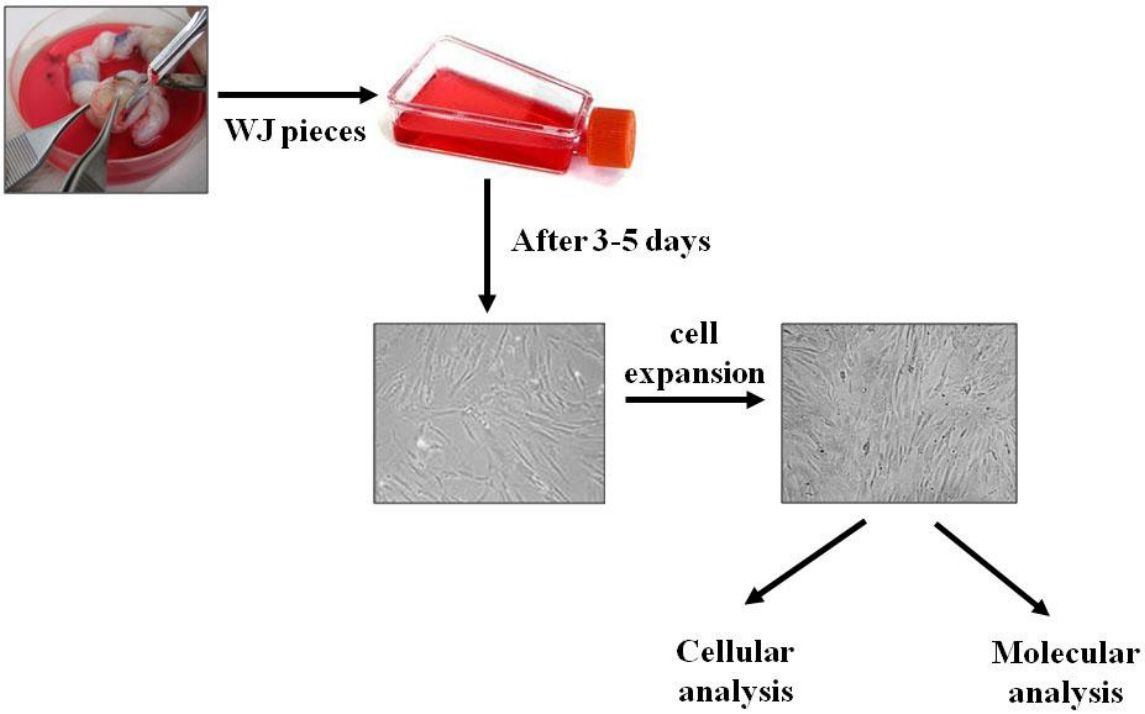


Figure 3. *Isolation and use of human* ***mesenchymal stem cells from Wharton's jelly (WJ-MSCs).*** MSCs were obtained from Wharton's jelly (WJ) umbilical cord. The cord was cut into small pieces and subjected to longitudinal incisions to obtain only the jelly mucoid tissue. WJ pieces were then rinsed and cultured in 25-cm^2 flask. About 3-5 days after isolation, WJ-MSCs form adherent colonies. As shown in this figure, cells present a spindle-shape morphology. Once WJ-MSCs reached confluency, they were used for cellular and molecular analysis.

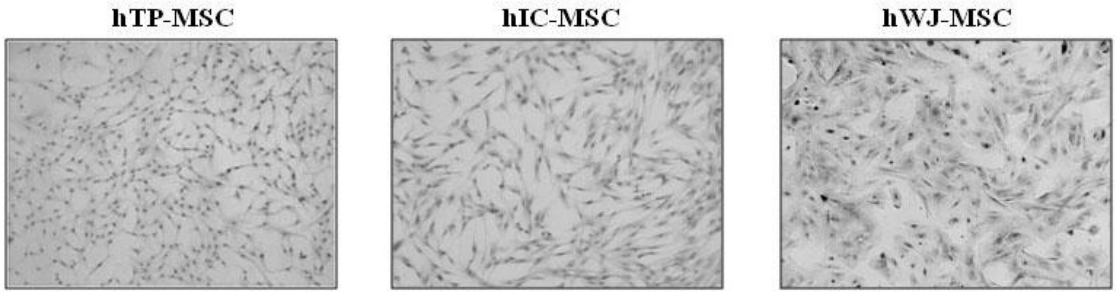


Figure 4. *Morphological analysis of hMSCs from different sources.* Optical micrographs of hMSCs isolated from tibial plateau (TP) trabecular bone, iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord. After one passage (P1), cells show a fibroblast-like morphology (original magnification: 10X).

To evaluate the characteristics of each MSC sample, the immunophenotypic profile of adherent cells obtained from the different sources was determined by testing a panel of surface markers using flow cytometry (see Figure 5). MSC from all samples were positive for CD90, CD73, CD105, CD146 (mesenchymal cell markers), but negative for CD31 (endothelial cell marker), CD3, CD14, CD34, CD45 (hematopoietic cell markers). In particular, we observed that the expression of CD90, CD73 and CD105 was not significantly different in all the three types of MSC analysed (total mean±SD 97±3), while the percentage of positive cells for CD146 was significantly lower in hTP-MSC (mean±SD 25.77±17.82) and in hWJ-MSC (mean±SD 23.4±19.09) compared to hIC-MSC (mean±SD 53.12 ± 23.01, P=0.000007 and P=0.01 respectively).

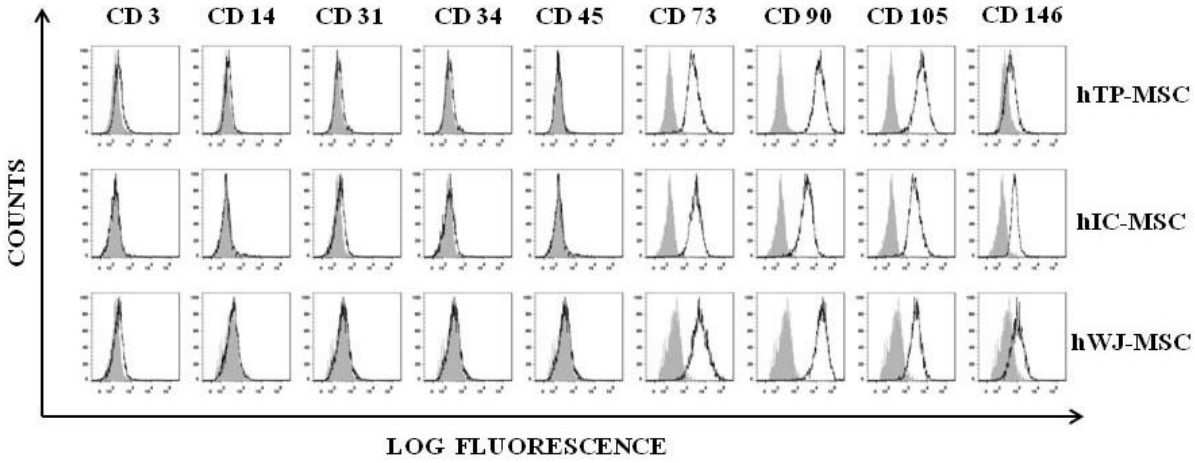


Figure 5. *Phenotypical characterization of hMSCs.* Flow cytometric analysis of a representative case of hTP-MSC, hIC-MSC and hWJ-MSC. Open histograms represent the isotype control antibody, dotted histograms represent anti-CD3, -CD14, -CD31, -CD34, -CD45, -CD73, -CD90, -CD105, -CD146 antibodies. X-axis, mean fluorescent channel; Y-axis, number of events.

Osteogenic potential of MSCs

We then investigated the osteogenic potential of hMSCs from the three sources. The experiments were performed after the 3rd to 4th passage at least, and then the cell

populations were differentiated. The osteogenic differentiation of expanded hMSCs was induced for 3 weeks by the addition of 100 nM dexamethasone, 10 mM β-glicerophosphate and 100 μM ascorbic acid to the culture medium. During this lapse of time, the cells lost their typical spindle-shape morphology turning into wider polygonal cells. At 21 days of differentiation, the cultures showed the presence of mineralized nodules following Alizarin red staining analysis (see Figure 6A). These data support the ability of hMSCs to differentiate towards osteogenic lineage when appropriately stimulated *in vitro*. Interestingly, mineralization occurred at day 7 only for hWJ-MSC while for hTP- and hIC-MSC started from day 14. In addition, we noticed that, both at day 14 and 21, mineralization was significantly increased in hTP- ($p=0.003$ and $p=0.006$, respectively) and hIC-MSC ($p=0.00001$ and $p=0.006$, respectively) compared to hWJ-MSC. Comparing hTP- and hIC-MSC we observed that, while at day 14 mineralization was significantly higher in hIC-MSC than in hTP-MSC ($p=0.008$), at day 21 it reached approximately the same values in both cell sources. The effectiveness of osteogenic differentiation was then assessed analysing Runx2 mRNA expression levels in all three hMSC populations (see Figure 6B). After 21 days in osteogenic medium, Runx2 expression showed a decrease in hTP-MSCs, an increase in hWJ-.MSCs, but remained constant in hIC-MSCs, suggesting that hMSCs from the three different sources are at different stage of maturation.

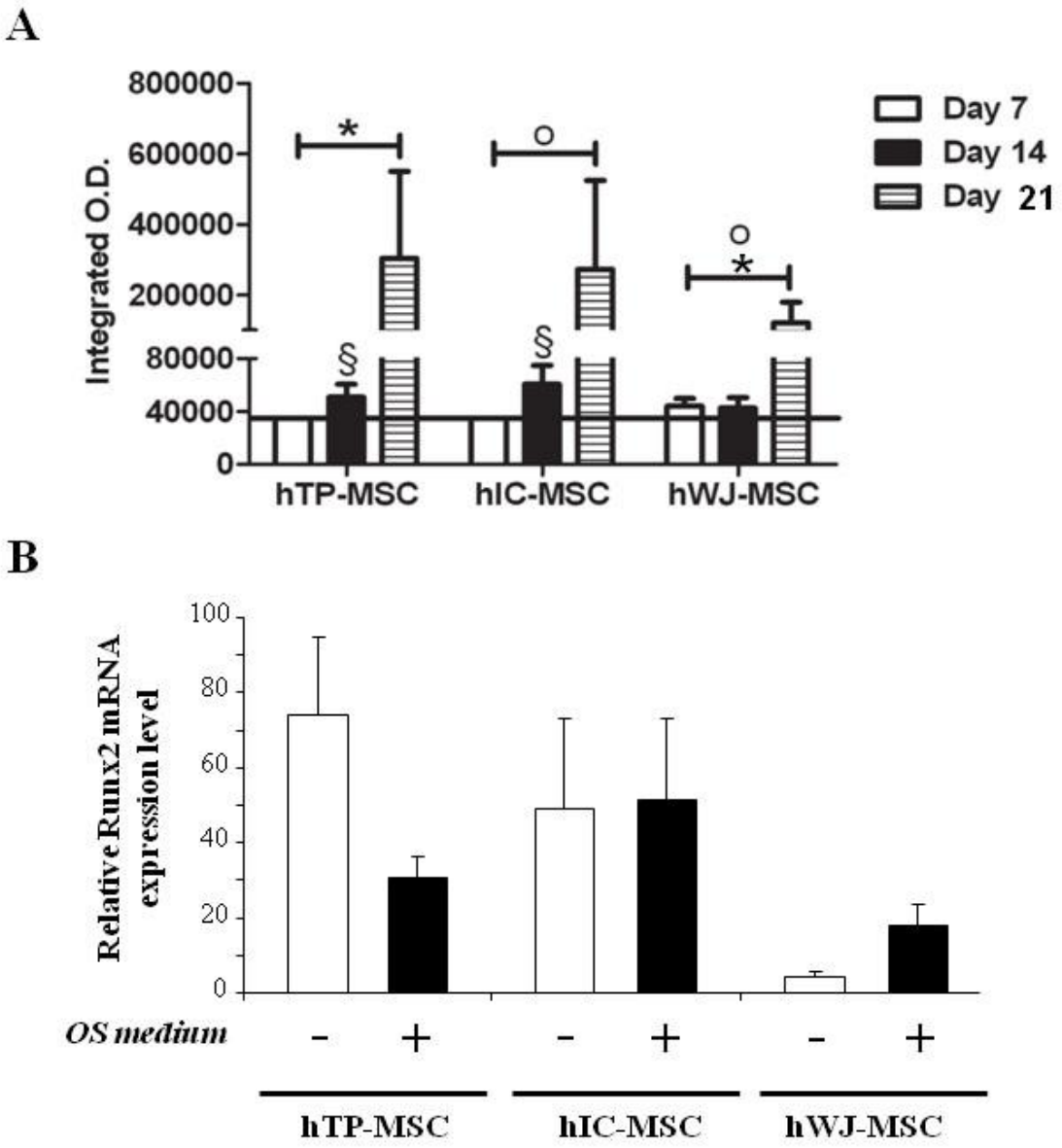


Figure 6. *Osteogenic differentiation of hMSCs.* A. Differentiation potential of hMSCs from the three sources was assessed by evaluating the mineralization of the cell cultures in the presence of osteogenic induction medium, up to 21 days. Data were expressed as integrated optical density (O.D.) ± SE. Statistical analysis was performed hTP-MSC *versus* hWJ-MSC *, hTP-MSC *versus* hIC-MSC § and hIC-MSC *versus* hWJ-MSC o, as described in "Results" section. B. Runx2 gene expression was evaluated in hMSCs isolated from tibial plateau (TP) trabecular bone, iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord and cultured up to 21 days in osteogenic medium (OS medium). mRNA level was revealed by quantitative RT-PCR analysis. RT-PCR results, were calculated using the ΔΔCt method. Data were expressed as fold difference value between the calibrator (Runx2 expression level in uninduced hWJ-MSCs) and the other samples. Data were expressed as median, box: 25-75 percentiles and whisker: minimum and maximum of non-outliers. It was not possible to apply statistical tests since the N was equal to 4 samples for each group analysed.

Osteogenic potential of WJ-MSCs and variabily between samples

Nevertheless the majority of WJ-MSC samples examined has shown the ability to differentiate into osteoblasts beginning from day 7 (see Figure 6A), however a very high heterogeneity in response to treatment with osteogenic medium was observed. This evidence, in agreement with the data obtained by other researchers (Markov *et al.*, 2007; Zhang *et al.*, 2009), prompted us to search a possible correlation between the osteogenic potential of WJ-MSCs and five different obstetric parameters, including baby's gender and birth weight, mother's age at delivery, gestational stage at parturition and mode of delivery. With the collaboration of Section of Obstetric and Gynaecological Clinic, Azienda Ospedaliero-Universitaria S, Anna, it was possible to obtain 60 fresh samples of human umbilical cords. Among the collected samples, we selected the 20 most homogeneous ones considering the percentage of CD90 presence and CD45 absence (≥ 99%), and we analyzed for each one the different obstetric parameters aforementioned (see Figure 7 table A).

We conducted analyses adjusting for two specific markers of osteoblast differentiation: the activity of alkaline phosphatase (ALP) and the expression levels of Runx2. As shown in Figure 7C, focusing on basal levels of ALP and Runx2, it has been possible to demonstrate that these parameters can be predictive of osteoblastic potential of WJ-MSCs. In fact, the samples with high basal levels of Runx2 and ALP are more prone to deposit mineral matrix if compared to WJ-MSC with low levels of these two proteins.

Next, we analyzed whether the basal levels of Runx2 and ALP correlate with the examined obstetrics factors (see Figure 8). We found that the infant gender and mode of delivery didn't significantly correlate ($P > 0.05$) with basal Runx2 expression and ALP activity. On the other hand, the age of the mother at delivery, has a significant impact on the basal ALP activity but doesn't affect Runx2 expression level. Samples collected from mothers which were <32 years old give origin to WJ-MSCs with high ALP activity (see Figure 8 upper graph). Interestingly, birth weight of the infant was shown to significatively impact on Runx2 basal expression level which, decreases with the decreasing of the baby's weight (see Figure 8 lower graph). The same relationship was found for the duration of

pregnancy. In fact, it was found that WJMSCs from babies born before the 37 weeks of gestation express lower basal level of Runx2 than the full term born (see Figure 8 middle graph).

As a whole, these findings led us to focus on two parameters, weeks of pregnancy and consequently birth weight of the baby, and Runx2 basal levels, subdividing the collected samples in the two subgroups reported in Figure 7 table A: subgroup I, premature birth with low levels of Runx2, and subgroup II, full term birth with high levels of Runx2. The ability of the samples belonging to these two subgroups to complete the event of cellular maturation, that is the deposition of mineralized matrix, was then compared. Two representative samples of the two subgroups demonstrate that, samples from subgroup I showed a null mineralization status also after 21 day of cell culture in osteogenic medium, whereas samples from subgroup II showed a high level of mineralization beginning from day 14 (see Figure 7B). These findings suggest that maximal WJ-MSCs osteoblastic potential can be obtained by primary cultures with Runx2 high basal levels, selected from the heaviest term babies.

A

	Sample	Mother's age (ys)	Weeks of pregnancy	Mode of delivery	Gender	Birth weight (Kg)	RUNX-2	ALP
subgroup I	1	26	29	CS	F	1.25	3.76±0.2	3.52±0.2
	2	31	32	CS	M	1.74	1.92±0.1	8.105±01
	22	33	35	CS	M	2.41	2.55±0.2	1.34±0.3
	23	33	35	CS	M	2.53	1	2.33±0.2
	30	32	35	SP	F	3.17	1.55±0.2	2.18±0.15
	35	35	37	CS	F	2.77	4.12±0.1	1.72±0.1
	41	30	34	SP	M	2.6	2.59±0.1	5.23±0.3
	43	27	33	CS	M	1.915	2.85±0.1	3.4±0.3
	44	27	33	CS	F	2.005	2.22±0.1	2.18±0.1
	53	31	37	CS	F	1.98	6.03±0.1	6.69±0.4
	60	27	29	CS	M	2.1	1.15±0.12	6.08±0.2
subgroup II	12	38	38	CS	F	2.6	1.67±0.2	3.68±0.1
	13	38	39	SP	M	3.45	2.06±0.01	7.38±0.2
	14	36	40	SP	F	3.6	12.85±0.1	2.43±0.2
	20	35	40	SP	M	3.18	7.28±0.2	2.62±0.1
	27	29	40	SP	M	3.4	9.94±0.01	4.8±0.15
	28	39	39	CS	M	3.62	9.8±0.1	4.06±0.2
	33	20	40	SP	F	3.02	2.85±0.2	2.23±0.2
	37	38	38	SP	F	3.05	10.31±0.2	4.6±0.1
	51	33	39	CS	M	3.27	2.14±0.2	5.75±0.1

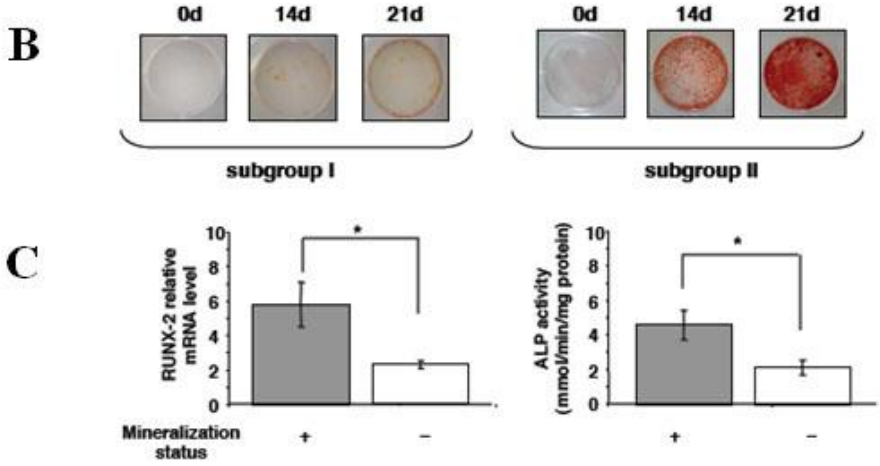


Figure 7. *Osteogenic potential of WJMSCs and variabily between samples.* In the table (A) clinical parameters, Runx2 expression and alkaline phosphatase activity (ALP) basal level are reported. As indicated, the samples were divided in two subgroups: subgroup I, premature birth; subgroup II, full term birth. F (female), M (male), CS (Caesarian delivery), SP (spontaneous delivery). B. The ability to deposit mineralized matrix was evaluated at the indicated times (0d, 14d, 21d) in two representative samples of each subgroup, by Alizarin red staining. C. The mineralization status (+, positive or -, negative) was correlated with the basal level (at day "0") of Runx2 expression (on the left) and ALP activity (on the right). * = $p < 0.05$.

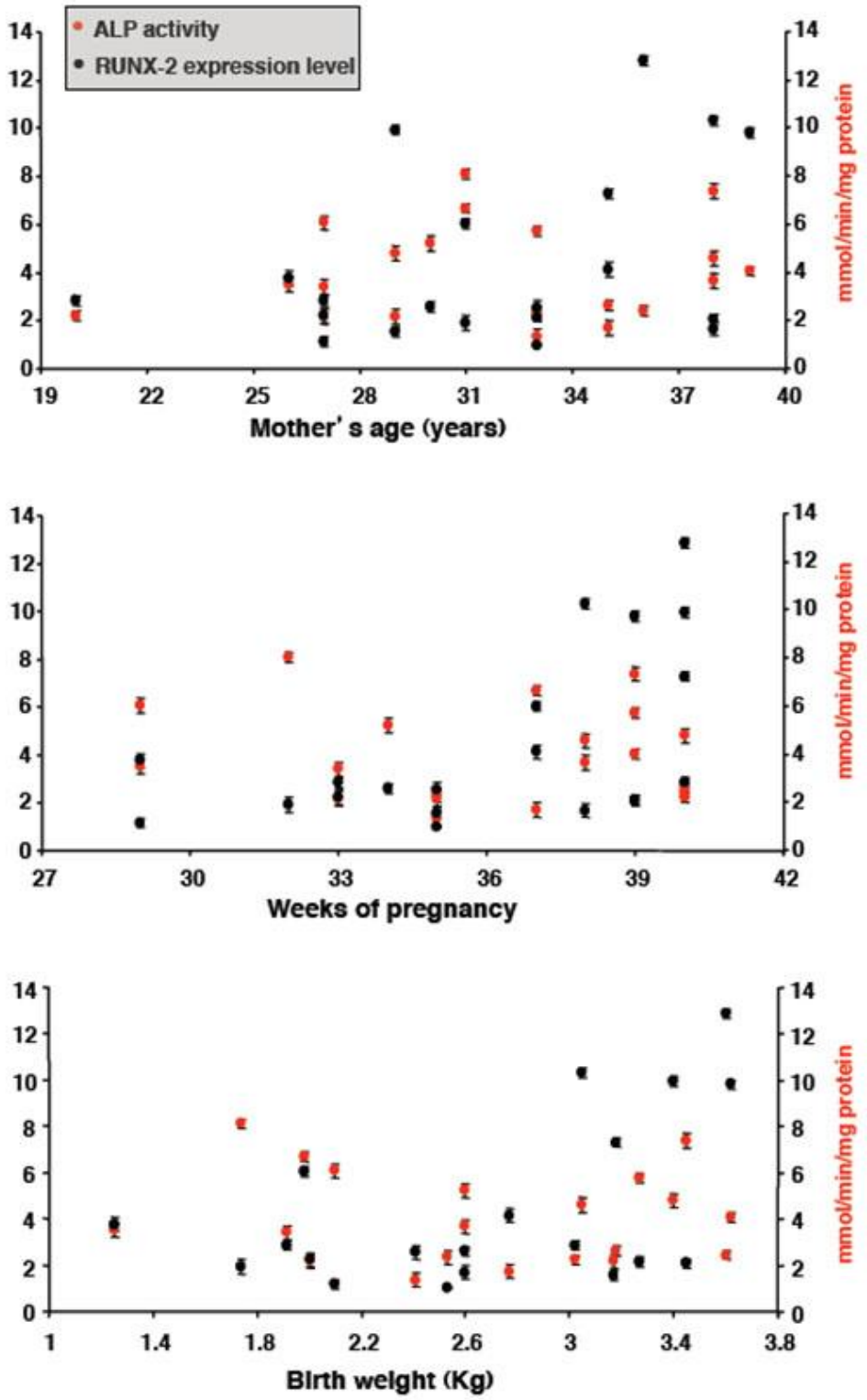


Figure 8. *Relationship between molecular and obstetric parameters in WJMSCs.* Basal levels of Runx2 expression and ALP activity were related to mother's age (upper graph), weeks of pregnancy (middle graph) and birth weight (lower graph) in 20 WJMSC samples.

Slug expression in human osteoblasts and their mesenchymal precursors

Slug expression levels were first assessed in osteoblastic cells by real-time RT-PCR analysis. To this aim the following cellular models were used: human craniofacial osteoblasts obtained from bone sample collected during nasal septum surgery, osteosarcoma cell lines (SaOS-2 and U2OS), osteoblast-like cell lines (CAL72 and Hobit), characterized by a different stage of differentiation. As negative control breast cancer cell line MCF7 was employed.

As shown in Figure 9, Slug mRNA level was expressed at a significant level in all types of osteoblastic cells analyzed, both in cell lines and in normal primary cells. In particular, the highest expression was observed in CAL72 cells and human primary osteoblasts. On the contrary, as expected (Hajra *et al.*, 2002), no Slug mRNA expression was detected in MCF7 cells.

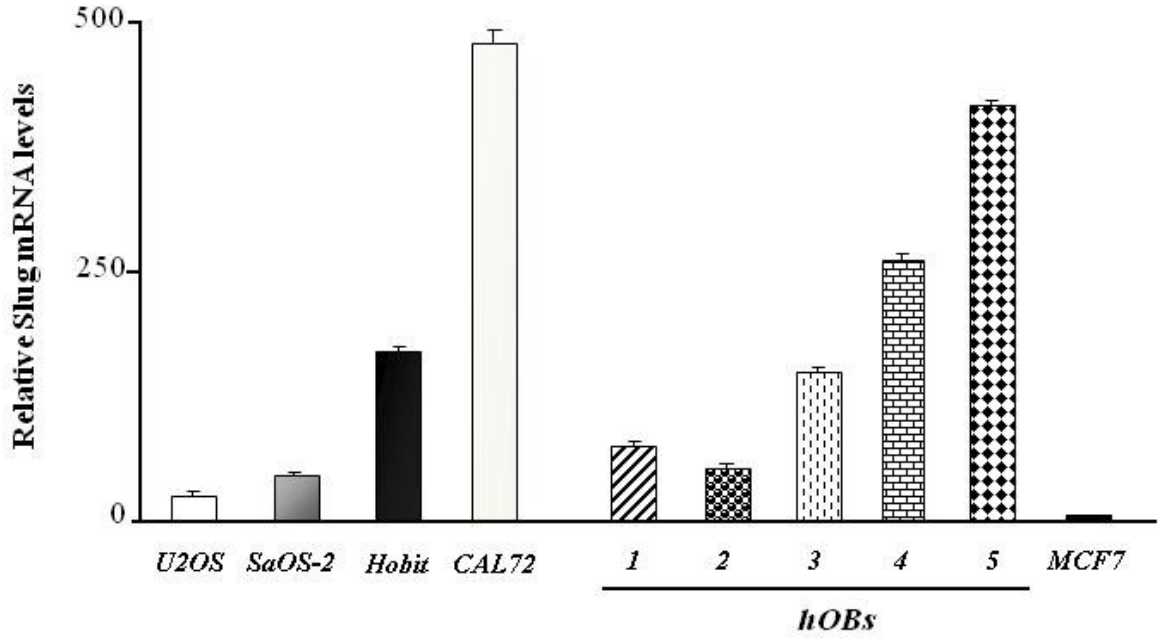


Figure 9. *Analysis of Slug expression by quantitative RT-PCR in different osteoblastic cells.* The level of Slug mRNA was examined by quantitative RT-PCR in four osteoblast-like cell lines (U2OS, SaOS-2, Hobit and CAL72), in MCF7 breast cancer cell line and in five human primary hOB samples. The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for Slug transcript analysis. The experiments were carried out in triplicate, the expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. ΔΔCt method was used to value the gene expression; standard error of the mean (SEM) was calculated.

The experiments have then carried on the investigation of Slug function and its expression regulation in a context as close as possible to the "*in vivo*" conditions, in human primary cells. Starting from evidence present in the literature, describing the role of Lef1 in osteoblast differentiation and the existence of a relationship between Lef-1, β-catenin and Slug in some epithelial-mesenchymal transition cellular models (Saegusa *et al.*, 2009; Hong *et al.*, 2009), we hypothesized a correlation between Lef-1 and Slug may in mature osteoblasts and their mesenchymal progenitors. To test this idea Slug and Lef1 expression were examined, at first, in hOBs obtained from five bone specimens (hOBs). All these samples were positive for ALP activity and were able to form mineralized nodules after 14 days in osteogenic condition (see Figure 2C).

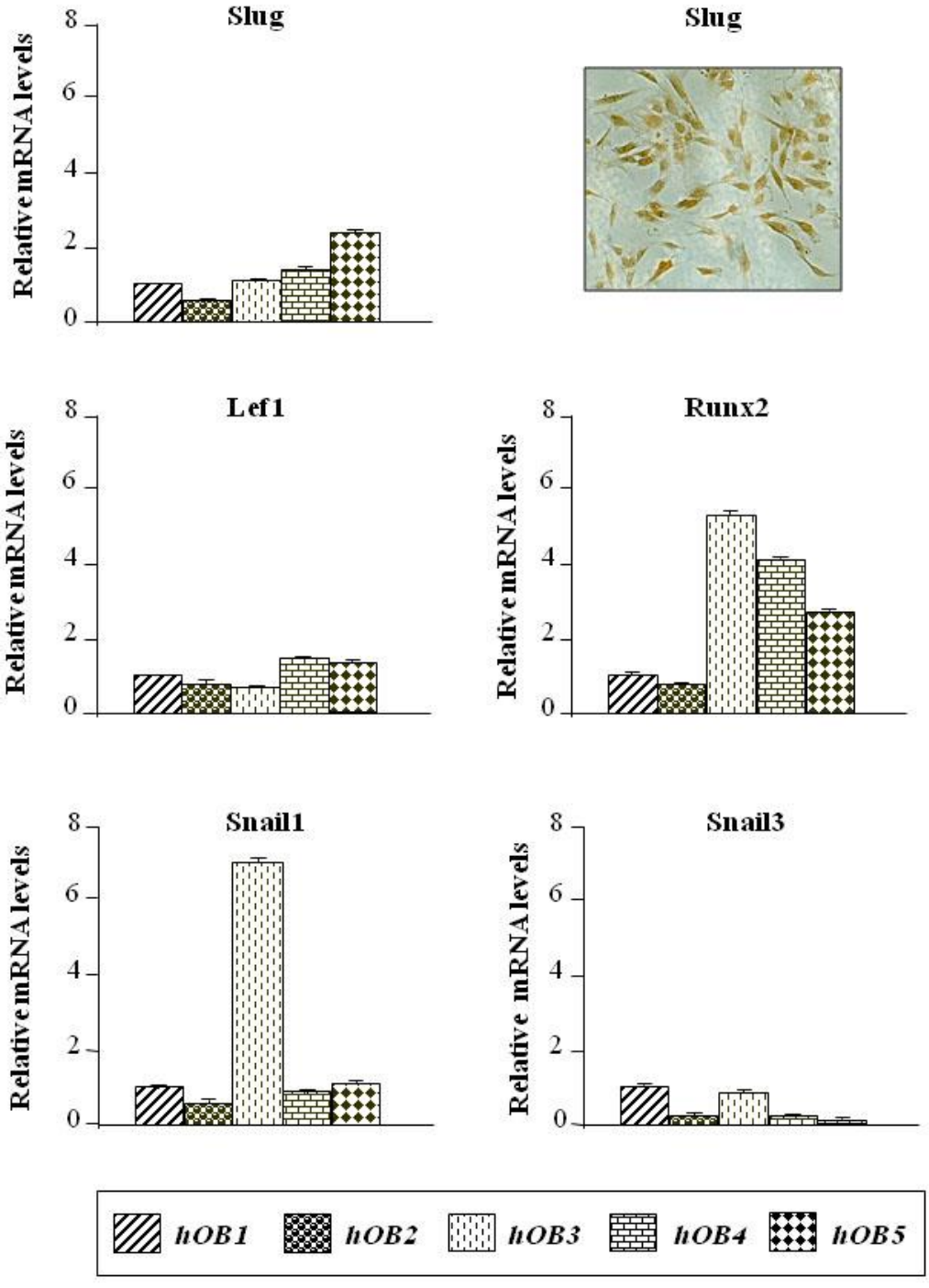
As shown in Figure 10A, Slug was detected at mRNA and protein level in all hOBs analyzed by RT-PCR and immunocytochemical analysis, respectively. Also Lef1 was observed in the same samples as well as Runx2, the master regulator in osteoblast development and bone formation (Marie., 2008).

Slug expression was then evaluated in uninduced hTP-MSCs, hIC-MSCs and WJ-MSCs by quantitative RT-PCR analysis. As shown in Figure 10B, basal Slug expression level was higher in hTP- and hIC-MSCs than in hWJ-MSCs. Furthermore, Slug expression trend was investigated during osteoblast differentiation. To this aim Slug mRNA levels were measured in hWJ-MSCs induced towards osteogenesis (see Figure 10C). We chose this source of hMSCs for its ability to anticipate the deposit of mineralized compared to hTP- and hIC-MSCs, as reported in Figure 6. As shown in Figure 10C, RNA was collected after 0, 7, 14, 21, and 28 days in culture and evaluated by quantitative RT-PCR. WJ-MSCs differentiate along the osteoblast lineage in osteogenic medium as confirmed by the positive staining for extracellular calcium deposition. Abundant Slug mRNA was detected in the cells at all times tested, and was induced as the cultures progressed. This was confirmed also at protein level by immunocytochemical analysis, thus showing that the positive signal increased during osteogenic differentiation of WJ-MSCs, both in the single cells and in the mineralized nodules (see Figure 10D). Lef-1 was less abundant, but significantly increased during the osteogenesis. Runx2 was also expressed at all stages, and

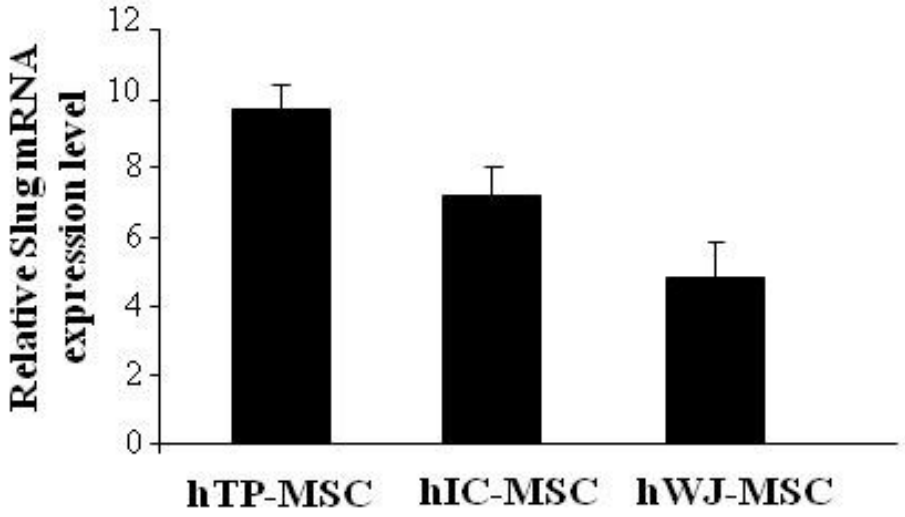
was induced as the cultures progressed, confirming that each time point represented increasingly mature osteoprogenitors.

To further characterize the potential involvement of Snail family members in osteogenesis, the expression of Snail1 and Snail3 was examined in the same set of experiments. Snail1 has been recently reported to act on the osteoblast population regulating bone cells differentiation and contributing to bone remodeling in mice (de Fructos *et al.*, 2009). In agreement with this previous study, we found that Snail1 was expressed at early stages of osteoblast differentiation and then downregulated for differentiation to proceed (see Figure 10C). In hOB Snail1 was expressed at substantial levels (see Figure 10A). The expression of Snail3 (Katoh *et al.*, 2003) was detectable at low levels in hOBs (see Figure 10A) and at very low levels in the hMSCs induced towards osteogenesis (see Figure 10C).

A


Slug
Slug
Relative mRNA levels
8
6
4
2
0
Lef1
Runx2
Snail1
Snail3
hOB1
hOB2
hOB3
hOB4
hOB5

B


Relative Slug mRNA expression level
12
10
8
6
4
2
0
hTP-MSC
hIC-MSC
hWJ-MSC

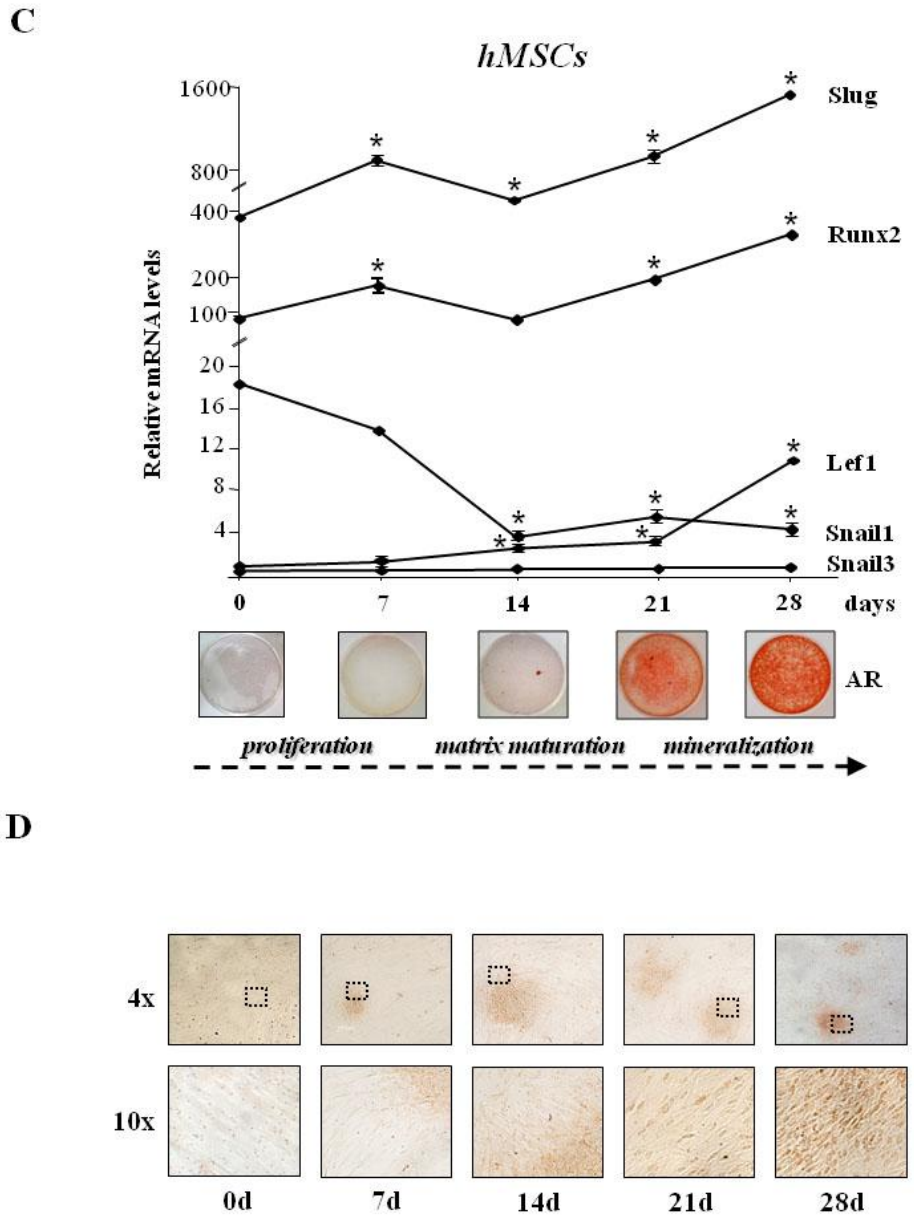


Figure 10. *Detection of Slug expression in hOBs and hMSCs.* The level of Slug, Lef1, Runx2, Snail1 and Snail3 expression was examined by quantitative RT-PCR in five hOB samples (A) and in three hMSC samples cultured up to 28 days in osteogenic medium (C). Slug expression level was also evaluated in uninduced hTP-MSCs, hIC-MSCs and WJ-MSCs (B). The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for Slug, Lef1, Runx2, Snail1 and Snail3 transcript analysis. The experiments were carried out in triplicate, the expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. ΔΔCt method was used to value the gene expression; standard error of the mean (SEM) was calculated. Slug expression was also examined by immunocytochemical analysis both in hOBs (A) and in osteogenic-induced WJMSCs at day 0, 7, 14, 21, and 28 days (D). Magnification 4X and 10X. The commitment to osteoblastic lineage of hMSCs was evaluated by Alizarin Red staining (AR) for extracellular calcium deposition (B). * = $p < 0.05$ (respect to day 0).

Chapter 2

Slug gene regulation in human osteoblasts.

Human Slug promoter characterization

As previously mentioned in the introduction, although the regulation of Slug expression and its role have been investigated extensively in many animal species, mechanisms governing Slug expression in humans are largely unknown. Indeed, there is little information regarding the characterization of Slug promoter and the role that specific proteins play in regulating its expression in normal adult human tissues.

In order to identify potential binding sites for transcription factors involved in Slug gene expression regulation, at first, we analyzed a region of approximately 2 Kb upstream of the transcription start site in the human Slug gene. This region was initially subjected to bioinformatic analysis based on the search for E-box sites for Slug protein and for binding sites for Runx2 and Lef1/Tcf transcription factors, which potentially can interact with Slug and modify its expression.

By using the programs Transcription Element Search Software TESS for transcription factor search and MatInspector 7.4 program, we identified ten E-box sites, four Runx2 binding sites and another seven potential consensus binding sites for the Lef/Tcf family, in addition to the previously identified Tcf binding site at -859/-855 position (Saegusa *et al.*, 2009; Hong *et al.*, 2009) (see Figure 1A).

Since most of the binding sites for the factors of our interest are located in the first thousand base pairs upstream of the transcription start site, we have focused on the analysis of this region. In addition to putative binding sites for Lef/Tcf, Runx2 and Slug (see

Figure 1B and Table 1), some other binding sites for transcription factors that play an important role in osteoblastic differentiation, such as ERα (estrogen receptor α) (Steward *et al.*, 2000 and AP-1 (activator protein-1) (McCabe *et al.*, 1996) have been identified in the same sequence.

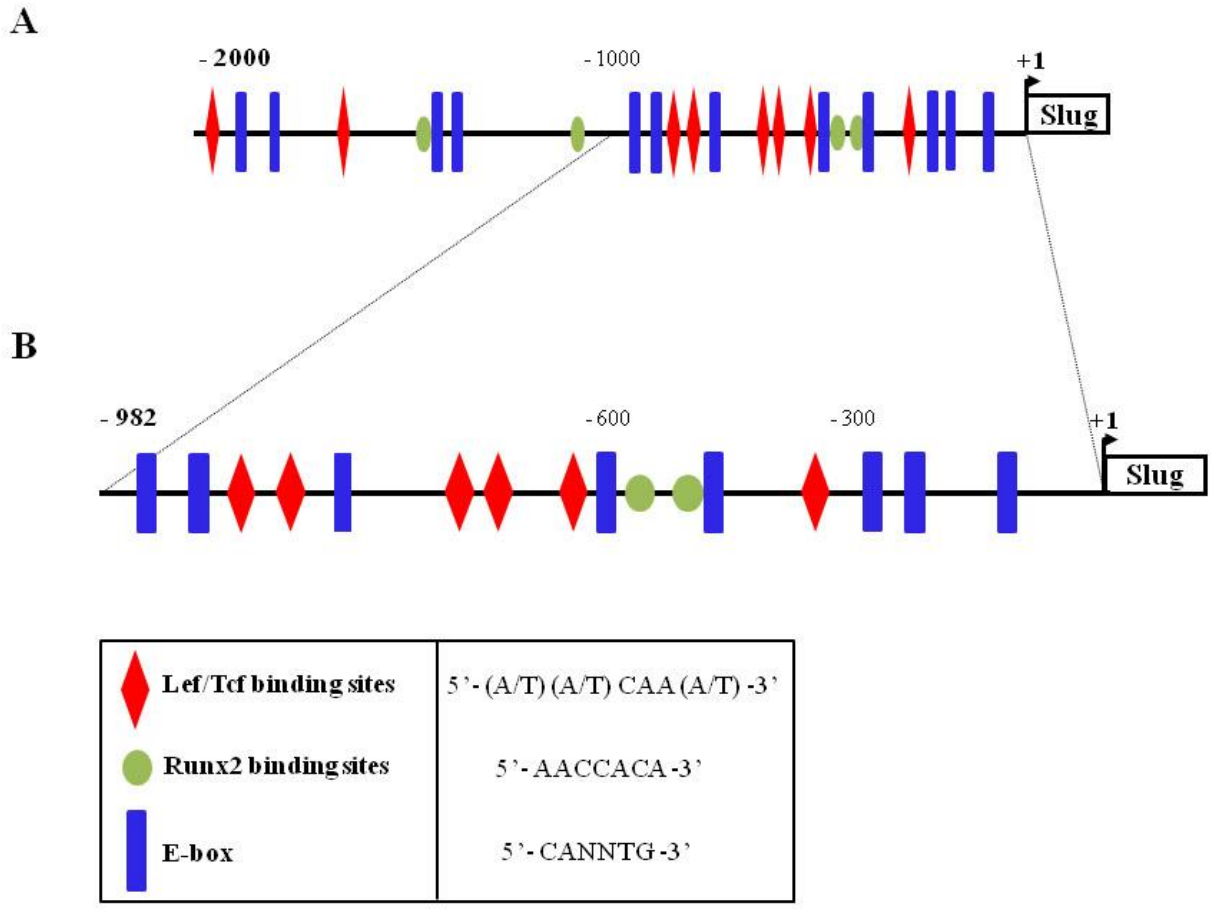


Figure 1. *Schematic representation of Slug promoter region under investigation.* A, B. Putative binding sites for Lef/Tcf, Runx2 and Slug transcription factors, identified by using bioinformatic analysis, are indicated. B. Each specific "consensus" sequences for these transcription factors, localized at -982/+1, are reporter in the table below.

TRANSCRIPTION FACTOR	POSITION	SEQUENCE
Lef/TCF	-310	ATTTTGA
	-610	TCTTTGT
	-637	TCAAAAG
	-667	CCTTTGT
	-849	TACAAAG
	-855	CTTTGCA
Runx2	-476	CACCACA
	-537	TGTGGTG
Slug	-107	CACCTG
	-224	CAGCTG
	-262	CAGCTG
	-472	CATCTG
	-589	CAGATG
	-882	CAGTTG
	-945	CAAATG

Table 1. Positions and putative binding site sequences for Lef/Tcf, Runx2 and Slug transcription factors, identfied in the Slug promoter region represented in Figure 1B.

Furthermore, the same region of about 1 Kb was analyzed to search for sequence homology between the different Slug gene promoters of different animal species so far characterized. The alignment revealed a degree of identity, corresponding to 8.3%, 46.3% and 54.5% of hology compared with *Xenopus*, chicken and mouse.

This first computing approach, together with the consideration that Snail/Slug genes regulation by Lef1/Tcf and Runx2 factors has been highlighted in other animal species (Vallin *et al.*, 2001; de Fructos *et al.*, 2009), led us to analyze the binding sites for these transcription factors in the human Slug promoter, in order to understand their possible role in modulating the expression of Slug in human osteoblasts.

Effect of Lef1 on Slug promoter activity

To understand the role of Lef1/Tcf in the regulation of Slug promoter activity, we chose to focus on an approximately 1 Kb fragment upstream of the transcription start site in the *Slug* gene since it contains six binding sites for the Lef/Tcf family, as aforementioned.

The sequence was cloned upstream of the Luc reporter gene in the pGL3-basic vector (see Figure 2), and the construct, (named 982 bp luc-construct), was assayed in five hOBs in presence of Lef1 expression plasmid (K14-myc-hLef1).

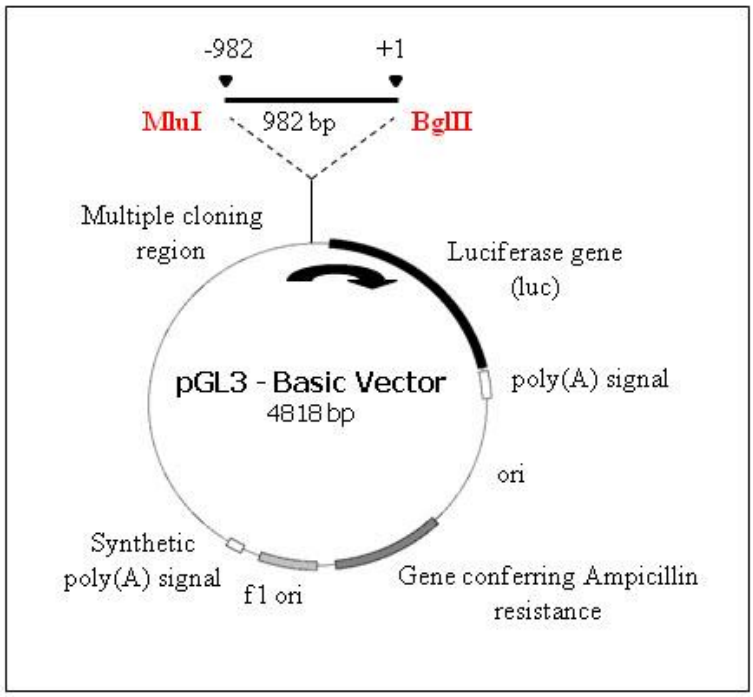


Figure 2. *Representation of the plasmid construct used for cloning the sequence under investigation.*The vector has an origin for replication in bacteria (ori), an origin for phage reliplication (f1 ori), a selection marker, represented by a gene conferring resistance to ampicillin, and a luciferase reporter gene. It also has a multiple cloning site, which was digested using restriction enzymes MluI and BglII and, in which the region to be analyzed was cloned.

As shown in Figure 3, transient transfection with the luciferase reporter 982 bp luc-construct (pGL3-Slug) resulted in an increase in luciferase activity relative to the empty, promoterless pGL3-basic vector, demonstrating that this DNA fragment contains

significant promoter activity in hOBs and in SaOS-2 cells. On the contrary, the same experiments performed in the non-osseous Slug-negative MCF7 breast cancer cell line revealed no promoter activity.

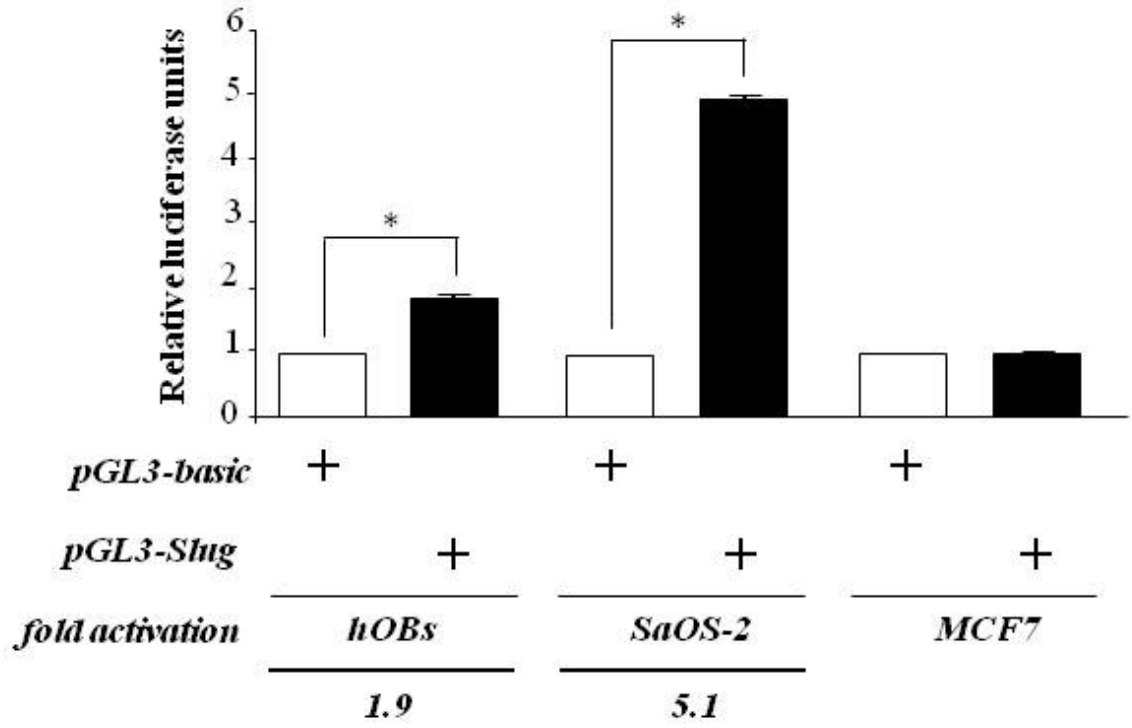


Figure 3. *Analysis of human Slug promoter activity.* The DNA construct containing the human Slug promoter region was cloned into upstream of the firefly luciferase (Luc) reporter gene. hOBs and SaOS-2 osteoblast-like cells were transfected with the pGL3-Slug Luc reporter vector containing the sequence from +1 to -982 of the human Slug promoter (pGL3-Slug 982 bp), and with the promoterless pGL3-basic vector. The results of reporter gene assays were normalized with protein concentration and β-gal activity for transfection efficiency and the data are represented as ratios of luciferase units to β-galactosidase units. MCF7 breast cancer cell line was used as negative control. All experiments were performed in triplicate and the average of the ratio of the reporter activity + SEM is shown. * = $p < 0.05$.

Cotransfection with plasmid encoding Lef1 produced a significant increase in Luc activity in hOBs as compared with cells containing the 982 bp luc-construct reporter plasmid. As expected, this increase was dramatic in Lef1 overexpressing SaOS-2 cells, because of a

higher intrinsic transfection facility of this cell line (see Figure 4). As a whole, these data indicate that Lef1 positively affects activity of Slug gene promoter in normal human osteoblasts.

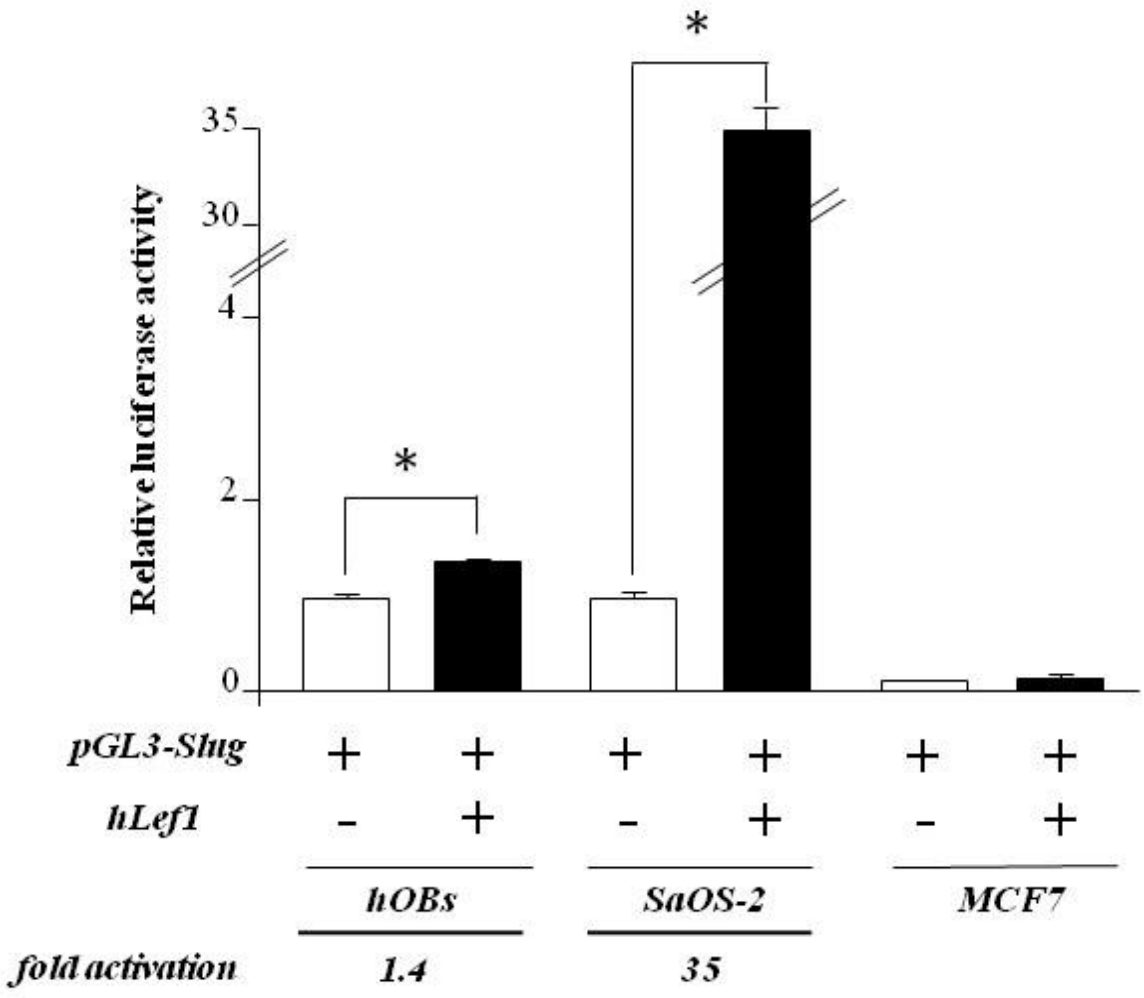
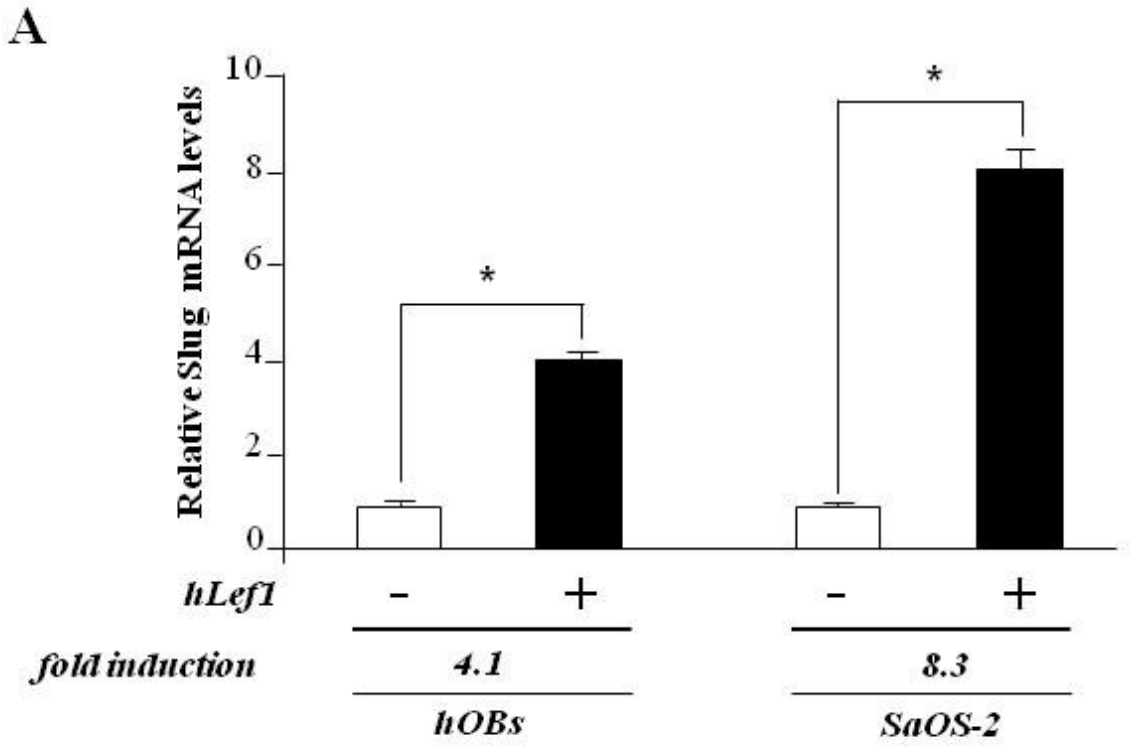


Figure 4. *Lef1 affects the activity of human Slug promoter.* The DNA construct containing the human Slug promoter region was cloned into upstream of the firefly luciferase (Luc) reporter gene. hOBs and SaOS-2 osteoblast-like cells were transfected with the pGL3-Slug Luc reporter vector containing the sequence from +1 to -982 of the human Slug promoter (pGL3-Slug 982 bp), in the absence (-) or presence (+) of 2.5 µg of hLef1 expression plasmid. The results of reporter gene assays were normalized with protein concentration and β-gal activity for transfection efficiency and the data are represented as ratios of luciferase units to β-galactosidase units. MCF7 breast cancer cell line was used as negative control. All experiments were performed in triplicate and the average of the ratio of the reporter activity + SEM is shown. * = $p < 0.05$.

Effect of Lef1 overexpression on Slug gene expression

hLef1 cDNA-transfected hOBs were then subjected to analysis of Slug gene expression. As shown in Figure 5, Slug expression significantly increased in Lef1 overexpressing cells, both at mRNA and protein level, as demonstrated by RT-PCR (see Figure 5A) and western blot analysis (see Figure 5B), respectively. As expected, forced expression of Lef1 increased Slug expression to higher levels in SaOS-2 cell line, in agreement with data obtained by luciferase assay.



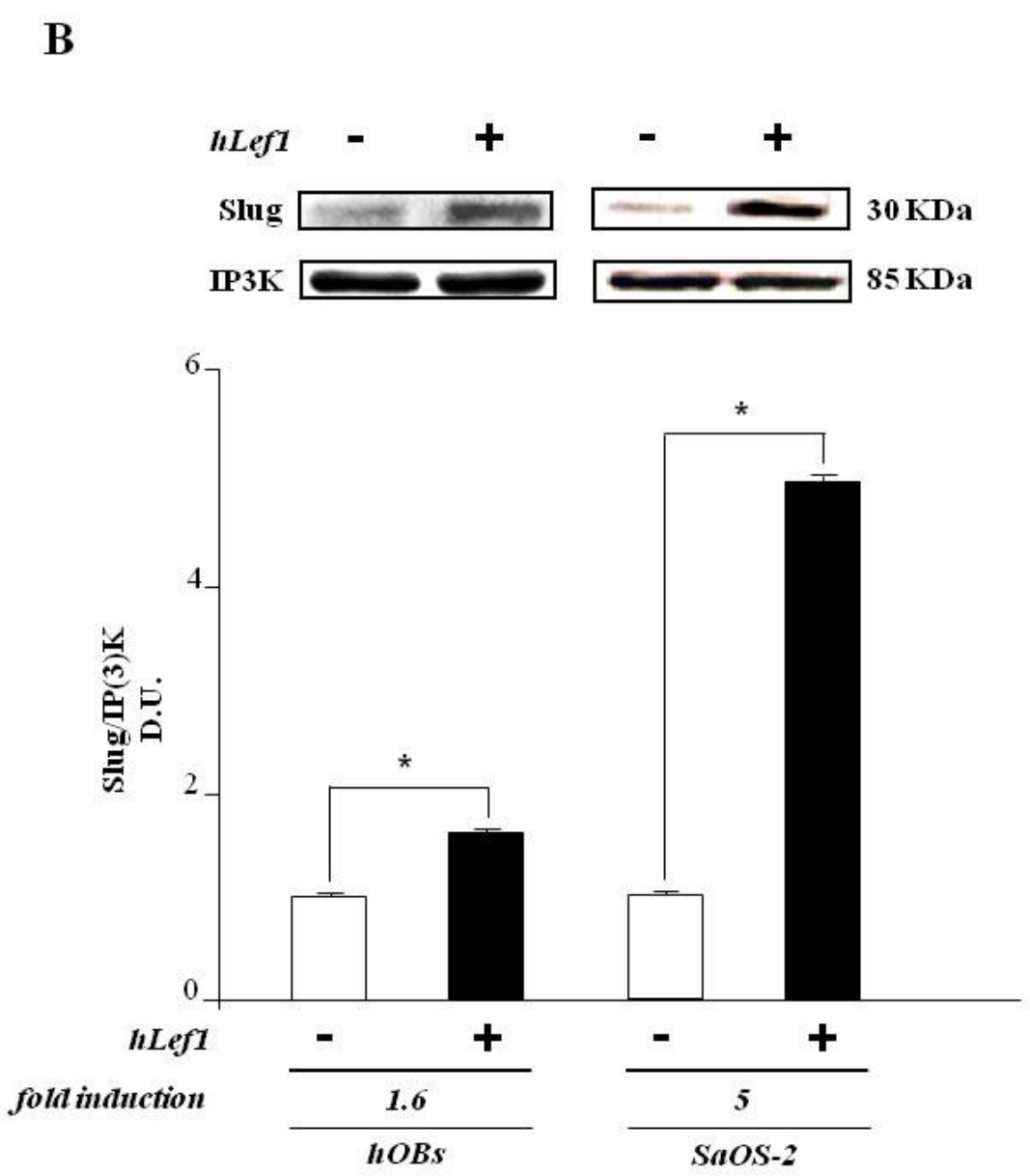


Figure 5. *Effect of Lef1 overexpression on Slug expression in hOBs.* The effect of Lef1 overexpression was examined at mRNA (A) and protein (B) level. A. Slug mRNA was evaluated by quantitative RT-PCR in hOBs and SaOS-2 osteoblast-like cells transfected with 2.5 µg of hLef11 (K14-myc-hLef1) expression plasmid. The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for Slug transcript analysis. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate. ΔΔCt method was used to compare gene expression data; standard error of the mean (SEM) was calculated. * = $p < 0.05$. B. Slug protein levels were examined by Western blot analysis in hOBs and SaOS-2 osteoblast-like cells transfected with 2.5 µg of hLef1 expression plasmid. Whole cell lysates were prepared and 10 µg of protein run on a 12% SDS-polyacrylamide gel. The proteins were visualized using Supersignal West Femto Substrate (Pierce). The quantitative presentation of the protein levels were performed by densitometric analysis using Anti-IP3K as control. D.U. = densitometric units. This experiment was repeated three times with similar results. A representative Slug Western blot analysis with size markers (KDa) is reported. * = $p < 0.05$.

"*In vivo*" recruitment of Lef1 on Slug gene promoter

Next, we investigated whether Lef1 could, "*in vivo*", physically bind with the human Slug promoter. Western blot analysis reveald that all analyzed hOBs and SaOS-2 cells expressed remarkable levels of Lef1 and other Tcf family members, including Tcf-1 and Tcf-4. All three transcription factors were investigated in the experiments described below (see Figure 6).

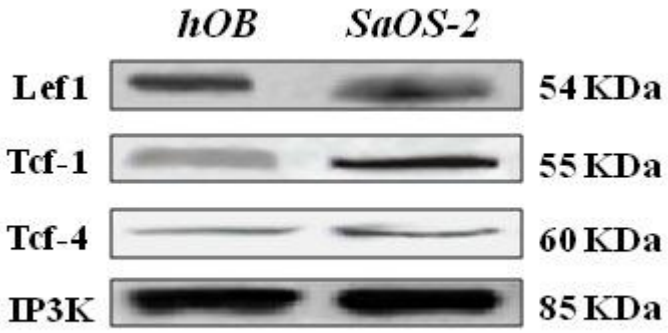


Figure 6. *Western blot analysis of Lef1, Tcf-1 and Tcf-4 expression levels in hOBs and SaOS-2 osteoblast-like cells.* 10 µg of whole cell lysates were assayed on a 12% SDS-polyacrylamide gel. The proteins were visualized using Supersignal West Femto Substrate (Pierce). Size markers are reported (KDa). IP3K was used as a loading control.

The "*in vivo*" transcription factors to the Slug promoter was studied by chromatin immunoprecipitation (ChIP) assays (see Figure 7B). To this aim, hOBs were exposed to formaldehyde to cross-link proteins and DNA, and were sonicated to fragment the chromatin. Specific antibody against Lef1, Tcf-1 and Tcf-4 were used to immunoprecipitate the protein-DNA complexes. After immunoprecipitation DNA was extracted from the beads and used as a template to generate specific PCR products. The presence of the promoter specific DNA region before immunoprecipitation was confirmed by PCR (input). In the Slug promoter fragment used for the reporter assay, three different regions were identified, and analyzed by a set of primers (see Figure 7A and Table 1 in "Materials and Methods" section) spanning the six consensus binding sites for the

Lef1/Tcf family. The amplified product sizes (bp) were 178 for region 1, 164 for region 2, and 165 for region 3. The results showed that all transcription factors analyzed were recruited to the Slug promoter even if at different levels (see Figure 7B). In particular: a) the promoter region 3, containing the previously identified Tcf binding site at -859/-855 position (Saegusa *et al.*, 2009; Hong *et al.*, 2009), was immunoprecipitated by Lef1 and Tcf-4 antibodies; b) Lef1 was mostly associated with the promoter region 1 and not at all with the promoter region 2; c) region 2 was occupied by Tcf-1 and Tcf-4 but not by Lef1; d) Tcf1 was less involved in the "*in vivo*" binding.

Therefore, the observation that the endogenous Slug gene expression may be increased by Lef1 was validated by the "*in vivo"* occupancy of the Lef/Tcf regulatory sites in the Slug gene promoter.

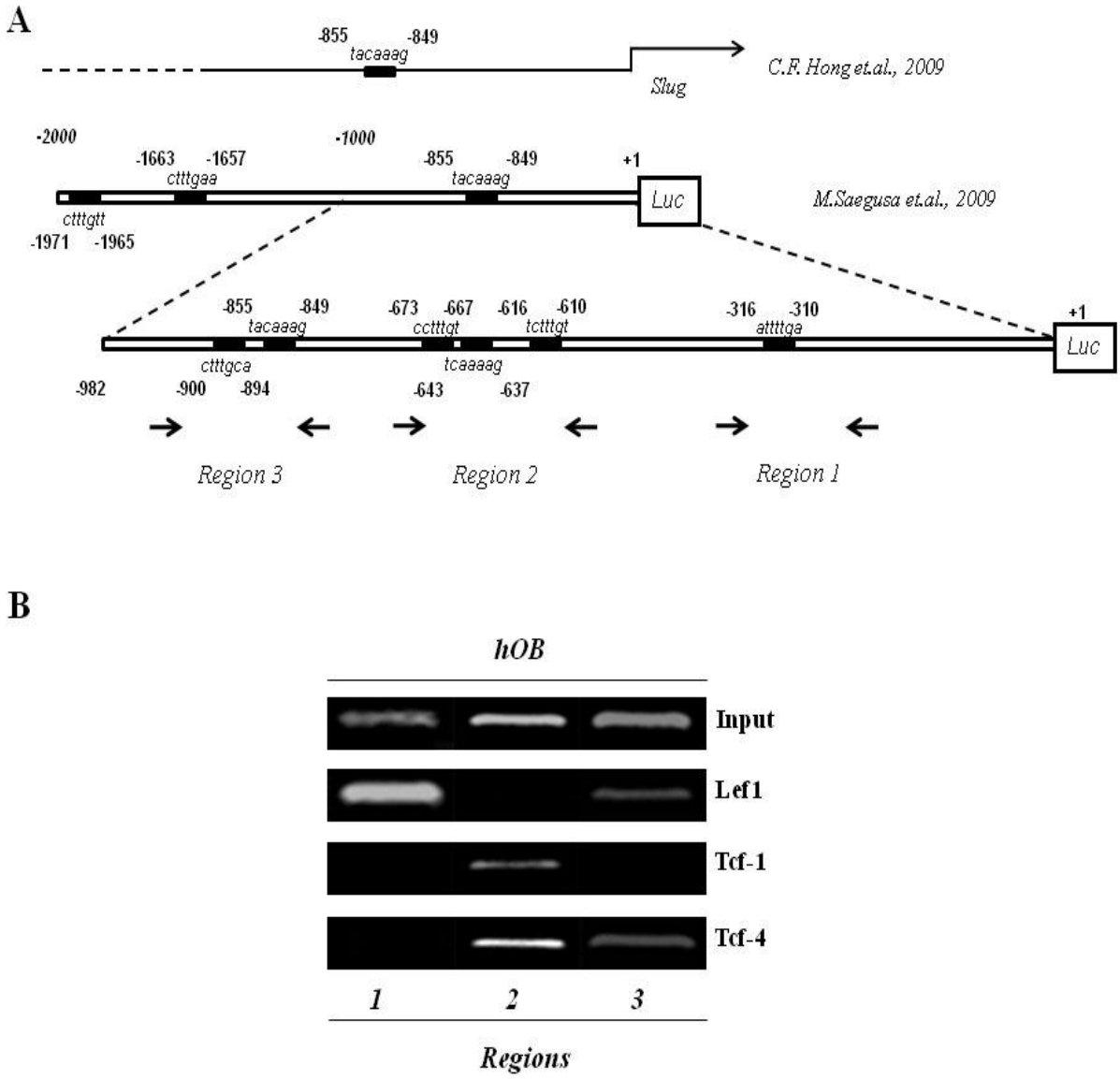


Figure 7. ***"In vivo" recruitment of Lef1 on Slug gene promoter.*** A. The Slug promoter region under investigation is reported. The positions of putative Lef/Tcf binding sites are enclosed by rectangles and are compared with those recently investigated by others. Positions of PCR primers used in ChIP experiments are also reported. B. Recruitment of Lef1/Tcf transcription factors to the human Slug promoter is demonstrated by "*in vivo*" chromatin immunoprecipitation (ChIP) binding assays. Soluble chromatin was prepared from hOBs and immunoprecipitated with the indicated specific antibodies against Lef1, Tcf-1, and Tcf-4. The associations of the transcription factors to bound precipitated DNA were monitored on the human Slug promoter regions 1, 2 and 3 by PCR with the primers indicated in the scheme. Input represents a positive control using the starting material (0.2%) prior to immunoprecipitation. Representative agarose gels are shown.

Regulation of Slug promoter activity through Wnt signaling activation by GSK-3β inhibitor

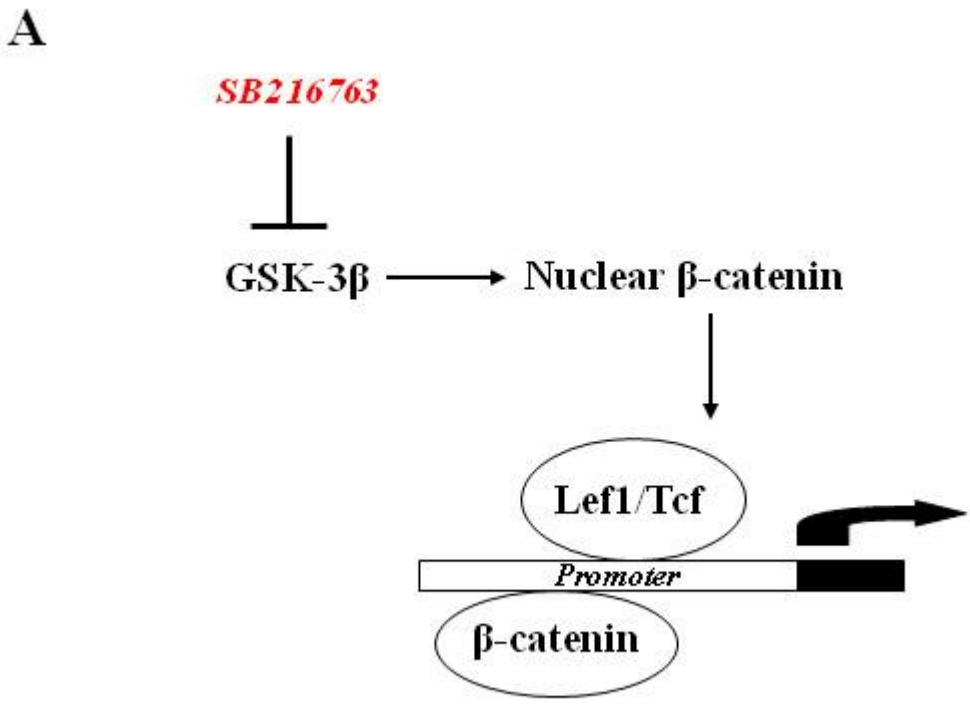
It has been demonstrated that β-catenin may act as co-factor promoting Lef/Tcf interaction with target DNA sequences in many cellular contexts (Shimizu *et al.*, 2008). In order to support the role of Lef/Tcf transcription factors in Slug expression regulation, we next investigated whether β-catenin activation was involved in Slug expression regulation. We used a treatment with a maleimide compound named SB216763, which was previously demonstrated to be efficient in β-catenin activation (see Figure 8A). This compound binds and specifically inhibits glycogen synthase kinase GSK-3β, a serine/threonine kinase, originally identified as a kinase that is involved in glucose metabolism. Recent research has determined that GSK-3β acts on a wide variety of substrates, including transcription factors, and is a key regulator in many signaling pathways (Cohen and Frame., 2001).

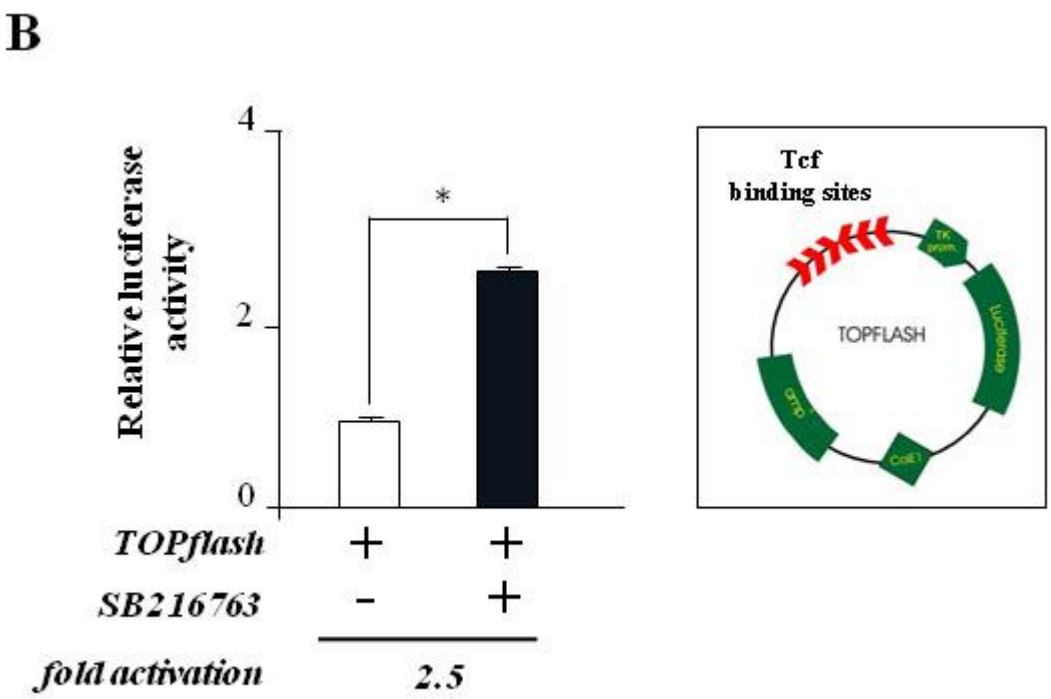
In particular and for what concerns our interests, this enzyme is known to be a key negative regulator of canonical Wnt/β-catenin and PI3K/Akt signalings (Patel *et al.*, 2004); hence, its inhibition activates Wnt signaling selectively via the β-catenin/Tcf pathway and results in relocation of stabilized β-catenin to the nucleus. We transfected hOBs with a β-catenin/Tcf transcription reporter construct named TOPflash reporter system to investigate the efficancy of SB216763 treatment.

As shown in Figure 8B an increase in TOPflash activity up to 4-fold was observed after SB216763 treatment, demonstrating the sensitivity of our experimental model to this approach. Therefore, as TOPflash has three Tcf-binding sites, it could be applied to represent the activation of the Wnt pathway in hOBs.

The efficancy of SB216763 treatment was confirmed at protein expression level. In fact, Western blot analysis showed an increase in β-catenin expression after SB216763 treatment (see Figure 8C). The same analysis demonstrated that the increase in β-catenin mediated by SB216763 was correlated with a significative increase in Slug and Runx2 protein expression level (see Figure 8C). Therefore, on the whole, this suggests that the canonical Wnt signaling positively affects Slug expression in normal human osteoblasts

via the β-catenin/Tcf pathway because, by potentiating β-catenin, Slug expression increases.





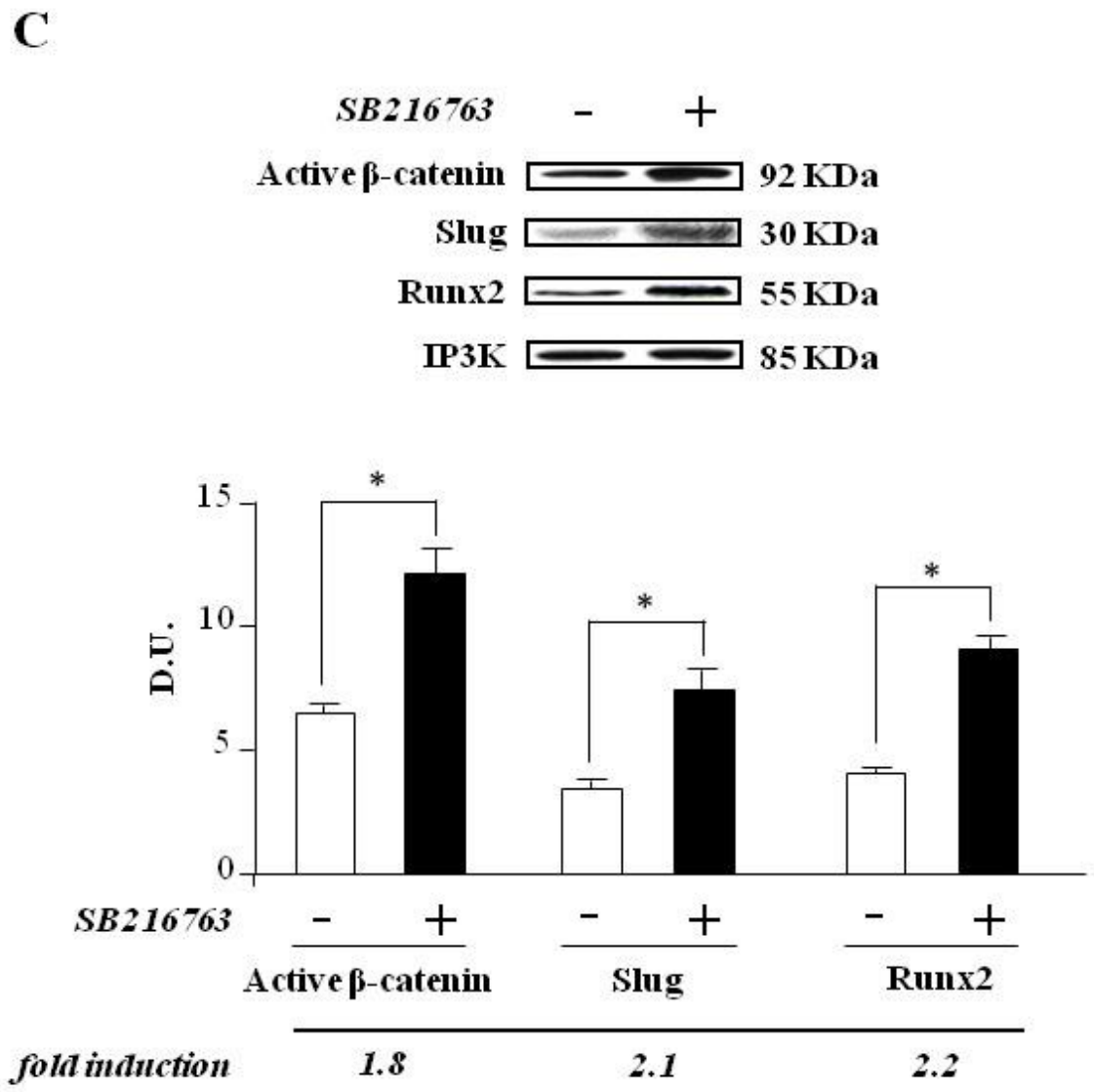


Figure 8. *Treatment of hOBs with the glycogen synthase kinase (GSK-3b) inhibitor, SB216763*. A. A scheme of SB216763 action mechanism is reported (see the text for details). B. Effect of SB216763 on the TOPflash reporter system. 24 h after transient transfection with the TOPflash plasmid, the cells were treated (+) or not (-) with SB216763 (10 μM) for 24 h prior to harvest. Luciferase activity was normalized to β-galactosidase activity in the same sample. The bars represent mean ± SEM. * = $p < 0.05$. On the right, schematic representation of TOPflash reporter plasmid containing two sets (with the second set in the reverse orientation) of three copies of the TCF binding site (in red) upstream of the Thymidine Kinase (TK) minimal promoter and Luciferase open reading frame. C. The levels of β-catenin expression, Slug and Runx2 were examined by Western blot in hOBs treated with SB216763 (10 μM) or with the only vehicle (-). The quantitative presentation of the protein levels was performed by densitometric analysis using Anti-IP3K as control. D.U. = densitometric units. A representative Western blot analysis with size markers (KDa) is reported. * = $p < 0.05$.

Runx2 interacts "*in vivo*" with Slug gene promoter

The results described above prompted us to investigate whether also Runx2 transcription factor may be directly involved in the control of Slug transcription in hOBs. To this aim, hOBs were exposed to formaldehyde to cross-link proteins and DNA, and sonicated to fragment the chromatin. Specific antibody against Runx2 was used to immunoprecipitate the protein–DNA complexes. The presence of the promoter-specific DNA region before immunoprecipitation was confirmed by PCR (input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate specific PCR products spanning the putative Runx2 binding sites localized at -537 and -476 from the transcription start site +1 in the Slug promoter, as indicated in Figure 1 and 9. Runx2 recruitment was assessed at the promoter region under investigation, as indicated in Figure 9, by using specific sets of primers (see Table 1 in "Materials and Methods" section). Runx2 occupancy was detected only at the region 4. As expected, no recruitment of this bone-specific transcription factor was observed in a sequence of Slug promoter, (region 5), lacking of Runx2 binding sites (see Figure 9).

These ChIP experiments demonstrated that Slug is also a Runx2 target gene in human osteoblasts.

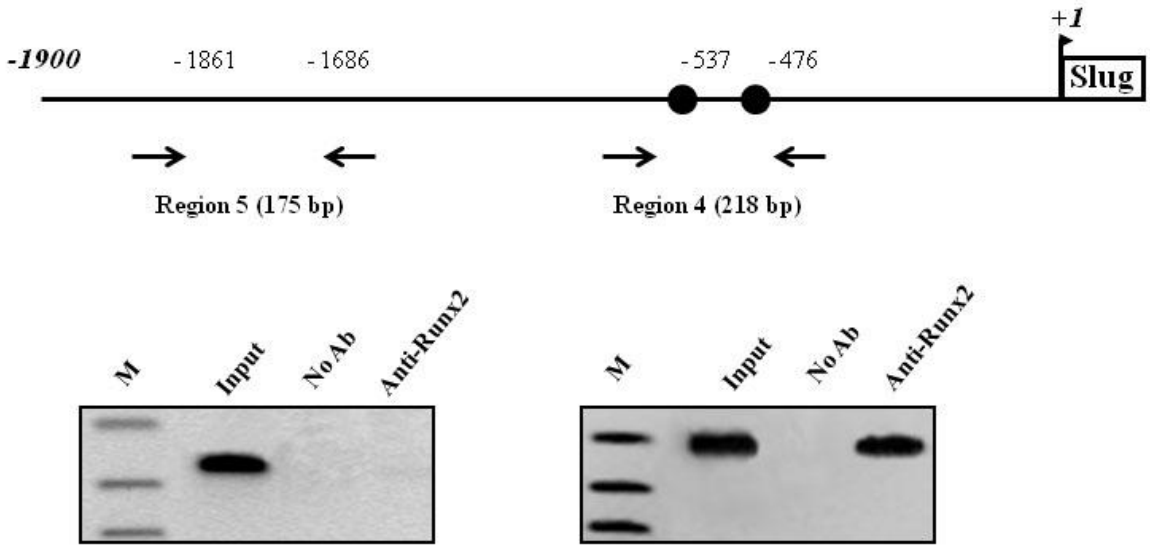


Figure 9. ***"In vivo" recruitment of Runx2 transcription factor on human Slug promoter.*** The Slug promoter region under investigation is reported (+1/-1900). The positions of the putative Runx2 consensus binding sites are enclosed by black circle. Recruitment of Runx2 transcription factor to the human Slug promoter is demonstrated by "*in vivo*" ChIP binding assays. Protein-DNA complexes were formaldehyde-cross-linked in hOBs in vivo. Chromatin fragments from these cells were subjected to immunoprecipitation with antibody against Runx2. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the reported primers, which positions are indicated with arrows. Region 5, lacking Runx2 binding sites, represents a negative control of ChIP experiments. PCR fragments were resolved through 1.5% agarose gels. No Ab represents a negative control. Input represents a positive control using the starting material (0.2%) prior to immunoprecipitation. The molecular weights of PCR fragments are shown in parentheses. M: molecular weight marker.

Chapter 3

Slug and osteogenesis.

Phenotypical characterization and osteogenic potential of human osteoblasts

Human primary osteoblast cultures (hOBs) were generated from bone chips removed from the tibial plateau as reported in ‘‘Materials and methods’’ section. We first analyzed a panel of nine phenotypic markers in cells at the second passage in culture. All osteoblasts were highly positive for the typical osteogenic markers, including Runx2, collagen type 1 (Col1a1), bone sialoprotein (BSP), and osteocalcin (OC), and weakly positive to alkaline phosphatase (ALP). In addition, the cells were positive for estrogen receptor alpha (ERα), a protein that is known to be associated to osteoblast differentiation. The samples were negative for a typical hematopoietic marker (CD45), only partially positive for a mesenchymal marker such as CD146, and positive for CD105. After this analysis, the cells that we used may be considered mature osteoblasts because they express low levels of CD146 and ALP, and high levels of CD105, as previously reported (Tonnarelli *et al.*, 2008). The percentage of positive cells for the markers analyzed by immunocytochemistry in five hOB samples is shown in Table 1, and the immunocytochemical staining of a representative sample is reported in Figure 1A. Next, the cells were characterized for their osteogenic capacity. All hOBs exhibited an evident extracellular matrix mineralization after 21 days of culture under osteogenic conditions (a representative sample is shown in Figure 1B).

Markers	Percentage of positive cells				
	hOB1	**hOB2**	**hOB3**	**hOB4**	**hOB5**
CD45	negative	negative	negative	negative	negative
ALP	5	15	15	12	15
CD146	2	2	4	2	4
CD105	100	100	100	100	100
Col1a1	50	60	60	60	60
Runx2	80	80	80	80	80
BSP	100	100	100	100	100
OC	60	80	90	100	80
ER-α	100	100	100	100	100

Table 1. *Immunocytochemical analysis of human primary osteoblasts (hOBs).*

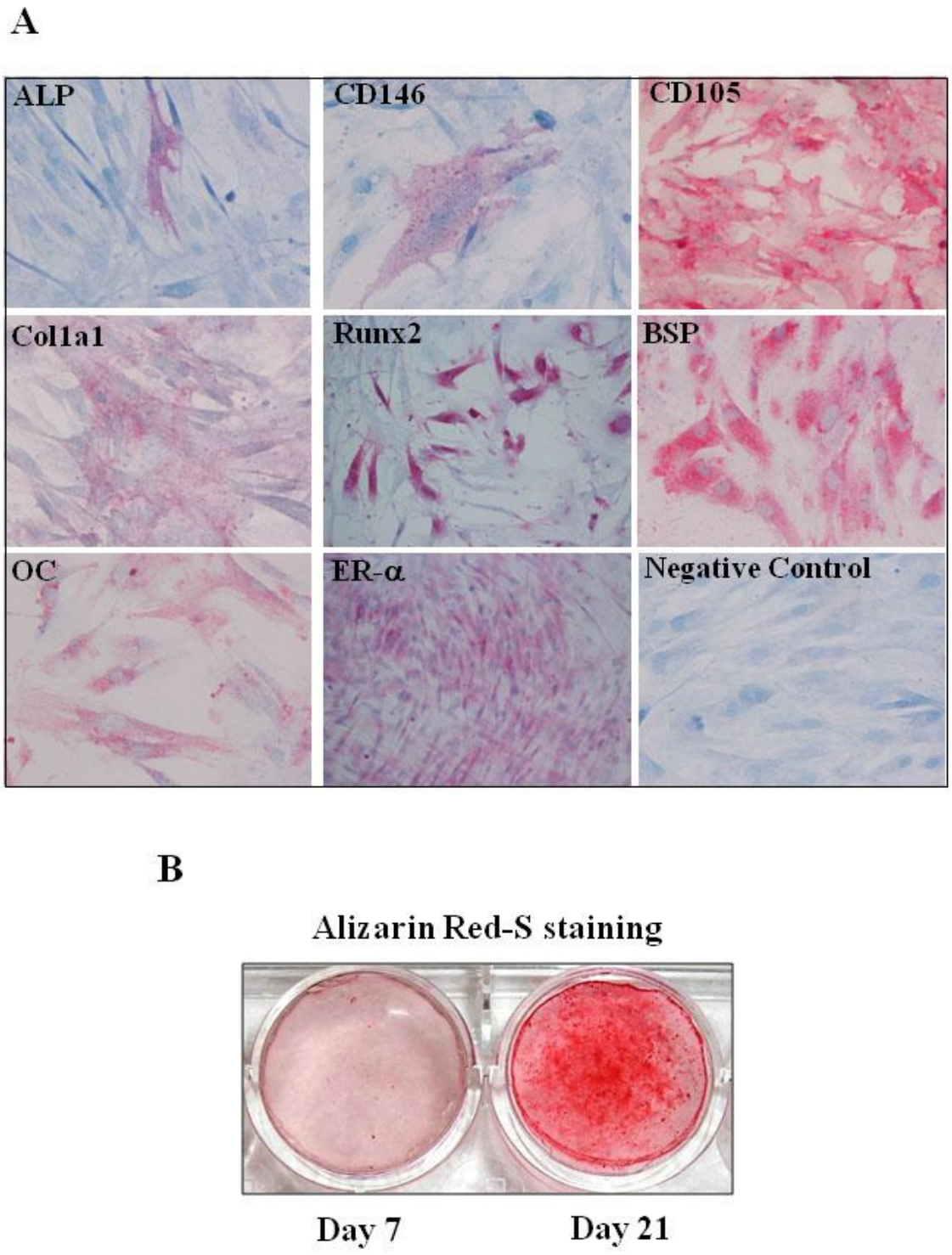


Figure 1. *Phenotypical characterization of hOBs.* Five hOB samples were subjected to immunocytochemical analysis for ALP, CD146, BSP, OC, CD105, Col1a1, Runx2, and ER-α phenotypical markers. A. The staining showed the local expression of the markers analyzed in a representative sample (X20 magnification). B. The deposition of extracellular matrix by cells treated with osteogenic medium. Mineral formation was examined by Alizarin Red S staining. The deposition of calcium salts was observed in osteogenic cultures at day 21.

Silencing of Slug gene expression by siRNA in hOBs

We firstly evaluated Slug expression in the five hOBs under investigation. As shown by western blot analysis performed on whole cell extracts (see Figure 2), all hOB samples express Slug protein at comparable levels, approximately the same that were found in Slug-positive MDA-MB-231 breast cancer cells (Tripathi *et al.*, 2005).

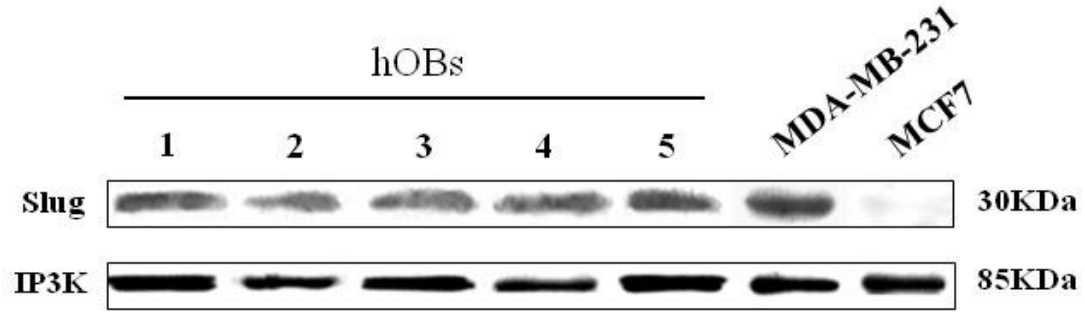


Figure 2. *Western blot analysis of endogenous Slug expression in hOBs.* 10 µg of whole cell lysates from five hOBs samples were assayed on a 12% SDS-polyacrylamide gel. The proteins were visualized using Supersignal Femto Substrate (Pierce). Size markers are reported (KDa). IP3K was used as a loading control. Positive and negative controls for Slug signal (Slug-positive MDA-MB-231 breast cancer cells, and Slug-negative MCF7 breast cancer cells, respectively) are shown.

In order, to test how Slug expression may be correlated with osteoblast phenotype, we used an indirect approach represented by a Slug knockdown through transient using siRNA. For this purpose, three siRNAs against Slug were designed, siRNA/Slug1, siRNA/Slug2, and siRNA/Slug3. Transfection of hOBs and osteosarcoma SaOS2 cell line with these siRNAs resulted in the down-regulation of Slug transcript by 30% (siRNA/Slug1 and siRNA/Slug3) or 80% (siRNA/Slug2) (see Figure 3).

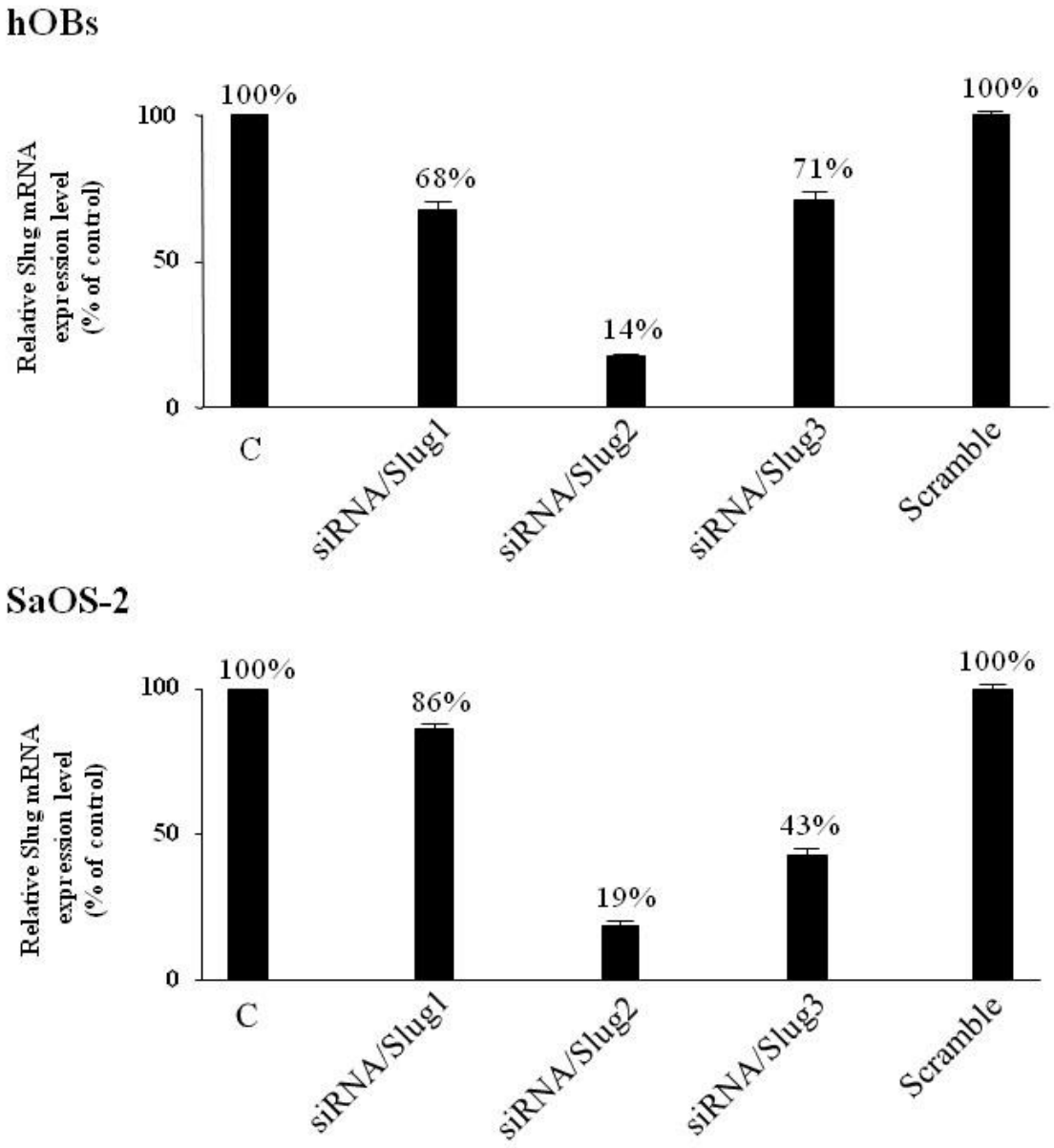


Figure 3. *Evaluation of Slug knockdown using three different siRNAs.* hOBs and SaOS-2 cells were transfected with three sets of Stealth siRNA duplexes against Slug (siRNA/Slug1, siRNA/Slug2, siRNA/Slug3) or a non relevant siRNA (scramble). Slug expression was determined after 6 days of treatment by quantitative RT-PCR. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug over control ratio. Results represent means ± SEM of six hOBs samples, and in SaOS-2 cell line rhe experiments were repeated three times.

Therefore, we chose to perform knockdown experiments treating the cells for 6 days with siRNA/Slug2 30 nM. As shown in Figure 4, western blot analysis confirmed the efficancy of siRNA/Slug2 that was able to inhibit protein expression by 80%.

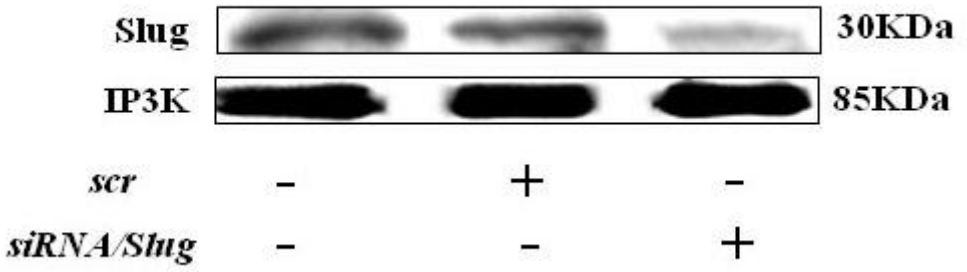


Figure 4. *Silencing of Slug gene expression by siRNA/Slug2 in hOBs.* hOBs were transfected with siRNA/Slug2 or a non-relevant siRNA (scr). Slug expression was determined at protein level as revealed by western blot analysis. Representative western blot of siRNA/Slug treated cells shows a specific decrease of endogenous Slug protein level.

Effect of Slug siRNA on Wnt signaling target genes in hOBs

In order to establish a role of Slug in osteoblasts, we firstly analyzed the effects of Slug silencing on the expression of mediators of a central pathway in bone metabolism, such as Wnt signaling.

As shown in Figure 5, Slug knockdown significantly reduced the protein levels of two important Wnt mediators, Lef1 and β-catenin, in all the analyzed samples. This evidence suggests that the levels of Slug may interfere with Wnt signaling modulating the levels of Lef1 and β-catenin transcription factors, and that Slug may consequently act by controlling the expression of specific genes in osteoblasts.

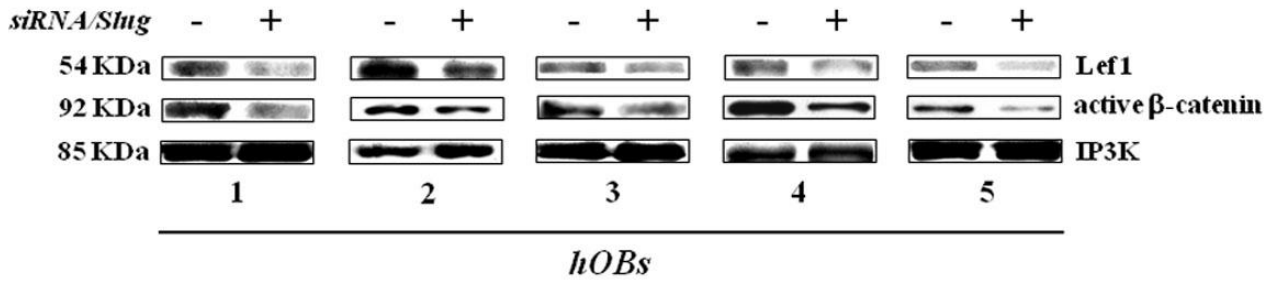
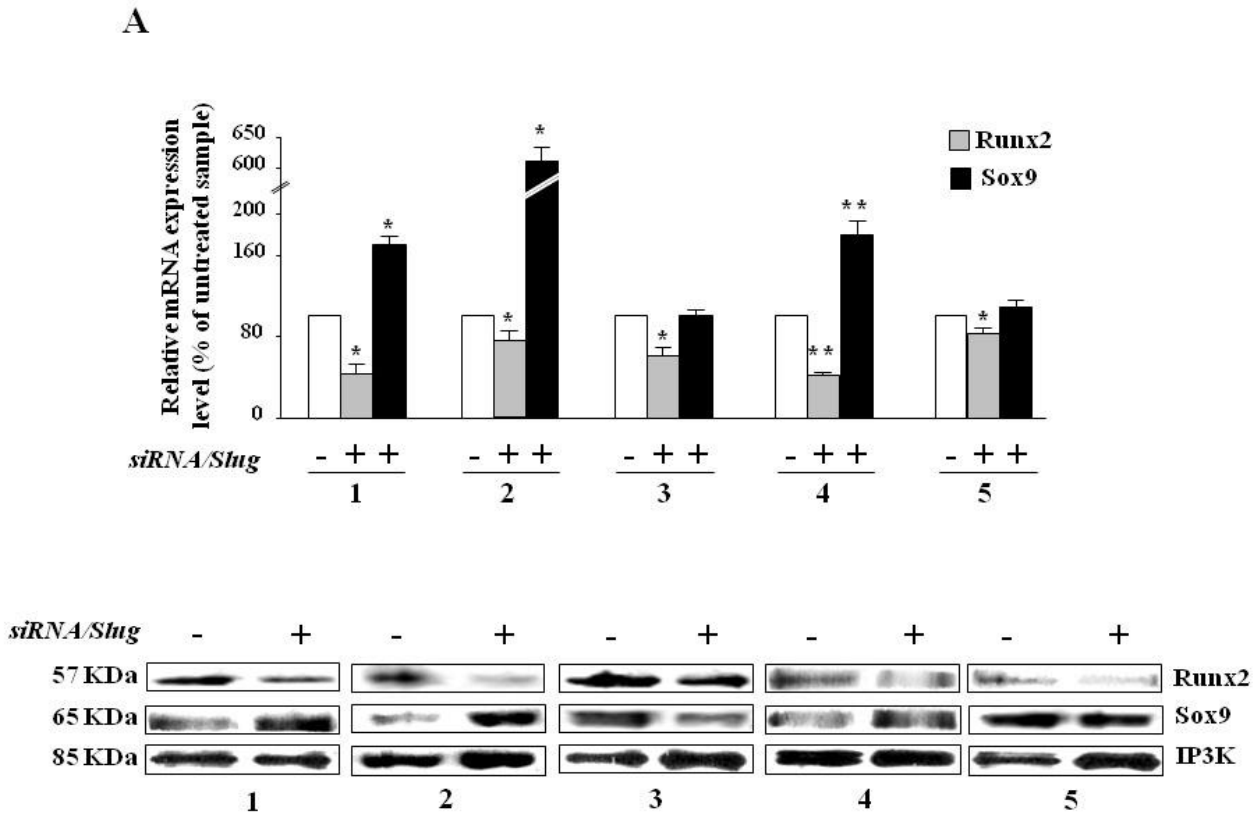


Figure 5. *Effect of Slug interference on Wnt protein mediators*. Western blot analysis of Lef1 and active β-catenin protein levels, after siRNA/Slug treatment in five hOB samples. Size markers are reported (KDa). IP3K was used as a loading control.

In order to strengthen this hypothesis, the expression of downstream target genes of canonical Wnt signaling such as Runx2, Sox9, osteocalcin, Rankl and c-myc were examined in Slug silenced cells. In Figure 6A the evaluation of Runx2 and Sox9 expression by RT-PCR and western blot is reported. Interestingly, the expression of Runx2, the master transcription regulator of osteoblasts previously identified as a Lef1/β-catenin target gene (Gaur *et al.*, 2005), was markedly decreased in Slug silenced cells compared to untreated osteoblasts. On the contrary, Slug knockdown induced expression of Sox9, a factor indispensable for chondrogenic development (Akiyama *et al.*, 2004), both at mRNA and protein level in four out five samples (see Figure 6A).

Accordingly, in four out five samples, the amount of secreted osteocalcin, which is a marker of late osteoblast differentiation positively modulated by Wnt signaling (Zhu *et al.*, 2008), was significantly reduced by Slug knockdown (see Figure 6B), as well as the expression of c-myc which is induced in response of activation of Wnt signaling (Katoh., 2008) (see Figure 6C). On the contrary, mRNA levels of the receptor activator of NFkB ligand (Rankl), the expression of which is repressed by Wnt signaling (Spencer *et al.*, 2006), was significantly increased in the siRNA/Slug hOBs treated samples (see Figure 6D). As a whole, these results suggest that Slug knockdown affects Wnt signaling and consequently its downstream target genes in human osteoblasts.

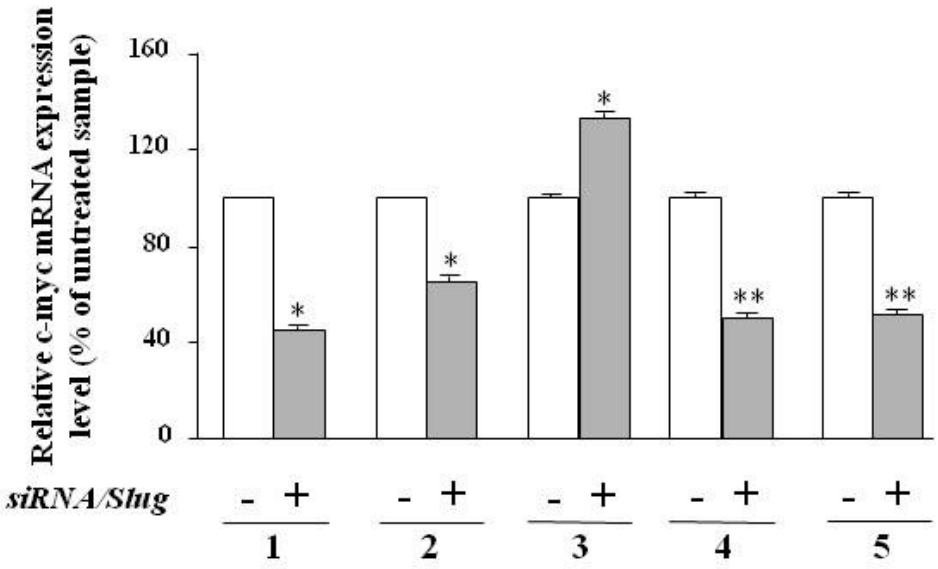

A
Relative mRNA expression level (% of untreated sample)
650
600
200
160
80
0
Runx2
Sox9
siRNA/Slug
- + +
1
2
3
4
5
siRNA/Slug
57 KDa
65 KDa
85 KDa
Runx2
Sox9
IP3K

B

Osteocalcin amount (ng/µg total protein ± S.D.)

	1	2	3	4	5
Control	102.52 ± 4.5	36.97 ± 5.49	32.61 ± 4.86	29.71 ± 6.7	38.55 ± 8.1
siRNA/Slug	78.41 ± 2.86	20.18 ± 1.54	18.87 ± 3.65	15.8 ± 2.19	81.92 ± 2.38

C



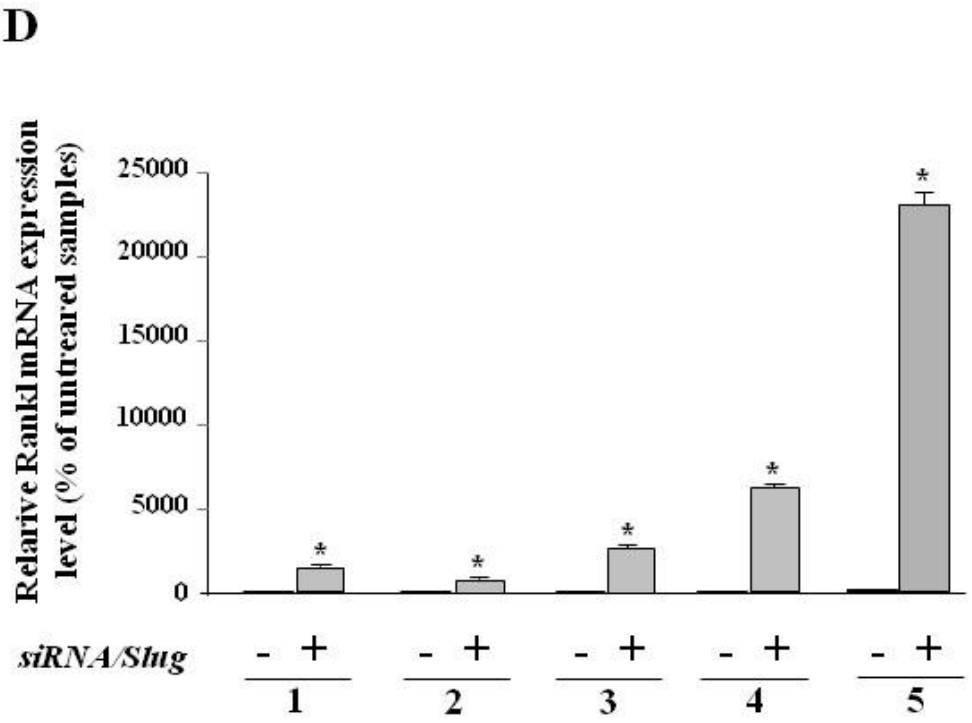


Figure 6. *Effect of Slug interference on Wnt signaling target genes in hOBs.* After 6 days of siRNA/Slug treatment the expression of bone-related Wnt target genes was analyzed in five hOB samples. A. Runx2 and Sox9 expression analysis in siRNA/Slug treated cells. mRNA and protein levels were determined by quantitative RT-PCR and western blot analysis, respectively. Osteocalcin (B), c-myc (C), and Rankl (D) expression analysis in siRNA/Slug treated cells. B. Osteocalcin protein was measured in cell culture supernatants by ELISA. The level of c-myc (C) and Rankl (D) expression was determined by quantitative RT-PCR. In all quantitative RT-PCR experiments the results, after correction to GAPDH content, are expressed as siRNA/Slug over control ratio. Results represent means ± SEM of triplicate determinations (*p < 0.05, **p < 0.01)

Effect of Slug siRNA on CXCL12 expression

In order to further delineate the function of Slug transcription factor in hOBs, we also evaluated the effect of Slug silencing on the expression of CXCL12, a chemokine which, together with its receptor CXCR4, has recently been found to play an important role in bone tissue remodeling and repair (Dominici *et al.*, 2009; Kitaori *et al.*, 2009).

We blocked the endogenous production of Slug by treating hOBs for 6 days with siRNA/Slug and obtained a strong inhibition of Slug mRNA, as revealed by quantitative RT-PCR (see Figure 7A). At the same time, we found an increase in CXCL12 mRNA (see

Figure 7A), but a significant decrease in CXCL12 protein in Slug-silenced cells, as seen by immunocytochemical (Figure 7B) and ELISA analysis (Figure 7C) by measuring CXCL12 intracellular expression and secretion, respectively. The absence of correlation between mRNA and protein levels of CXCL12 in Slug-silenced cells suggests that Slug is differently involved in determining CXCL12 expression at mRNA and protein level.

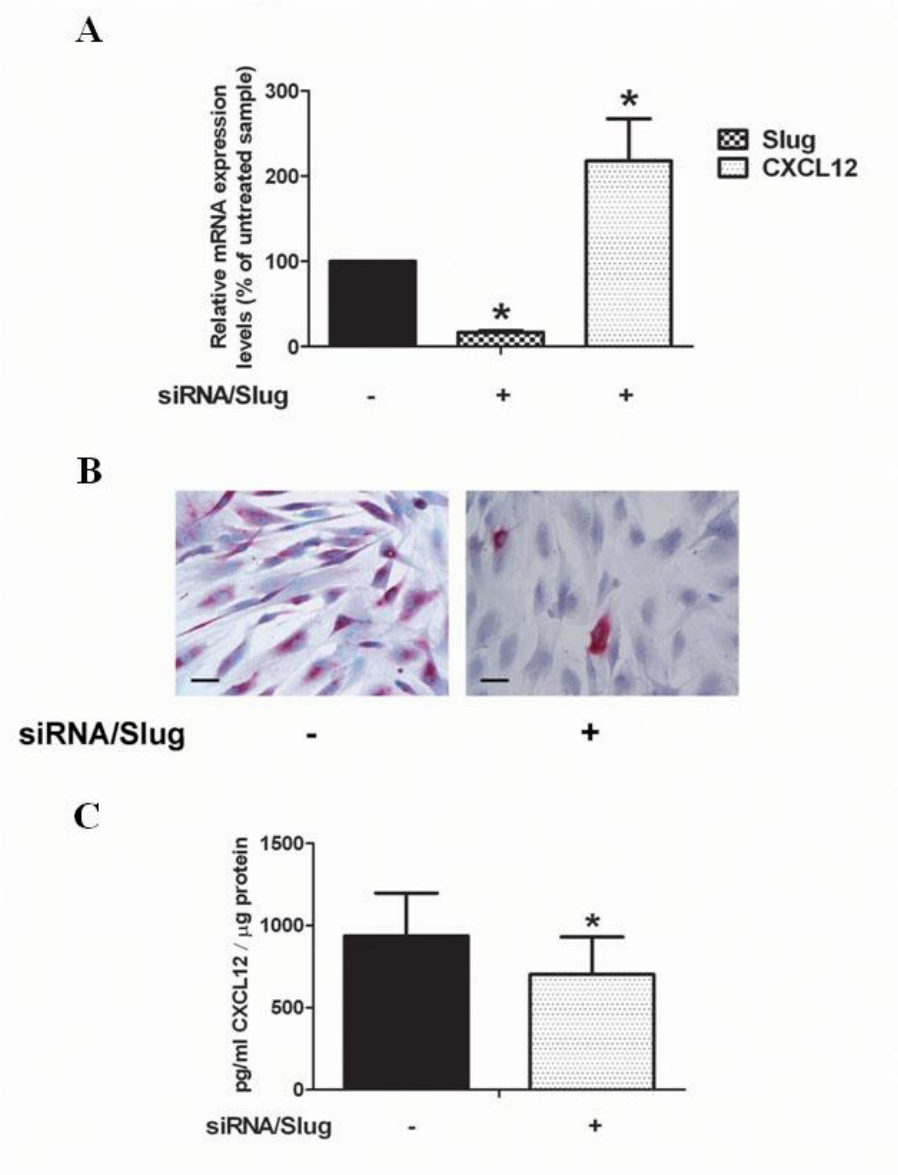
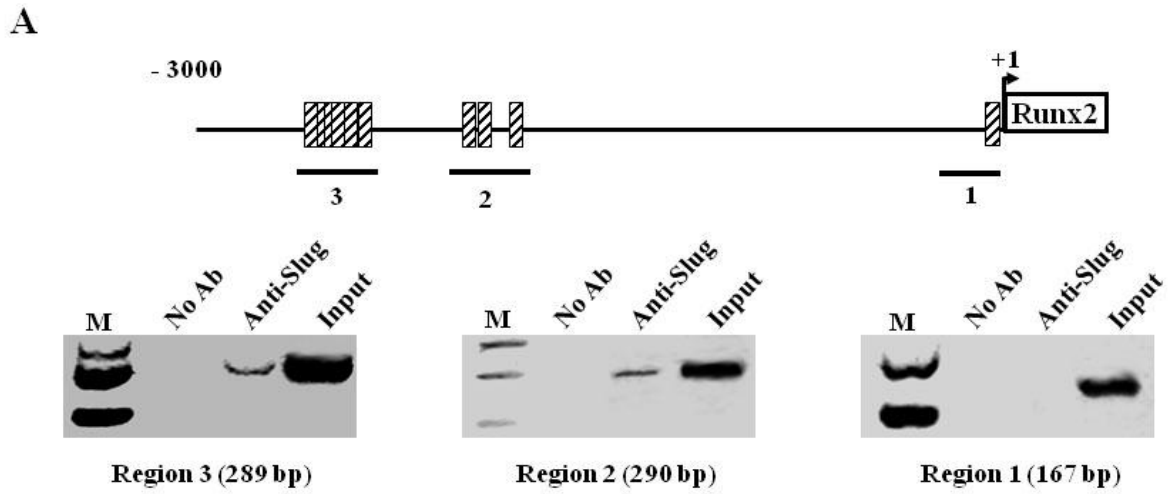
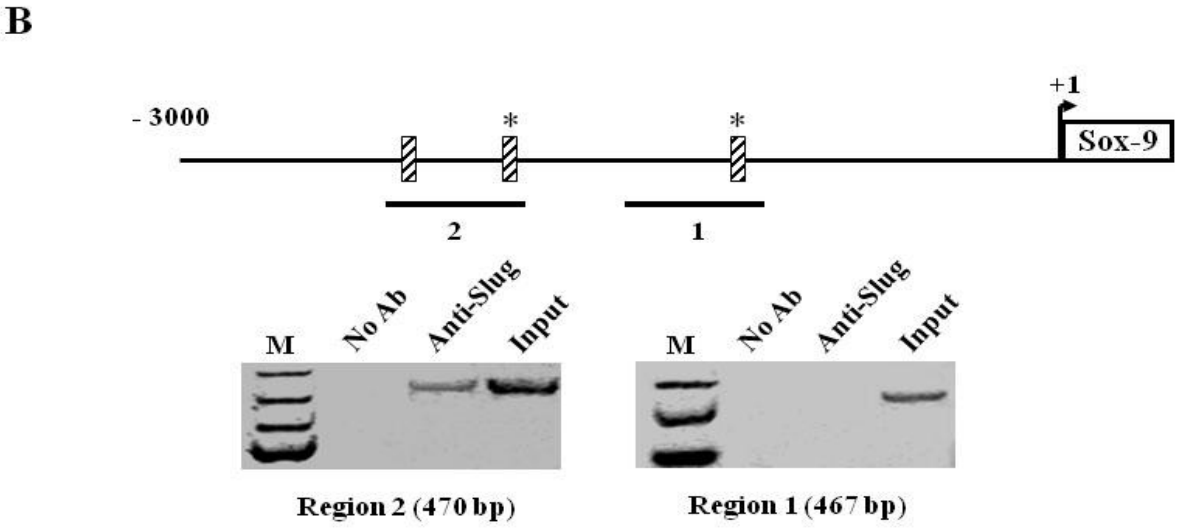


Figure 7. *Evaluation of Slug knockdown effect on CXCL12 expression.* A. Evaluation of Slug and CXCL12 mRNA expression both in control (-) and in siRNA/Slug silenced (+) hOBs. Data were expressed as relative mRNA expression levels (% of untreated sample) ± s.e.m. * $p < 0.05$. B. Immunocytochemical analysis of CXCL12 in control (-) and in siRNA/Slug silenced (+) hOBs. Scale bar = 25µm. C. CXCL12 protein secreted levels in control (-) and in siRNA/Slug silenced (+) hOBs. Data were expressed as pg/ml CXCL12/µg protein ± s.e.m.

"In vivo" recruitment of Slug on Runx2, Sox9 and CXCL12 gene promoters

In order to correlate the effect of Slug silencing on gene expression of specific genes with molecular events directly controlled by Slug, chromatin immunoprecipitation (ChIP) experiments were then performed. The promoters of Runx2, Sox9 and CXCL12 were chosen for this analysis. TFSEARCH program (www.cbrc.jp/research/db/TFSEARCH.html) revealed the presence of many putative Slug binding sites (E boxes) in the promoter of all three genes (see Figure 8). In order to determine the *in vivo* association between Slug and the promoter sequences, hOBs were exposed to formaldehyde to cross-link proteins and DNA, and sonicated to fragment the chromatin. Specific antibody against Slug was used to immunoprecipitate the protein–DNA complexes and verify the functionality of these sites. The presence of the promoter-specific DNA region before immunoprecipitation was confirmed by PCR (input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate specific PCR products spanning the putative Slug binding sites from -3000 bp to +1 bp in the promoter of Runx2 and Sox9 genes and from -2000 bp to +1 bp in the CXCL12 gene promoter. Slug recruitment was assessed at the different promoter regions, as indicated in Figure 8, by using specific sets of primers (see Table 1 in "Materials and Methods" section). These experiments allowed us to determine specific Slug occupancy: Slug was recruited at the region 2 and 3 of Runx2 gene (see Figure 8A), at the region 2 of Sox9 gene (see Figure 8B), and at the region 1 and 2 of CXCL12 gene. This was confirmed reapiting these ChIP experiments four times: this demonstrated that Runx2, Sox9 and CXCL12 are Slug target genes in human osteoblasts.


A
- 3000
+1
Runx2
3
2
1
M
No Ab
Anti-Slug
Input
Region 3 (289 bp)
Region 2 (290 bp)
Region 1 (167 bp)


B
- 3000
+1
Sox-9
*
*
2
1
M
No Ab
Anti-Slug
Input
Region 2 (470 bp)
Region 1 (467 bp)

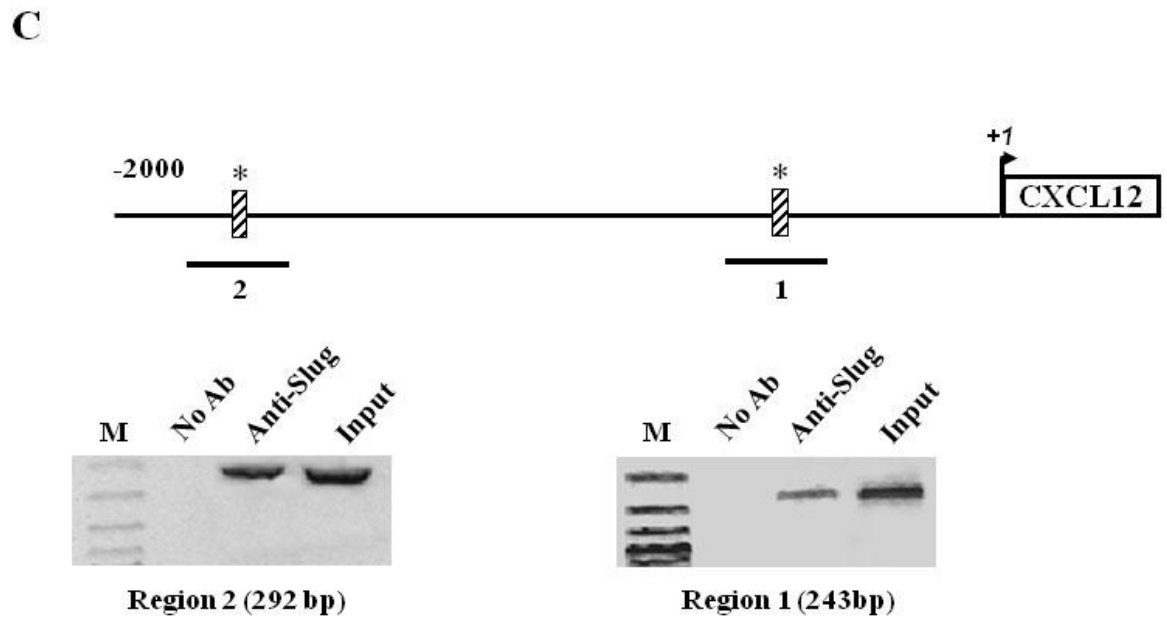


Figure 8. ***"In vivo" recruitment of Slug on Runx2, Sox-9 and CXCL12 gene promoters.*** Protein–DNA complexes were formaldehyde-cross-linked in hOBs "*in vivo*". Chromatin fragments from these cells were subjected to immunoprecipitation with antibody against Slug. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the primers reported in Table 1of "Materials and Methods" section, and specific for the indicated promoter regions. PCR fragments were resolved in 1.5% agarose gels. Aliquots of chromatin taken before immunoprecipitation were used as Input positive controls whereas chromatin eluted from immunoprecipitations lacking antibody were used as no antibody (NoAb) controls. The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the reported regions of Runx2 (A), Sox9 (B), CXCL12 (C) promoters. Representative agarose gels are shown. The specific molecular weights of PCR fragments are shown in parentheses. The relative positions of Slug putative binding sites (striped boxes) are indicated. *Sites showing 100% homology with consensus-binding site (CAGGTG). M: molecular weight marker.

Slug knockdown inhibits maturation of osteoblasts and supports differentiation of chondrocytes

The effect of Slug silencing was then examined at cellular functional level investigating the osteoblast maturation in terms of ALP activity and mineralized matrix deposition. As shown in Figure 9A, there was a significative decrease of ALP activity after 6 days of siRNA/Slug treatment. Accordingly, Slug silenced cells showed a reduced mineralization ability; in fact, 14 days after the initiation of osteoblast induction in osteogenic medium, a reduction up to 86% of mineral deposition was observed in Slug silenced cells in comparison with non-silenced cells (see Figure 9B). This decrease in osteoblast maturation was accompanied by a significative decrease of other correlated markers including Col1a1 and classical Runx2 target genes such as osteopontin and osteocalcin, all analyzed by quantitative RT-PCR (see Figure 9C).

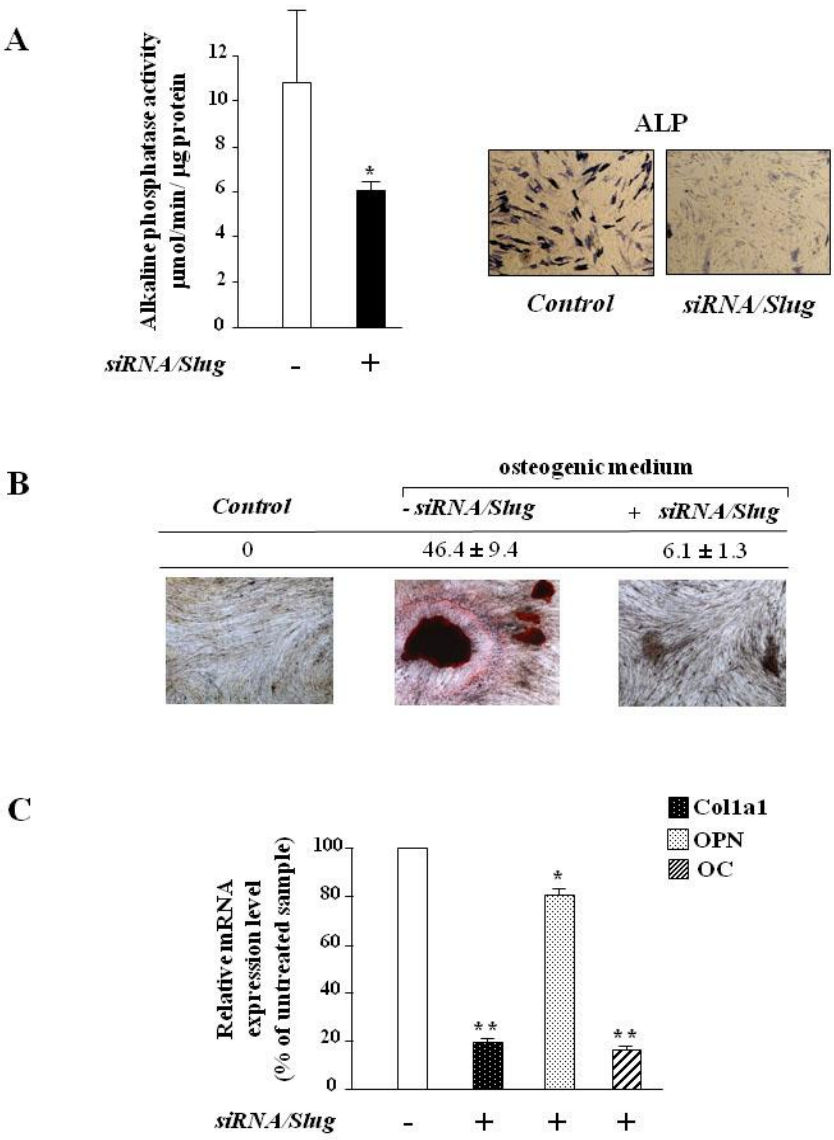


Figure 9. *Effect of Slug depletion on osteoblast maturation.* After siRNA/Slug treatment the cells were analyzed for the presence of alkaline phosphatase activity (ALP), formation of mineralized matrix and expression of osteoblast maturation markers. A. Alkaline phosphatase activity was evaluated by PNPP hydrolysis and ALP Leukocyte kit. The presence of sites of ALP activity appeared as blue cytoplasmic staining as shown in the reported representative sample (X20 magnification). B. Mineral formation was examined by Alizarin Red S staining in the cells cultured with β-glycerophosphate, ascorbic acid, and dexamethasone (osteogenic medium). The deposition of calcium salts was observed in osteogenic cultures at day 14, but not in control cells (not cultured in osteogenic medium), and was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer (''Quantity one'' software, Biorad). The ratio of the surface to the number of nodules in a representative hOB sample is reported. C. The expression of Col1a1, osteopontin (OPN) and osteocalcin (OC) was determined by quantitative RT-PCR in the cells cultured in osteogenic medium. The results, after correction to GAPDH content, are expressed as siRNA/Slug over control ratio. Results represent means ± SEM of triplicate determinations (*p< 0.05, **p< 0.01).

Since we have here demonstrated that Slug knockdown induced expression of Sox9 in hOBs, investigations were then performed to evaluate the potential pro-chondrogenic effect of Slug knockdown in human undifferentiated chondrocytes at passage 6–8. In these conditions, the cells express very low level of Collagen type 2 (Okazaki *et al.*, 2002). Slug mRNA expression was blocked in these cells with the same approach and effectiveness as in hOBs (see Figure 10). Runx2 and Sox9 expression was then tested by quantitative RT-PCR analysis. As shown in Figure 11A, a significative upregulation of both these genes, required for chondrocyte maturation (Komori., 2009), was found in all analyzed samples after Slug silencing. This effect was accompanied by a significant increase of Collagen type 2 evaluated by immunocytochemistry (see Figure 11B).

These preliminary findings demonstrate that when endogenous Slug levels are suppressed, chondrocytes are inclined to differentiate, supporting the idea that Slug may play a critical role as pro-chondrogenic factor.

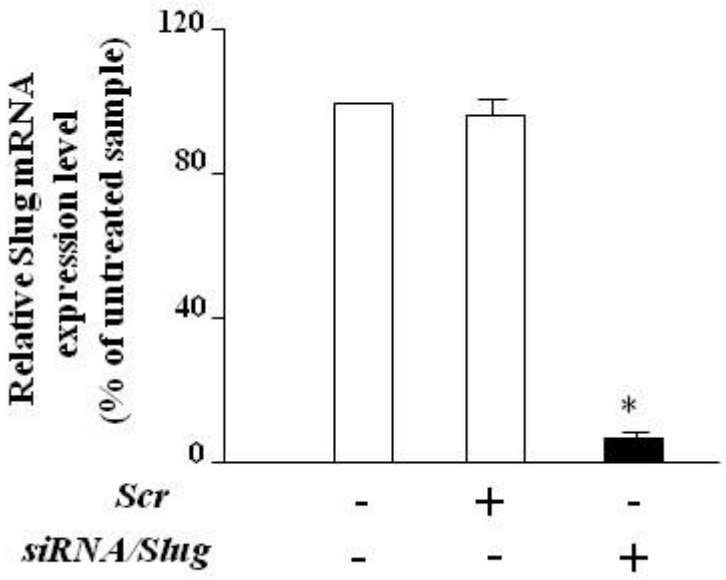


Figure 10. *Silencing of Slug gene expression by siRNA/Slug in undifferentiated chondrocytes.* Undifferentiated chondrocytes were transfected with siRNA/Slug or a non-relevant siRNA (scr). Slug expression was determined at mRNA, and revealed by quantitative RT-PCR. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug over control ratio. Results represent means ± SEM of three chondrocytes samples (*$p < 0.05$).

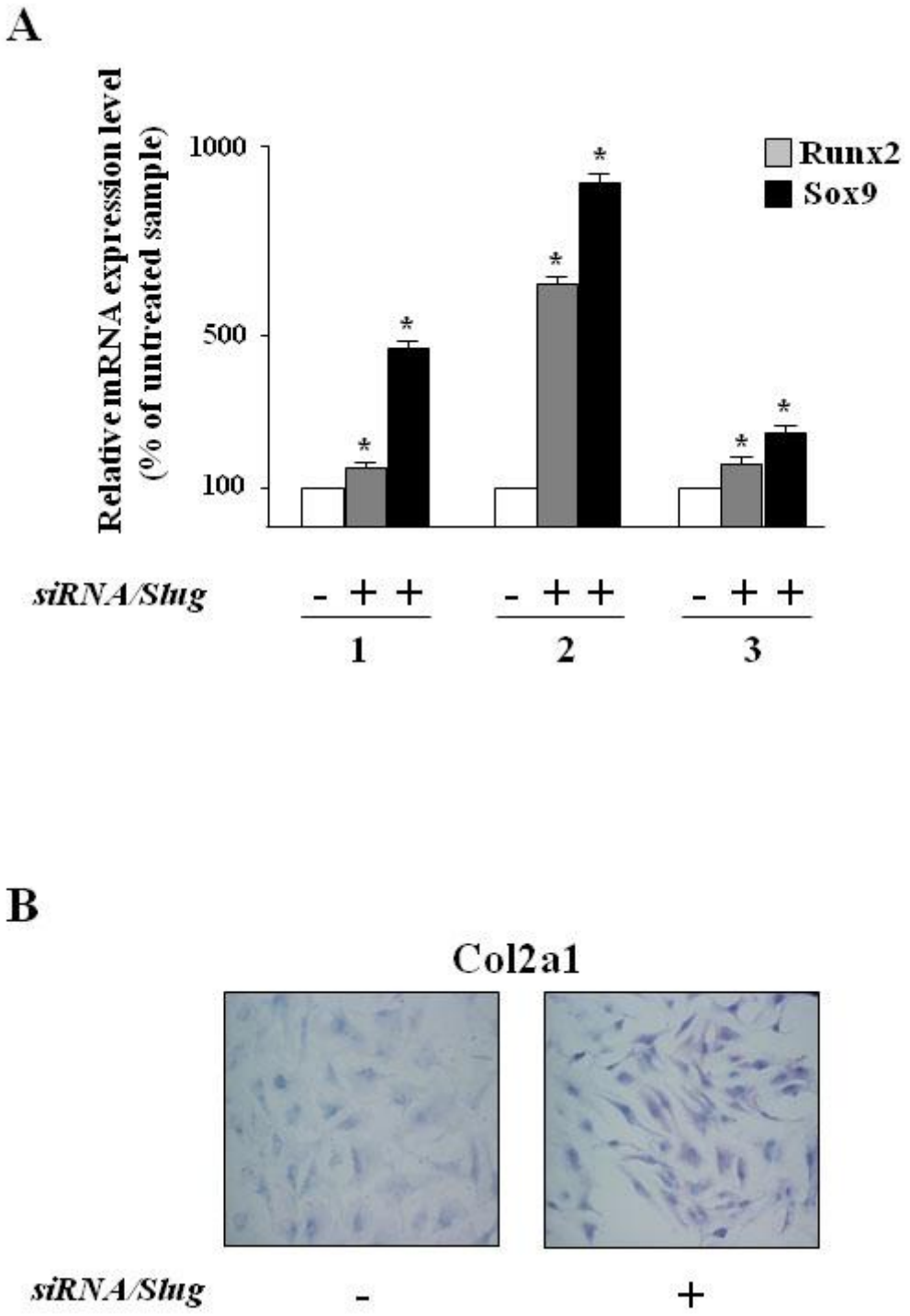


Figure 11. *Effect of Slug silencing on factors involved in chondrocytes differentiation.* A. Runx2 and Sox9 expression analysis in siRNA/Slug treated cells. mRNA levels of these samples were determined by quantitative RT-PCR analysis. Results means ± SEM of triplicate determinations (*p< 0.05). B. Collagen type 2 expression analysis in siRNA/Slug treated cells. The presence of Col2a1 protein was determined by immunocytochemistry and appeared as violet cytoplasmic staining, as shown in the reported representative sample (X20 magnification).

Chapter 4

Role of Slug transcription factor in human mesenchymal stem cells.

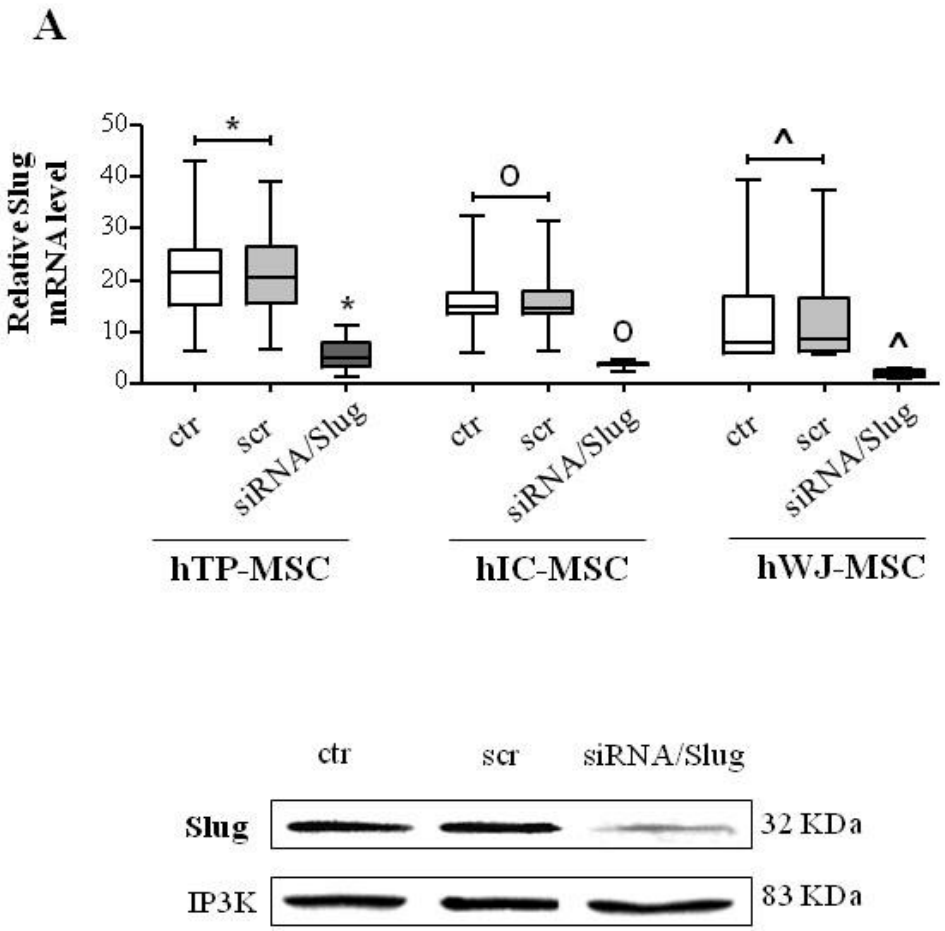
Silencing of Slug gene expression by siRNA in hMSCs

The informations derived from experiments on hOBs, led us to investigate whether the depletion of a transcription factor such as Slug, had a role in determining the fate of uninduced MSC progenitors. Therefore, we blocked Slug endogenous production in hMSCs using the same siRNA approach employed for hOBs and depicted in the previous chapter. The hMSCs isolated from tiabial plateau (TP) trabecular bone, iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord, described in chapter 1, were treated for 6 days with siRNA/Slug 30 nM. The efficiency of the treatment was tested in all hMSCs by quantitative real time RT-PCR (see Figure 1A) which revealed a decrease in Slug mRNA expression by about 80% ($p<0.05$). The efficiency of Slug silencing was also validated at protein level by western blot analysis (see Figure 1A).

Phase contrast microscopy observations suggested that transfected hMSCs slightly reduced their proliferation capacity, changed their morphology by becoming rounded in response to siRNA/Slug treatment, but remain viable (see Figure 1B).

To further explore the effects of Slug knockdown on cell function, we performed the scratch-wound healing assay. Scratching of the hMSC monolayer triggers a migratory event similar to the events that happen in fracture healing. We found that Slug-depleted cells had an impaired ability to close the wounded area compared with control and

scrambled cells. After 48 hours, gap closure in Slug-depleted hMSCs was significantly reduced because migration from the border of the wound was very slow (see Figure 1C).



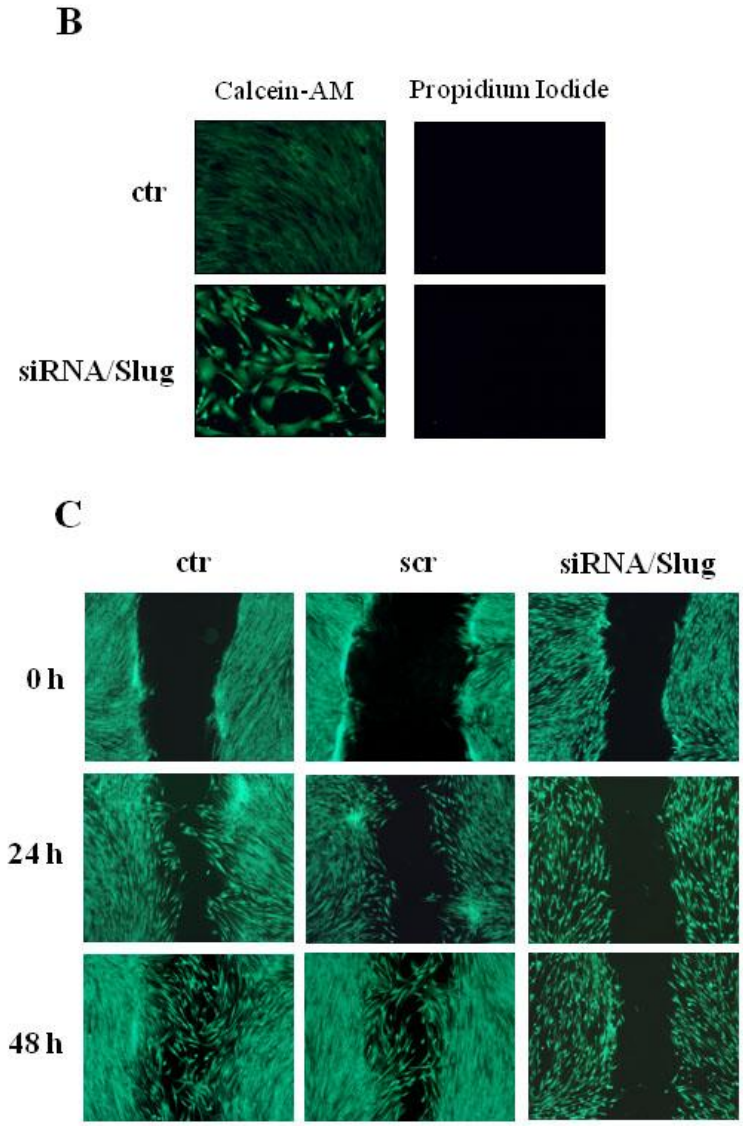


Figure 1. *Silencing of Slug gene expression by siSlug in hMSCs.* A. hMSCs were transfected with siRNA/Slug or a nonrelevant siRNA (scr). Slug expression was determined both at mRNA and protein level, and revealed by quantitative RT-PCR and western blot analysis, respectively. RT-PCR results were calculated using the ΔΔCt method, using GAPDH as the housekeeping gene, and WJ-MSCs siRNA/Slug transfected sample as the calibrator. Statistical analysis was performed control and a nonrelevant siRNA *versus* siRNA/Slug-silenced cells (* ,o, and ^ for hTP-MSC, hIC-MSC and hWJMSC respectively), as described in "Results" section. On the bottom, representative western blot of siRNA/Slug treated cells shows a specific decrease in endogenous protein level. 10 μg of whole cell lysates were assayed on a 12% SDS-polyacrylamide gel. The proteins were visualized using Supersignal Femto Substrate (Pierce). Size markers are reported (KDa). IP3K was used as loading control. B. Morphological alterations and viability of siRNA/Slug transfected cells, determined by double staining assay with Calcein-AM and propidium bromide (10X magnification). C. Analysis of hMSCs migration by in vitro scratch assay. hMSCs were transfected with 30 nM siRNA/Slug or a nonrelevant siRNA (scr). 72h after silencing treatment, hMSCs monolayers were scratch wounded with a pipet tip (0h), and observed over the indicated time periods, 0h, 24h, and 48h (4X magnification). Images show that siRNA/Slug treated cells exhibited a reduced capacity to cover the scratch area compared to control cells.

Effect of Slug siRNA on the expression of specific genes involved in osteochondroprogenitors differentiation

To investigate whether Slug has a specific role in determining the molecular signature of hMSCs, we analyzed the effect of Slug knockdown on the expression of transcription factors which are required in the control of the differentiation program of osteochondroprogenitors. These genes include: i. Runx2, a member of runt family playing a pivotal role in osteoblast differentiation decision and hypertrophic chondrocyte maturation (Komori., 2009; Kim *et al.*, 1999); ii. Sox9, which is particularly necessary for chondrogenic differentiation commitment (Lefebvre., 1998; Bi *et al.*, 1999); iii. Sox5 and Sox6 whose main function in chondrocytes is to boost the ability of Sox9 to activate major chondrocyte markers (Han *et al.*, 2008); iiii. STAT1 which inhibits chondrocyte proliferation and regulates bone development (Xiao *et al.*,2004; Goldring *et al.*, 2006).

After Slug silencing, all genes, except Runx2 showed the same modulation that was independent of cell type (see Figure 2). Slug-silenced cells always showed an increase in Sox9 and Sox5 when compared to the control condition. In particular, this upregulation was significant for hTP-MSCs (for Sox9 $p=0.035$; for Sox5 $p=0.027$) and hWJ-MSCs (for Sox9 $p=0.027$; for Sox5 $p=0.027$). On the contrary, Sox6 and STAT1 expression declined in all hMSCs treated with siRNA/Slug compared to the control. In particular, downregulation of Sox6 was significant for hWJ-MSCs ($p=0.027$), while downregulation of STAT1 was significant for hTP-MSCs ($p=0.027$) and hIC-MSCs ($p=0.043$).

Finally, Runx2 expression did not significantly change after the Slug knockdown, even if slightly increased in hIC-MSCs, and decreased in hWJ-MSCs.

This trend suggests that Slug acts as a negative regulator of Sox9 and Sox5 expression, and as a positive regulator of Sox6 and STAT1 genes, in hMSCs. On the contrary, as regards Runx2, the role of Slug seems to be influenced by cell type. This is in agreement with data previously shown in human primary osteoblasts, demonstrating that the same Slug knockdown increased Sox9 expression, but decreased Runx2. This different role of Slug in

mature committed osteoblasts and in their undifferentiated progenitors suggests a cell stage-specific mechanism of control of Runx2 and osteoblast differentiation by Slug.

Furthermore the human genomic DNA sequences belonging to 5' regulatory regions of Runx2, Sox9, Sox5, Sox6 and STAT1 were analyzed for the presence of putative Slug binding sites (E boxes) (Nieto., 2002) by TFSEARCH predicting transcription factor binding sites program (www.cbrc.jp/research/db/TFSEARCH.html). This analysis revealed the presence of E boxes in the promoter regions of all five genes (see Figure 2), suggesting that they could be potential Slug target genes.

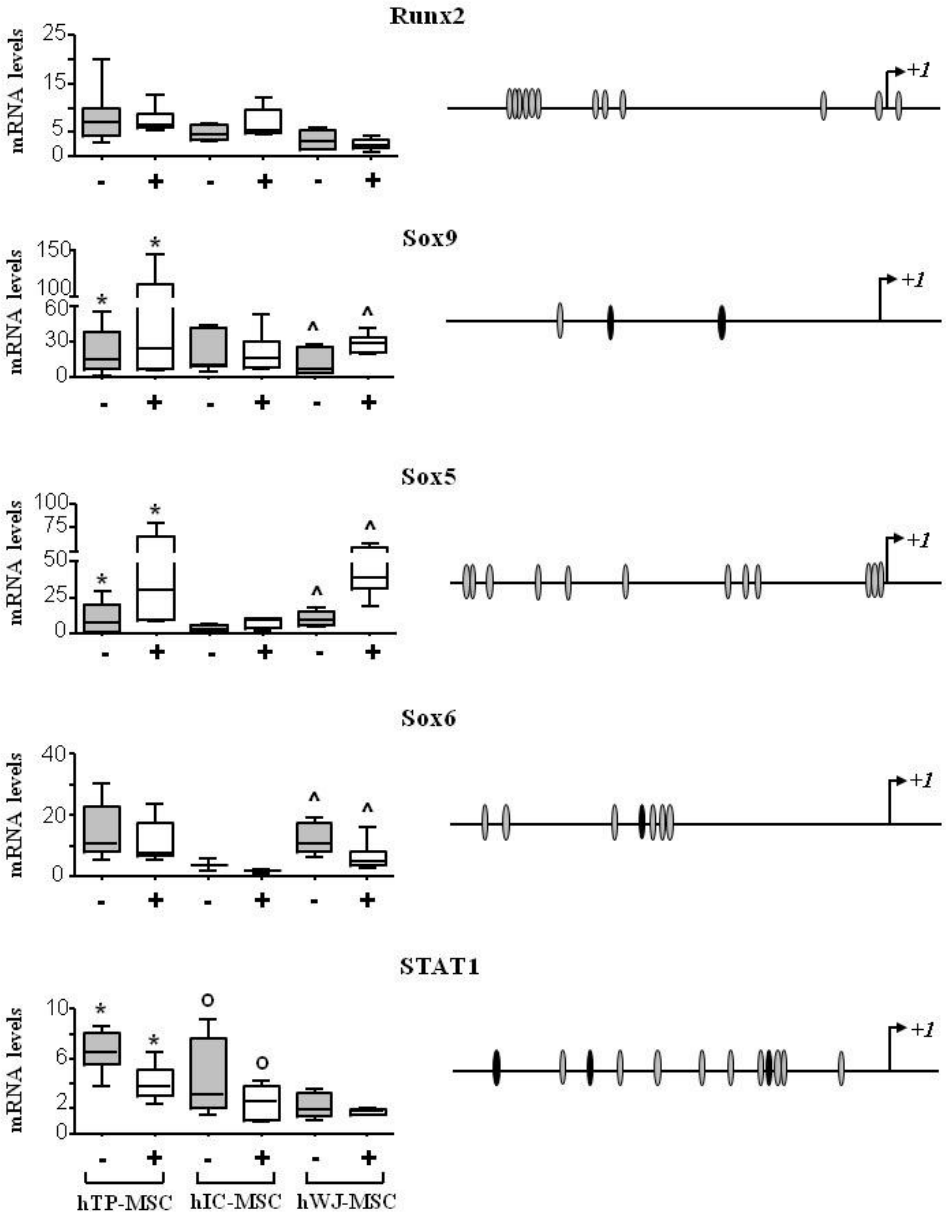


Figure 2. *Effect of Slug knockdown on the expression of specific genes.* On the left, Runx2, Sox9, Sox5, Sox6 and STAT1 gene expression after siSlug treatment in hMSCs isolated from tibial plateau (TP) trabecular bone iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord. mRNA level was revealed by quantitative RT-PCR analysis. RT-PCR results, were calculated using the ΔΔCt method, and the sample with the highest ΔCt as the calibrator for each gene analysis. Statistical analysis was performed control *versus* siSlug-silenced cells (* , o, and ^ for hTP-MSC, hIC-MSC and hWJ-MSC respectively) as described in "Results" section. On the right, schematic representation of Runx2, Sox9, Sox5, Sox6 and STAT1 human gene promoters (3000 bp upstream +1 transcription site). Using the TFSEARCH predicting transcription factor binding sites program, several potential Slug binding motifs have been identified in the promoter regions of all five genes (gray ovals). Sites showing 100% homology with consensus-binding site (CAGGTG/CACCTG) are indicated with black ovals.

"In vivo" recruitment of Slug on Runx2 and Sox9 gene promoters

On the basis of these findings, we tried to dissect the relationship between Slug and some of the analyzed genes by investigating *in vivo* Slug recruitment at specific promoter sequences. We focused our attention on the promoter of Runx2 and Sox9 genes, and the functionality of their E boxes was analyzed by ChIP (see Figure 3) on six samples of hMSCs from the three sources. Occupancy of these E boxes by Slug was compared among hMSCs. ChIP data were collectively considered for the presence or absence of PCR signals and the different promoter regions containing the E boxes were characterized for their high, low or no ability to recruit Slug. The results revealed that Slug can, *in vivo*, associate with multiple sites across Runx2 and Sox9 promoters to a different extent regardless of the hMSCs source.

Considering Runx2 gene, Slug associated with region 2 and 3 to a similar extent, but more localized to a discrete region proximal to the transcription start site (region 1). Interestingly, the E boxes within region 1 are highly conserved, as revealed by the alignment of sequences from rat, mouse and human (see Figure 3A).

Furthermore, with regard to Slug occupancy of Sox9 promoter, region 2 was the only one involved in the interaction, while no chromatin was immunoprecipitated from the region 1 (see Figure 3B). The alignment of Sox9 sequence promoters from rat, mouse and human revealed no significant homology.

As a whole, these ChIP experiments revealed that both Runx2 and Sox9 are Slug target genes in hMSCs, and that the E boxes present in these promoters are differently involved in the Slug recruitment.

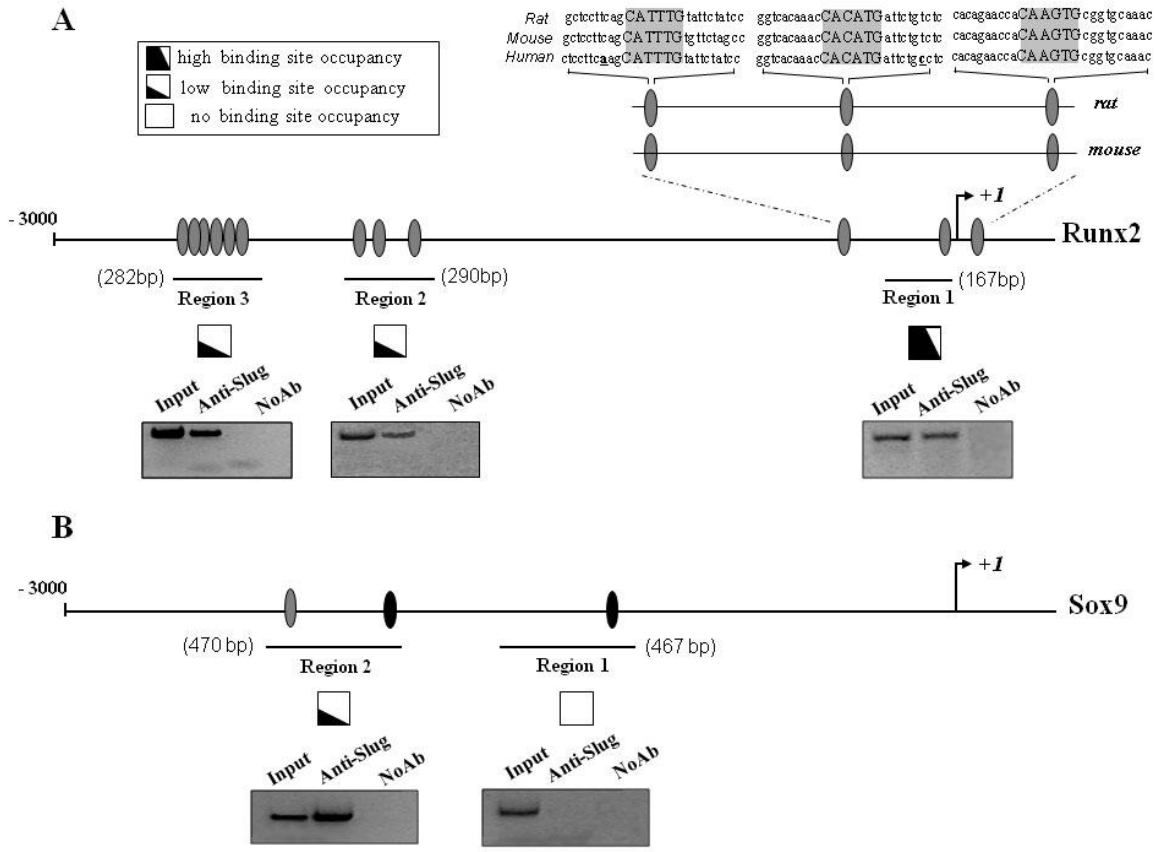


Figure 3. *In vivo recruitment of Slug on Runx2 and Sox9 human gene promoters.* Protein–DNA complexes were *in vivo* formaldehyde-cross-linked in six samples of hMSC isolated from tibial plateau (TP) trabecular bone, iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord. Chromatin fragments were subjected to immunoprecipitation with antibody against Slug. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the primers pairs spanning the reported regions of Runx2 and Sox9 promoters. Aliquots of chromatin taken before immunoprecipitation were used as input positive controls whereas chromatin eluted from immunoprecipitations lacking antibody were used as no antibody controls (NoAb). PCR fragments of all samples analyzed were resolved in 1.5% agarose gels and subjected to densitometric analysis for a semi quantitative determination of occupancy of binding sites. High, low or no ability to recruit Slug is represented by the extent of the black portion of the square. A representative PCR analysis is reported below each investigated region. The relative positions of Slug putative binding sites (gray ovals) are indicated. Sites showing 100% homology with consensus-binding site (CAGGTG/CACCTG) are indicated with black ovals. The alignment of sequences from rat, mouse and human of Runx2 promoter region 1 is reported, showing the highly conserved E boxes.

DISCUSSION

The complexity of bone cell functions, appears from several molecular and cellular studies, both in terms of differentiation and bone development, and for alterations of this tissue in various diseases. The proper bone formation and the maintenance of bone homeostasis are regulated by different signaling pathways, known in detail only minimally. These signaling pathways, interacting each other, modulate different target genes, which play a specific function during differentiation and proliferation of osteoblasts, osteoclasts and their precursors. Understanding the mechanisms that control bone formation and transcription factors that regulate the expression of specific genes in osteoprogenitors and bone mature cells, is the target of much research aimed to develop effective therapeutic strategies, in order to solve many pathological conditions as well as osteogenic abnormalities, such as critical sized defects, non-union fractures, and vertebral interventions. These research make use of experimental strategies and opportune models of study in order to transfer as much as possible, the results from the laboratory to the clinic.
In this context, bone tissue engineering represents a new approach for bone tissue repair and regeneration. The regeneration of bone tissue is a multidisciplinary approach that is based on the synergistic interaction between human bone cells, various types of scaffolds and the concerted action of both positive and negative regulatory factors which control osteoblast commitment, differentiation, and functions. In particular, the search for new crucial molecules for the commitment of MSCs toward osteogenic lineage and the understanding of role that specific transcription factors play in determining osteoblast phenotype, are important issues in bone regeneration and repair field.

These considerations have guided the setting of the experiments described in this thesis that focused on the study of Slug protein. Through a series of *in vitro* and *in vivo* experiments, we investigated, for the first time, the expression and role of Slug gene in human normal osteoblasts and their mesenchymal precursors, characterizing:

- the regulatory elements located in its promoter;
- the effect of its silencing;
- its correlation with Wnt pathway and osteogenesis process.

We started from the consideration that alterations in gene regulation are often associated with the onset of various pathological conditions or the difficulty of a cell population to react to a tissue trauma. A broad area of molecular biology is aimed, to identify and correct such modifications by the recognition of specific binding sequences for transcription factors in the genome and the design of new therapeutic molecules, including antisense oligonucleotides, decoy molecules, siRNA, antagomir and vectors for gene therapy in an attempt to restore the physiological condition.

With regard to Slug gene, as described in the introduction of this thesis, possible alterations in the regulation machinery, which determines Slug absence, have been associated with defects in embryonic development (Hemavanthy *et al.*, 2000), while its overexpression was found in many types of cancer (Barrallo-Gimeno *et al.*, 2005). To date, for what concerns adult bone tissue, Slug, indeed, is considered exclusively a marker of malignancy and, consequently, an attractive potential target for therapeutic modulation of bone metastasis and osteosarcoma invasiveness (Guo *et al.*, 2007), blocking its expression and potentially repressing any detrimental downstream effects. However, there are no data about Slug expression and regulation in human normal osteoblasts. Therefore, we started investigating in this direction supported by the evidence that: 1. Slug positively affects differentiation and migration of osteoblasts from neural crest in the craniofacial compartment and 2. the process of fracture repair mimics the embryonic bone development where a critical role is played by Slug.

In this study we have demonstrated that the transcription factor Slug:

- is present at substantial levels in normal human osteoblasts and their mesenchymal precursors;
- is positively correlated with osteogenesis;

- is positively regulated by Lef1 and Runx2 osteoblastic transcription factors which are directly *in vivo* recruited in the Slug promoter.

In particular, our Slug promoter study revealed in a region of approximately 1 Kb upstream of the transcription start site, the presence of two putative binding sites for Runx2 and, at least, six potential consensus binding sites for the Lef/Tcf family, and not just only one at -859/-855 position, as recently reported (Saegusa *et al.*, 2009; Hong *et al.*, 2009). We found that the sequence regions containing these sites are all involved in the *in vivo* recruitment of Runx2 and Lef/Tcf factors, including Lef1, Tcf-1 and Tcf-4, in human normal osteoblasts. On the contrary, the research of other groups focused on cancer experimental models demonstrated, for example, the ability of Tcf binding site at -859/-855, to recruit Tcf-4 in SW480 human colon cancer (Hong *et al.*, 2009), but not in Hec251 endometrial cancer cell line (Saegusa *et al.*, 2009) where, Slug expression seems regulated by β-catenin without the involvement of Lef/Tcf factors. Other studies in animal experimental models provide evidence that Slug promoter in *Xenopus* and mouse is directly activated by β-catenin/Tcf complexes through the binding sequences (Sakai *et al.*, 2005; Vallin *et al.*, 2001), and that Slug promoter activity may be inhibited by dominant negative Tcf (Conacci-Sorrell *et al.*, 2003). Therefore, combined with these reports, our results strengthen the hypothesis that, directly or indirectly, Slug and Lef1 are strictly correlated in many cellular events, including osteoblast differentiation, mediated by Wnt/β-catenin signalling.

A relationship between Slug and Wnt signalling was also confirmed by our experiments with SB216763, a compound able to increase β-catenin levels suppressing GSK-3β activity. Interestingly, SB216763-treated osteoblasts showed a significative Slug gene expression increase. This finding correlates positively Slug with osteoblast function, being β-catenin largely investigated in association with osteogenesis process. It is largely reported that β-catenin Lef/Tcf transcription factor family cooperate to promote the expression of several genes through the recruitment of other factors to form a transcriptionally active complex (Nusse., 1999; Case *et al.*, 2008). In the bone context Lef1

is reported to have an important role in osteoblast maturation for its ability in the regulation of expression of genes involved in the stimulation of bone formation, such as Runx2 and Col11a1 (Gaur *et al.*, 2005; Kahler *et al.*, 2008). In addition, an age- and gender- dependent role for Lef1 in regulating bone formation *in vivo* has recently been described (Noh *et al.*, 2009). The discovery that Slug expression is upregulated during osteogenesis, is positively correlated with the expression of Runx2 and Lef1, and its regulation is under the control of Runx2 and Lef1, corroborates the proved function of Runx2 in bone tissue and the role of Lef/Tcf transcription factors in osteoblasts. Therefore, our results highlight the mechanisms by which Lef1 may affect maturation and differentiation of these cells, suggesting a new role of Slug.

Slug knockdown experiments were particularly informative in support of this hypothesis, and the suppression of Slug mediated by siRNA increases Runx2 expression, weakens Wnt/β-catenin signaling, decreases ALP activity, as well as osteoblast mineralization, osteopontin, osteocalcin, and Col1a1, demonstrating that Slug may be considered a novel osteogenic factor. It is important to underline that the specificity of the Slug silencing effects is attested also by the expression of Rankl which is suppressed by Wnt signaling in osteoblasts (Spencer *et al.*, 2006) and is enhanced in response to Slug knockdown, further supporting the link between Slug and Wnt.

The investigation of the link between Slug and another important osteoblast marker, the CXCL12 chemokine, has allowed us to highlight a potential dual action level of Slug as modulator of gene expression. Unexpectely, Slug-silenced osteoblasts showed an increase of CXCL12 mRNA but a decrease of CXCL12 protein. CXCL12, together with its receptor CXCR4, has recently been suggested to be involved in the bone remodeling process (Ponomaryov *et al.*, 2000; Dominici *et al.*, 2009; Otsuro *et al.*, 2008), in the recruitment of MSCs to the fracture site during skeletal repair (Kitaori *et al.*, 2009); also it is highly expressed and secreted by regenerating/proliferating osteoblasts after irradiation in mouse models (Dominici *et al.*, 2009). Although a recent partial characterization of the gene

promoter suggested that both the constitutive and induced expression of CXCL12 is finely regulated at transcriptional level (Garcia-Moruja *et al.*, 2005; Calonge *et al.*, 2010), the molecular mechanisms involved in directing the correct levels of this chemokine remain unclear. In our study, we found that Slug knockdown in hOBs induced a consistent decrease of CXCL12 protein even if the corresponding mRNA increased. One explanation for this apparent discrepancy found for mRNA and protein expression of CXCL12 in Slug-silenced cells may be the presence of numerous potential Slug targets in the cell and, consequently, the wide spectrum of effects generated by its silencing. Therefore, our findings suggest possible different roles for Slug and consequently at least two different main effects of Slug knockdown: a) Slug may act as a repressor of CXCL12 gene transcription and its silencing removes the negative control by increasing CXCL12 mRNA levels, b) Slug may have an indirect positive impact on components of specific pathways correlated with expression, stability secretion and activity of CXCL12 and its silencing results in a decrease in CXCL12 protein. The first hypothesis, supported by the fact that Slug is specifically recruited to CXCL12 promoter *in vivo* as shown by chromatin immunoprecipitation analysis, suggests that high levels of CXCL12 mRNA are not sufficient to assure high levels of CXCL12 protein. Therefore, we may conclude that the amount of CXCL12 protein depends on several factors and mechanisms including Slug-mediated events. Nevertheless, since the remarkable final phenotypic effect mediated by Slug silencing is the decrease in CXCL12 protein expression and secretion, it may be concluded that the presence of Slug is required for CXCL12 protein expression: this support the hypothesis that both proteins are crucial for osteoblast phenotype and strengthen the critical role of Slug in osteoblasts as we have propose. This is the first study where the relationship between CXCL12 and Slug is analyzed in a bone cellular context. In agreement with our observations, the potential strict correlation between CXCL12 signaling and Slug activity has been shown in experimental models that can be related to bone. For example, CXCL12 and Slug are both involved in neural crest cell (NCC) migration. Recent evidence suggests that both knockdown and an over-expression of

CXCL12 and its receptor leads to migration and important craniofacial defects for patterning the craniofacial skeleton (Olesnicky Killian *et al.*, 2009).

Another important evidence from our results on Slug silenced cells has opened a possible new scenario about Slug function. This concerns the effect of Slug silencing on Sox9 expression. We observed that Slug silencing potentiates the expression of Sox9 both in hOBs and in undifferentiated chondrocytes. This is particularly interesting in the context of maturation and differentiation of these cells because Sox9 is an indispensable factor for chondrogenic development (Akyama *et al.*, 2004; Komori., 2009), activating cartilage-specific genes, but acts also as a transcriptional repressor for osteoblast differentiation (Zhou *et al.*, 2006), interacting with Runx2 and repressing its function. Therefore, our findings may suggest not only that Slug is required for supporting osteogenic maturation, but also that Slug suppression may have a potential pro-chondrogenic effect. Thus, Slug could affect phenotypical changes in response to alteration of its expression levels favoring the dominance of Runx2 function over Sox9 or vice versa. This is in agreement with several observations demonstrating that, in order to achieve differentiation towards a desired lineage, it is important to direct the stem cell differentiation with correct levels of transcription factors (Soltanoff *et al.*, 2009; Davis and Zur Nieden, 2008).
In this context, the concept of manipulating specific transcription factors is creating great interest. Overexpression or depletion of transcription factors, including Runx2, Osterix, Sox9, Twist1, NFATc1, Foxo1, Sp1, HIF-1 c/EBP, and Rex-1, was recently performed in MSCs (Lin *et al.*, 2006; Miraoui *et al.*, 2010; Tominaga *et al.*, 2009; Deng *et al.,* 2008; Wan *et al.*, 2010; Bhandari *et al.*, 2010). This approach is largely based on the knowledge of the molecular networks implicated in osteogenic differentiation, and, at the same time, it allows the identification of the role of a specific
transcription factors in mediating the fate and maturation of hMSCs. These considerations are part of a wider field of observations including those sustaining that the level of modulation of several osteoblastic genes may differ depending on culture conditions and source from which the cells are taken (Mosna *et al.*, 2010; Hsieh *et al.*, 2010; Noel *et al.*,

2008; Mannello *et al.*, 2007; da Silva Meirelles *et al.*, 2008). This may have significant implications for cell-based bone tissue regeneration, particularly when much evidence in literature suggests that the basal expression levels of specific genes in uninduced MSCs may have a crucial role in this scenario (Karsenty., 2008; Marie., 2008; Satija *et al.*, 2007). To corroborate this hypothesis and further Slug knowledge, we performed a series of experiments with hMSCs isolated from tibial plateau (TP) trabecular bone, iliac crest (IC) bone marrow and Wharton's jelly (WJ) umbilical cord opportunely selected.

Because we had the opportunity to have a large number of WJ samples, it was possible design a series of experiments *ad hoc* to discriminate among different WJ-MSC samples those will have a positive outcome towards osteoblastic differentiation. Comparing the presence of ALP and Runx2 osteoblastic markers with different obstetric parameters, (baby's gender and birth weight, mother's age at delivey, gestational stage at parturition and mode of delivery), we found, in fact, that the highest degree of osteoblastic potential has been shown by WJ-MSCs with Runx2 transcription factor high basal levels, selected from umbilical cords of the heaviest term babies. In addition, our results suggest that it is preferred to recruit the samples from full term borns without paying attention to mother's age.

Therefore, even if further evaluation is required, our hypothesis is that our findings may help in selecting the optimal umbilical cord donors and in collecting high potential Wharton's jelly-derived osteoprogenitors efficiently. The analysis of the basal level of Runx2 and ALP activity could allow to quickly test an high number of mesenchymal precursors cultured *in vitro* and select the more suitable to potentially use for bone tissue engineering application.

Harking to experiments performed in hTP-MSCs, IC-MSCs and WJ-MSCs, we demonstrated that the cell response to a same *in vitro* microenvironment may be different. Firstly, we found that hMSCs isolated from the three sources possess similar surface marker profiles, but the basal expression levels of transcription factors which control differentiation program of osteochondroprogenitors, such as Runx2, Sox9, Sox5, Sox6,

STAT1 and Slug, are different. In particular, hWJ-MSCs showed the lowest expression levels of Runx2, Sox9, STAT1 and Slug. Furthermore, investigations of the osteogenic potential of hMSCs isolated from the three sources highlighted that osteogenically induced hWJ-MSCs showed the characteristic staining of bone-like nodules beginning from day 7, whereas the same signs of mineralization were evident in hTP- and hIC-MSCs only from day 14. Nevertheless, hWJ-MSCs were not able to reach at day 21 the level of mineralization that we found in the other hMSCs. These data indicate that there is a variability in the extent of osteogenic differentiation among the analyzed hMSCs. This is confirmed by the fact that Runx2 expression showed a trend to decrease in hTP-MSCs, remained constant in hIC-MSCs, but increased in hWJ-MSCs after 21 days in osteogenic medium. It is, in fact, well established that, during bone development, Runx2 induces osteoblast differentiation and increases the number of immature osteoblasts, which form immature bone, whereas Runx2 expression has to be downregulated for differentiation into mature osteoblasts, which form mature bone (Komori., 2010; Maruyama *et al.*, 2007). On the contrary, hWJ-MSCs which do not reach a complete "end-stage" differentiation, quickly respond to osteogenic medium. This is in accordance with many other previously reported features of umbilical cord-derived stem cells (Hsieh *et al.*, 2010; Moretti *et al.*, 2010; Troyer and Weiss, 2008).

We may speculate that, thanks to the ease of accessibility, lack of ethical concerns, and abundant cell number, hWJ-MSCs may be a particularly promising cell population, supporting new concepts in cellular therapy. These cells appear to possess the proper stage of development that makes them preferable candidates when a bone regeneration under endogenous factors control is required. In many cases, in order to promote tissue integration, it is, in fact, critical that the pre-differentiated osteogenic progenitors to be implanted are able not only to differentiate, but also to interact with the endogenous microenviroment and respond to local differentiation signals *in vivo*. This capability can be carried on by cells that have not completely reached the terminal differentiation, such as the hWJ-MSCs here described. Conversely, it is conceivable that tibial plateau or iliac crest, from which we obtained the other hMSCs, are already committed compartments

containing stem cells with a higher maturation stage and particularly prone to form mature bone. Probably, these kind of cells are to be preferred when large bone defects have to be repaired.

Another important issue that bone tissue regeneration and repair through therapeutic use of cells have to take into account, is that the achievement of pre-differentiated osteogenic progenitors requires the employment of induction media containing exogenous recombinant growth factors, fetal bovine serum, hormones and other reagents whose effects on long-term *in vivo* differentiation are not known, and which may be potentially negative by transmitting infectious agents and triggering an immune response. Therefore, in the light of these considerations, it is interesting to investigate the possibility that the change in the levels of specific gene transcription can replace the standard method of induction to differentiation. In this regard and in order to further investigate the properties of the hTP-MSCs, hIC-MSCs and hWJ-MSCs, another aspect we considered in this thesis has been the possibility to affect the behaviour of hMSCs by using gene silencing approach without exposing cells to induction media. In particular, the informations derived from experiments on hOBs, led us to investigate whether the depletion of a transcription factor such as Slug, had a role in determining the fate of uninduced MSC progenitors. Our results obtained from RT-PCR analysis depicted a complex scenario where all Slug-silenced hMSCs from the three sources showed generally a higher expression of Sox9 and Sox5, and a lower expression of Sox6 and STAT1 in comparison with control cells. This suggests that Slug acts as a negative regulator of Sox9 and Sox5 expression, and as a positive regulator of Sox6 and STAT1 genes. Sox5, Sox6 and Sox9 constitute the so-called SOX trio and are essential factors for the development of embryonic cartilage, and are mainly associated with the commitment of undifferentiated MSCs into chondrocytes (Ikeda *et al.*, 2004). In particular, Sox9 is the first essential transcription factor for chondrocyte differentiation (Lefebvre., 1998; Bi *et al.*, 1999). Sox 5 and Sox6 are indicated as downstream genes of Sox9 in chondrocytes, but are not absolutely necessary for chondrocyte differentiation even if they strongly potentiate Sox9 activity (Han *et al.*, 2008; Ikeda *et al.*, 2004). Recent studies in MSCs have demonstrated that SOX trio family

members may be differently regulated. For example, BMP-2, which was reported to control chondrogenic differentiation, increased Sox6 and Sox9, but not Sox5 mRNA expression (Fernandez-Lloris *et al.*, 2003). As a whole, our results strengthen the evidence that a specific factor may differently affect SOX trio regulation, suggesting also a novel role for Slug transcription factor. In future studies, we will determine whether Slug acts directly on Sox5 and Sox6 promoters or the up-regulation of Sox5 by Slug gene silencing was mediated by Sox9.

Another interesting finding after Slug silencing is STAT1 downregulation. This result demonstrates the specificity of knockdown treatment and suggests that STAT1 expression is positively regulated by Slug. Accordingly, in chondrogenesis, STAT1 acts downstream in relation to Slug and, as Slug, negatively regulates the proliferation of chondroprogenitors (Goldring, 2006). Conversely, a recent study demonstrates that STAT1 is a negative regulator for osteoblast differentiation, and suggests that inhibition of STAT1 activity may be beneficial for skeletal fracture treatment (Tajima *et al.*, 2010).

As a whole, these findings support the hypothesis that 1. modulating the expression of one or more specific transcription factors a preferential selection of osteo- or chondro-precursors may be obtained; and 2. although the mechanisms of this process are currently unknown, nevertheless it seems that they are dependent on experimental models and developmental stages of the cells. Therefore, to clarify the role of specific transcription factors in osteochondroprogenitors and bone mature cells, and to understand the possible discrepancies between the data collected so far, further investigations are required. These observations are consistent with the results obtained regarding Runx2 in Slug-silenced hMSCs. In fact, concerning Runx2, the role of Slug seems to be influenced by cell type. We found that, after Slug knockdown, Runx2 expression did not significantly change. This can be explained by the evidence that Runx2 has a broader spectrum of phenotype control of a cell in comparison with SOX trio or STAT1. In fact, in addition to its role in osteoblast differentiation, Runx2 also promotes chondrocyte maturation (Kim *et al.*, 1999; Goldring., 2006). This confirms that, for certain genes, including Runx2, their

susceptibility to be modulated in expression levels depends on gene function, and is strictly correlated with heterogeneity and properties of MSC population.

Based on findings obtained, Slug might represent a new regulation factor required for human osteoblast differentiation and maturation, although further studies are required to elucidate its regulation and its role in MSCs fate determination. This is can properly done with appropriate manipulation of gene expression and using three–dimentional culture conditions which, overall, might affect cell behaviour leading to significant improvement in the efficiency of tissue engineering and enhancing the therapeutic value of stem cells/osteoprogenitors for the restoration of bone defects.

CONCLUSIONS AND FUTURE WORK

The experimental approach and data described in this thesis take place in a very broad view that regards the understanding of the molecular mechanisms of osteogenesis process, and the development of alternative strategies to induce efficient differentiation of hMSCs in bone and cartilage tissue engineering and repair.

Summarizing, the development of effective cellular-based therapies for regenerative medicine is based on the achievement of several objectives:

- Development of new strategies to select most potential cells donors/sites could be helpful in expanding and obtaining larger cell population, overcoming the problem connected to the small number of stem cells in adult tissue stem cell niche;
- *In vitro* manipulation of hMSCs could allow the control of their self-renewal, proliferation, and senescence. On the other hand, strategies to prevent and avoid hMSCs transformation have to be developed, in order to ensure safe use in short and long term therapies with these cells;
- Development of multicompartimental biomaterial systems for the realization of "Smart 3-D systems" containing osteoprogenitors and specific biomolecules, such as nutrients, growth factors and pre-differentiating agents promoting the biological response to bone and cartilage tissue engineering applications;
- Appropriate manipulation of gene expression to modificate cell characteristics, improving the efficiency of tissue engineering and enhancing the therapeutic value of stem cells/osteochondroprogenitors for the restoration of bone defects.

All these efforts and increasing knowledge around human osteochondroprogenitors and mature cells through basic research experiments will be of considerable clinical significance, especially in terms of developing novel mechanisms of achieving tissue regeneration.

In particular, in these years, much attention has been paid to discovery the pathways and factors implicated in the regulation of hMSCs differentiation process. At present, a great number of experimental strategies are addressed to investigate the role of epigenetic mechanisms and key transcription factors implicated in the control of stem cell properties, as well as in the chondrogenic or osteoblast committed status, in order to identify potential therapeutic molecules. In bone tissue context, much research are based on the targeting of hMSCs with constructs that cause permanent expression of transcription factors (e.g., Satb2, Hoxa2, Runx2, osterix, APC/β-catenin/LefTcf-1) pertaining to osteoblastogenesis and/or overexpression of miR- (e.g., miR-29b) upregulated in osteoblasts, or the use of miR- antagonists, in stable and inducible viral mono- or polycistronic constructs, that target miR- species (e.g., miR-125b, MiR-133, and miR-135a) downregulated in osteoblasts.

Regarding the work of this thesis, we may conclude that our results have identified Slug as a new osteogenic factor suggesting that it may be considered a novel therapeutic target for bone tissue engineering. Slug suppression, in fact, might have a therapeutic value in diseases that involve the formation of ectopic bone such as osteopetrosis and ankylosing spondylitis or in pathologies that require chondrocytes differentiation such as osteoarthritis.

Nevertheless, further investigations have to be performed in order to determine the role of other transcription factors that take part in Slug regulation and function in hMSCs, and to identify the biological phenomena affected by Slug knockdown.

For these purposes, the molecular aspects of our future work will be based on these main objectives:

- the identification of new potential transcription factors involved in Slug gene expression regulation;
- the identification of new Slug target genes;
- understanding of Slug function in mesenchymal stem cells commitment, by Slug stably-inducible knocked down cells which we are now creating with lentiviral systems:

- the identification of new osteoblast-specific proteins correlated with Wnt signaling, during hMSCs osteoblastogenesis.

REFERENCES

Acloque H., Adams M.S., Fishwick K., *et al*. (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest*. 119(6):1438-1449.

Ai M., Holmen S.L., Van Hul W., *et al.* (2005). Reduced affinity to and inhibition by DKK1 form a common mechanism by which high bone mass-associated missense mutations in LRP5 affect canonical Wnt signaling. *Mol Cell Biol*., 25:4946-4955.

Akiyama H., Chaboissier M.C., Martin J.F., *et al.* (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathways and is required for expression of Sox5 and Sox6. *Genes Dev*., 16(21):2813-2828.

Akiyama H., Kim J.E., Nakashima K., *et al*. (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci USA.,* 102:14665–14670.

Akiyama H., Lyons J.P., Mori-Akiyama Y., *et al*. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.,* 18:1072–1087.

Alam T., Sollinger H.W. (2002). Glucose-regulated insulin production in hepatocytes. *Transplantation*., 74(12):1781-1787.

Alberga A., Boulay J.L., Kempe E. (1991). The *snail* gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germ layers. *Development*., 111:983-992.

Alberts B., Johnson A., Lewis J., *et al.* (2002). Molecular Biology of the Cell, 4th ed. New York:Garland.

Allison M.R., Poulsom R., Forbes S., *et al*. (2002). An introduction to stem cells. *J Pathol.,* 197:419–423.

Alves C.C., Carneiro F., Hoefler H., *et al*. (2009). Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers. *Front Biosci*., 14:3035-3050).

Anjos-Afonso F., Bonnet D. (2007). Non-hematopoietic/endothelial SSEA-1+ cells define the most primitive progenitors in the adult murine bone marrow mesenchymal compartment. *Blood.,* 109: 1298–1306.

Arnoux V., Nassour M., L'Helgoualc'h A., *et al.* (2008). Erk5 controls Slug expression and keratinocyte activation during wound healing. *Mol Biol Cell.*, 19(11):4738-4749.

Ashraf S.I., Hu X., Roote J., *et al.* The mesoderm determinant snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis. *EMBO J.*, 18(22):6426-6438.

Ashton B.A., Abdullar F., Cave J., *et al.* (1985). Characterization of cells with high alkaline phosphatase activity derived from human bone and marrow: preliminary assessment of their osteogenicity. *Bone.*, 6:313-319.

Ashton B.A., Allen T.D., Howlett C.R., *et al.* (1980). Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. *Clin Orthop Relat Res.*, 151:294-307.

Attisano L., Wrana J.L. (2002). Signal transducion by the TGF-beta superfamily. *Science.*, 296:1646-1647.

Aubin J.E., Liu F. (1996). The osteoblast lineage. In: Bilezikian J.P., Riasz L.G., Rodan G.A. (eds) *Principles of Bone Biology. Academic Presss*, San Diego, CA, pp 51-67.

Awad H.A., Butler D.L., Boivin G.P., *et al.* (1999). Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng.,* 5:267–277.

Aybar M.J., Nieto M.A., Mayor R. (2003). Snail precedes slug in the genetic cascade required for the specification and migration of the Xenopus neural crest. *Development.*, 130(3):483-494.

Babij P., Zhao W., Small C., *et al.* (2003). High bone mass in mice expressing a mutant LRP5 gene. *J Bone Miner Res.,* 18(6):960- 974.

Baggiolini M., Dewald B., Moser B. (1997). Human chemokines: an update. *Annu Rev Immunol.*, 15:675-705.

Bailey C.K., Misra S., Mittal M.K., *et al.* (2007). Human SLUG does not directly bind to CtBP1. *Biochem Biophys Res Commun.*, 353(3):661-664.

Bajada S., Mazakova I., Richardson J.B., et al. (2008). Updates on stem cells and their applications in regenerative medicine. *J Tissue Eng Regen Med.*, 2:169-183.

Baksh D., Boland G.M., Tuan R.S. (2007). Cross-talk between Wnt signaling pathways in human mesenchymal stem cells leads to functional antagonism during osteogenic differentiation. *J Cell Biochem.*, 101:1109–1124.

Baksh D., Tuan R.S. (2007). Canonical and non-canonical Wnts differentially affect the development potential of primary isolate of human bone marrow mesenchymal stem cells. *J Cell Physiol.*, 212:817–826.

Baksh D., Yao R., Tuan R.S. (2007). Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells.,* 25(6):1384-1392.

Balemans W., Ebeling M., Patel N., *et al.* (2001). Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet.,* 10(5):537-543.

Barker N. (2008). The canonical Wnt/beta-catenin signalling pathway. *Methods Mol Biol.*, 468:5-15.

Barrallo-Gimeno A., Nieto M.A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development.*, 132:3151-3161.

Bartholomew A., Sturgeon C., Siatskas M., *et al.* (2002). Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol.*, 30:42-48.

Basch M.L., Bronner-Fraser M. (2006). Neural crest inducing signals. *Adv Exp Med Biol.*, 589:24-31.

Battula V.L., Treml S., Bareiss P.M., *et al.* (2009). Isolation of functionally distinct mesenchymal stem cells subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1 (MSCA-1). *Haematologica.,* 94:173–184.

Behrens J., (2000). Cross-regulation of the Wnt signaling pathway: a role of MAP kinases. *J Cell Sci.*, 113(Pt 6):911-919.
Bennett C.N., Longo K.A., Wright W.S., *et al.* (2005) Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci USA.,* 102:3324–3329.

Bennett C.N., Ouyang H., Ma Y.L., *et al.* (2007) Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation. *J Bone Miner Res.,* 22:1924–1932.

Bernardo M.E., Locatelli F., Fibbe W.E. (2009). Mesenchymal stromal cells. *Ann N Y Acad Sci.*, 1176:101-117.

Beyer N.N., da Silva M.L. (2006). Mesenchymal stem cells: isolation, *in vitro* expansion and characterization. *Handb Exp Pharmacol.,* 174:249–282.

Bhabavati S., Xu W. (2004). Isolation and enrichment of skeletal muscle progenitor cells from mouse bone marrow. *Biochem Biophys Res Commun .,*21:119–124.

Bhandari D.R., Seo K.W., Roh K.H., *et al.* (2010). REX-1 expression and p38 MAPK activation status can determine proliferation/differentiation fates in human mesenchymal stem cells. *PLoS One.*, 5(5):e10493.

Bi W., Deng J.M., Zhang Z., *et al.* (1999). Sox9 is required for cartilage formation. *Nat Genet.*, 22:85-89.

Bianco P., Riminucci M., Gronthos S., *et al.* (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells.*, 19(3):180-192.

Bluteau G., Luder H.U., De Bari C., *et al.* (2008). Stem cells for tooth engineering. *Eur Cell Mater.*, 16:1-9.

Bodine P.V., Zhao W., Kharode Y.P., *et al.* (2004). The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol. Endocrinol.*, 18(5):1222-1237.

Boland G.M., Perkins G., Hall D.J., *et al.* (2004). Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. *J Cell Biochem.*, 93:1210–1230.

Borycki A., Brown A.M., Emerson C.P Jr. (2000). Shh and Wnt signaling pathways converge to control Gli gene activation in avian somites. *Development.,* 127:2075–2087.

Boulay J.L., Dennefeld C., Alberga A. (1987). The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers. *Nature.*, 330(6146):395-398.

Boyce B.F., Xing L. (2007). Biology of RANK, RANKL and osteoprotegerin. *Arthritis Res Ther.*, Suppl 1:S1.

Boyce BF., Xing L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys.*, 473(2):139-46.

Boyden L.M., Mao J., Belsky J., *et al.* (2002). High bone density due to a mutation in LDL-receptor-related protein 5. *N Engl J Med.*, 346:1513–1521.

Boyer B., Vallés A.M., Edme N. (2000). Induction and regulation of epithelial-mesenchymal transitions. *Biochem Pharmacol.*, 60(8):1091-1099.

Bridgewater L.C., Lefebvre V., de Crombrugghe B. (1998). Chondrocyte-specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer. *J Biol Chem.,* 273:14998–15006.

Bronner-Fraser M. (1995). Origins and developmental potential of the neural crest. *Exp Cell Res.*, 218(2):405-417.

Brugnoli F., Lambertini E., Varin-Blank N., *et al.* (2010). Vav1 and PU.1 are recruited to the CD11b promoter in APL-derived promyelocytes: Role of Vav1 in modulating PU.1-containing complexes during ATRA-induced differentiation. *Exp Cell Res.*, 316:38-47.

Brunkow M.E., Gardner J.C., Van Ness J., *et al.* (2001). Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am. J. Hum. Genet.,* 68(3):577-589.

Buckwalter J.A., Mankin H.J. (1998). Articular cartilage: degeneration and osteoarthrosis, repair, regeneration, and transplantation. *Instr Course Lect.,* 47:487–504.

Buhring H.J., Battula V.L., Treml S., *et al.* (2007). Novel markers for the prospective isolation of human MSC. *Ann N Y Acad Sci.,* 1106:262–271.

Byers R.J., Brown J., Brandwood C., *et al.* (1999). Osteoblastic differentiation and mRNA analysis of STRO-1-positive human bone marrow stromal cells using primary in vitro culture and poly (A) PCR. *J Pathol.*, 187:374-381.

Cadigan K.M., Nusse R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.*,11(24):3286-3305.

Cai Y., Chia W., Yang X. (2001). A family of Snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate *Drosophila* neuroblast asymmetric divisions. *EMBO J.*, 20(7):1704–1714.

Calonge E., Alonso-Lobo J.M., Escandon C., *et al.* (2010). c/EBPbeta is a major regulatory element driving transcriptional activation of the CXCL12 promoter. *J Mol Biol.*, 396:463-472.

Can A., Karahuseyinoglu S. (2007). Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells.*, 25:2886-2895.

Canalis E., Economides A.N., Gazzerro E. (2003). Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev.*, 24:218-235.

Cano A., Perez-Moreno M.A., Rodrigo I., *et al.* (2000).The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol.*, 2(2):76-83.

Cao X., Chen D. (2005). The BMP signaling and in vivo bone formation. *Gene.*, 357:1-8.

Caplan, A.I. 1994. The mesengenic process. *Clin Plast Surg.,* 21:429–435.

Caplan A.I., Boyan B.D. (1994). Endochondral bone formation: the lineage cascade. In: Hall B.K., ed. Mechanisms of Bone Development and Growth. Boca Raton, Fla: CRC Press. *Bone.*, 8:2-46.

Carl T.F., Dufton C., Hanken J., *et al.* (1999). Inhibition of neural crest migration in *Xenopus* using antisense Slug RNA. *Dev Biol.*, 213:101–115.

Case N., Ma M., Sen B., *et al.* (2008). Beta-catenin levels influence rapid mechanical responses in osteoblasts. *J Biol Chem.*, 283(43):29196-29205.

Caterson, E.J., Nesti L.J., Danielson K.J., *et al.*(2002). Human marrow derived mesenchymal progenitor cells: isolation culture, expansion and analysis of differentiation. *Mol Biotechnol.,* 20:245–256.

Cavallo R.A., Cox R.T., Moline M.M., *et al*. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature.,*395(6702):604-608.

Chandler H.L., Colitz C.M., Lu P., *et al*. (2006). The role of the slug transcription factor in cell migration during corneal re-epithelialization in the dog. *Exp Eye Res*., 84(3):400-411.

Chau J.F., Leong W.F., Li B. (2009) Signaling pathways governing osteoblast proliferation, differentiation and function. *Histol Histopathol*., 24: 1593-1606.

Cheng A., Genever P.G. (2010). Sox9 determines Runx2 transactivity by directing intracellular degradation. *J Bone Miner Res*., 25(12):2404-2413.

Chen L., Deng C.X. (2005). Roles of FGF signaling in skeletal development and human genetic diseases. *Front Biosci*., 10:1961-1976.

Chen M., Xie H.Q., Deng L., *et al*. (2008). Stromal cell-derived factor-1 promotes bone marrow-derived cells differentiation to cardiomyocyte phenotype in vitro. *Cell Prolif*., 41:336-347.

Chen X.D., Qian H.Y., Neff L., *et al*. (1999). Thy-1 antigen expression by cells in the osteoblast lineage. *J Bone Miner Res*., 14:362-375.

Chen Y., Alman B.A. (2009). Wnt pathway, an essential role in bone regeneration. *J Cell Biochem*., 106 (3):353-362.

Chen Y., Whetstone H.C., Youn A., *et al*. (2007b). Beta-catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. *J Biol Chem.,* 282:526–533.

Cheng H., Jiang W., Phillips F.M., *et al*. (2003). Osteogenic activity of the fourteen types of human bone morphogenic proteins (BMPs). J Bone Joint Surg Am., 85-A:1544-1552.

Chiba S. (2006). Notch signaling in stem cell systems. *Stem Cells*., 24:2437-2447.

Cho H.H., Kim Y.J., Kim S.J., *et al*. (2006). Endogenous Wnt signaling promotes proliferation and suppresses osteogenic differentiation in human adipose derived stromal cells. *Tissue Eng*., 12:111–121.

Choi J., Park S.Y., Joo C.K. (2007). Transforming growth factor-beta1 represses E-cadherin production via slug expression in lens epithelial cells. *Invest Ophthalmol Vis Sci*., 48(6):2708-2718.

Clarkson E.D. (2001) Fetal tissue transplantation for patients with Parkinson's disease: a database of published clinical results. *Drugs Aging.,* 18:773–785

Clement-Lacroix P., Ai M., Morvan F., *et al.* (2005). Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci USA.,* 102(48):17406-17411.

Clevers H. (2006). Wnt/beta-catenin signaling in Development and disease. *Cell.,*127(3):469-480.

Cohen M.E., Yin M., Paznekas W.A., *et al.* (1998). Human SLUG gene organization, expression, and chromosome map location on 8q. *Genomics.*, 51(3):468-471.

Cohen P., Frame S (2001). The renaissance of GSK3. *Nat Rev Mol Cell Biol.*, 2(10):769-776.

Compagni A., Logan M., Klein R., *et al.* (2003). Control of skeletal patterning by ephrinB1-EphB interactions. *Dev Cell.*, 5:217-230.

Conacci-Sorrell M., Simcha I., Ben-Yedidia T., *et al.* (2003). Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. *J Cell Biol.*, 163(4):847-857.

Conconi M.T., Burra P., Di Liddo R., *et al.* (2006). CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. *Int J Mol Med.*, 18(6):1089-1096.

Cool S.M., Nurcombe V. (2006). Heparan sulfate regulation of progenitor cell fate. *J Cell Biochem.*, 99:1040-1051.

Cool S., Jackson R., Pincus P., *et al.* (2002). Fibroblast growth factor receptor 4 (FGFR4) expression in newborn murine calvaria and primary osteoblast cultures. *Int J Dev Biol.*, 46:519-523.

Coulombe P.A. (2003). Wound epithelialization: accelerating the pace of discovery. *J Invest Dermatol.*, 121(2):219-230.

Croce J.C., McClay D.R. (2006). The canonical Wnt pathway in embryonic axis polarity. *Semin Cell Dev Biol.*, 17(2):168-174.

D'Apuzzo M., Rolink A., Loetscher M., *et al.* (1997). The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur J Immunol.*, 27:1788-1793.

Dao D.Y., Yang X., Flick L.M., *et al.* (2010). Axin2 regulates chondrocyte maturation and axial skeletal Development. *J Orthop Res.,* 28(1):89-95.

Silva Meirelles L., Caplan A.I., Beyer Nardi N. (2008). In search of the in vivo identity of mesenchymal stem cells. *Stem Cells.*, 26:2287-2299.

da Silva Meirelles L., Chagastelles P.C., Nardi N.B. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci.*, 119:2204-2213.

da Silva Meirelles L., Nardi N.B. (2003). Murine marrow-derived mesenchymal stem cells: isolation, *in vitro* expansion, and characterization. *Br J Hematol.*, 123:702-711.

Davis L.A., Zur Nieden N.I. (2008). Mesodermal fate decisions of a stem cell: the Wnt switch. *Cell Mol Life Sci.,* 65:2658–2674.

Davis R.J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell.*, 103(2):239-252.

Day T.F., Guo X., Garrett-Beal L., *et al.* (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell.*, 8:739–750.

Day T.F, Yang Y. (2008). Wnt and hedgehog signaling pathways in bone development. *J Bone Joint Surg Am 90.,* (Suppl 1):19–24.

Deasy B.M., Jankowski R.J., Huard J. (2001). Muscle-derived stem cells: characterization and potential for cell-mediated therapy. *Blood Cells Mol Dis.,* 27:924–933.

De Bari C., Dell'Accio F. (2008). Cell therapy: a challenge in modern medicine. *Biomed Mater Eng.*, 18:S11-17.

De Bari C., Dell'Accio F., Karystinou A., *et al.* (2008). A biomarker-based mathematical model to predict bone-forming potency of human synovial and periosteal mesenchymal stem cells. *Arthritis Rheum.*, 58:240-250.

De Bari C., Dell'Accio F., Tylzanowski P., *et al.* (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum.,* 44:1928–1942.

De Bari C., Dell'Accio F., Vanlauwe J., *et al.* (2006). Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. *Arthritis Rheum.*, 54:1209-1221.

De Boer J., Wang H.J., Van Blitterswijk C. (2004b). Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells. *Tissue Eng.*, 10:393–401.

De Coppi P., Bartsch G Jr., Siddiqui M.M., *et al.* (2007). Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol., 25:100-106.

De Craene B., van Roy F., Berx G. (2005). Unraveling signalling cascades for the Snail family of transcription factors. *Cell Signal.*, 17(5):535-547.

De Ferrari G.V., Inestrosa N.V. (2000). Wnt signaling function in Alzheimer's disease. *Brain Res Rev.*, 33:1-12.

de Fructos C.A., Dacquin R., Vega S., *et al.* (2009). Snail1 controls bone mass by regulating Runx2 and VDR expression during osteoblast differentiation. *EMBO J.*, 28(6):686-696.

de Frutos C.A., Vega S., Manzanares M., *et al.* (2007). Snail1 is a transcriptional effector of FGFR3 signaling during chondrogenesis and achondroplasias. *Dev Cell.*, 13(6):872-883.

del Barrio M.G., Nieto M.A. (2002). Overexpression of Snail family members highlights their ability to promote chick neural crest formation. *Development.*, 129(7):1583-15693.

Dell'Accio F., De Bari C., Eltawil N.M., *et al.* (2008). Identification of the molecular response of articular cartilage to injury, by microarray screening: Wnt-16 expression and signaling after injury and in osteoarthritis. *Arthritis Rheum.*, 58:1410-1421.

Deng Z.L., Sharff K.A., Tang N., *et al.* (2008). Regulation of osteogenic differentiation during skeletal development. *Front Biosci.*, 13:2001-2021).

Dennis J.E., Merriam A., Awadallah A., *et al.* (1999). A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. *J Bone Miner Res.*, 14:700-709.

Deregowski V., Gazzerro E., Priest L., *et al.* (2006). Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not bone morphogenic protein signaling. *J Biol Chem.*, 281:6203-6210.

Derynck R., Akhurst R.J., Balmain A. (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet.*, 29:117-129.

Dexter T.M., Allen T.D., Lajtha L.G. (1977). Conditions controlling the proliferation of hematopoietic stem cells *in vitro. J Cell Physiol.*, 91:335-344.

Dhasarathy A., Kajita M., Wade P.A. (2007). The transcription factor Snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-α. *Mol Endocr.*, 21(12): 2907-2918.

D'Ippolito G., Schiller P.C., Ricordi C., *et al.* (1999). Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *J Bone Miner Res.*, 14(7):1115-22.

Di Girolamo C.M., Stokes D., Colter D., *et al.* (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br. J. Haematol.,* 107:275–281.

Di Naro E., Ghezzi F., Raio L., *et al.* (2001). Umbilical cord morphology and pregnancy outcome. *Ultrasound Obstet Gynecol.*,17(3):224-8.

Di Nicola M., Carlo-Stella C., Magni M., *et al.* (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular nonspecific mitogen stimuli. *Blood.*, 99:3838-3843.

Dobreva G., Chahrour M., Dautzenberg M., *et al.* (2006). SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. *Cell.*, 125:(5):971–986.

Dombrowski C. Song S.J., Chuan P., *et al.* (2009). Heparan sulfate mediates the proliferation and differentiation of rat mesenchymal stem cells. Stem Cells Dev., 18(4):661-670.

Domínguez D., Montserrat-Sentís B., Virgós-Soler A., *et al.* (2003). Phosphorylation Regulates the Subcellular Location and Activity of the Snail Transcriptional Repressor. *Mol Cell Biol.*, 23(14): 5078–5089.

Dominici M., Le BK Mueller I., Slaper-Cortenbach I., *et al.* (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international Society for Cellular Therapy position statement. *Cytotherapy.*, 8:315-317.

Dominici M., Rasini V., Bussolari R., *et al.* (2009). Restoration and reversibile expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. *Blood.,* 114:2333-2343.

Donald P.L., Haynesworth S.E., Buder S.P., *et al.* (1996). Human and animal mesenchymal progenitor cells from bone marrow: identification of serum for optimal selection and proliferation. *In Vitro Cell Dev Biol Anim.,* 32:602.

Dorsky R.I., Chang W.S., Rapaport D.H., *et al.* Regulation of neuronal diversity in the Xenopus retina by Delta signalling. *Nature.*, 385(6611):67-70.

Downey P.A., Siegel M.I. (2006). Bone biology and the clinical implications for osteoporosis. *Phys Ther.*, 86(1):77-91.

Ducy P., Zhang R., Geoffroy V., *et al.* (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell.*, 89(5):747–754.

Eames B.F., Sharpe P.T., Helms J.A. (2004). Hierarchy revealed in the specification of three skeletal fates by Sox9 and Runx2. *Dev Biol* 274:188–200.

Edmondson S.R., Thumiger S.P., Werther G.A., *et al.* (2003). Epidermal homeostasis: the role of the growth hormone and insulin-like growth factor systems. *Endocr Rev.*, 24(6):737-64.

Eeckhoute J., Carroll J.S., Geistlinger T.R., *et al.* (2006). A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. *Genes Dev.*, 20(18):2513-2526.

Ehebauer M., Hayward P., Arias A. (2006). Notch, a universal arbiter of cell fate decisions. *Science.*, 314:1414-1415.

Eijken M., Meijer I.M., Westbroek I., *et al.* (2008). Wnt signaling acts and is regulated in a human osteoblast differentiation dependent manner. *J Cell Biochem.*, 104:568-579.

Elloul S., Silins I., Tropé C.G., *et al.* (2006). Expression of E-cadherin transcriptional regulators in ovarian carcinoma. *Virchows Arch.*, 449(5):520-528.

El Tamer M.K., Reis R.L. (2009). Progenitor and stem cells for bone and cartilage regeneration. *J Tissue Eng and Regen Med.*, 3:327-337.

Endres M., Hutmacher D.W., Salgado A.J., *et al.* (2003). Osteogenic induction of human bone marrow-derived mesenchymal progenitor cells in novel synthetic polymer–hydrogel matrices. *Tissue Eng.,* 9:689–702.

Erices A., Conget P., Minguel J.J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol.,* 109:235–242.

Erlebacher A., Filvaro E.H., Gitelman S.E., *et al.* (1995). Toward a molecular understanding of skeletal development. *Cell.*, 80:371-378.

Essex L.J., Mayor R., Sargent M.G. (1993). Expression of Xenopus snail in mesoderm and prospective neural fold ectoderm. *Dev Dyn.*, 198(2):108-122.

Etheridge S.L., Spencer G.J., Heath D.J., *et al.* (2004). Expression profiling and functional analysis of Wnt signaling mechanisms in mesenchymal stem cells. *Stem Cells.*, 22:849–860.

Evans M.J., Kaufman M.H. (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature.,* 292:154–156.

Fernandez-Lloris R., Vinals F., Lopez-Rovira T., *et al.* (2003). Induction of the Sry-related factor SOX6 contributes to bone morphogenetic protein-2-induced chondroblastic differentiation of C3H10T1/2 cells. *Mol Endocrinol.*, 17:1332-1343.

Fernandez-Salguero P.M., Hilbert D.M., Rudikoff S., *et al.* (1996). Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol Appl Pharmacol.*, 140(1):173-179.

Filanti C., Dickson G.R., Di Martino D., *et al.* (2000). The expression of metalloproteinase-2, -9, and -14 and of tissue inhibitors-1 and -2 is developmentally modulated during osteogenesis in vitro, the mature osteoblastic phenotype expressing metalloproteinase-14. *J Bone Miner Res.*, 15:2154-2168.

Fischer L., Boland G., Tuan R.S. (2002). Wnt-3A enhances bone morphogenetic protein-2 mediated chondrogenesis of murine C3H10T1/2 mesenchymal cells. *J Biol Chem.,* 277:30870-30878.

Franc S., Rousseau J.C., Garrone R., *et al.* (1998). Microfibrillar composition of umbilical cord matrix: characterization of fibrillin, collagen VI and intact collagen V. *Placenta.*, 19(1):95-104.

Freeman T.B. (1997). From transplants to gene therapy for Parkinson's disease. *Exp Neurol.,* 144:47–50.

Friedenstein A.J. (1976). Precursor cells of mechanocytes. *Int Rev Cytol.*, 47:327-359.

Friedenstein A.J., Gorskaja J.F., Kilagina N.N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.*, 4:267-274.

Friedman R., Betancur M., Boissel L., *et al.* (2007). Umbilical cord mesenchymal stem cells: Adjuvant for human cell transplantation. *Biol Blood Marrow Transplant.*, 13:1477-1486.

Fujisawa-Sehara A., Sogawa K., Nishi C., *et al.* (1986). Regulatory DNA elements localized remotely upstream from the drug-metabolizing cytochrome P-450c gene. *Nucleic Acids Res.*, 14(3):1465-1477.

Fujita N., Jaye D.L., Kajita M., *et al.* (2003). MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. *Cell.*, 113(2):207-219.

Fukuchi Y., Nakajima H., Sugiyama D., *et al.* (2004). Human placenta derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells.,* 22:649–658.

Fuse N., Hirose S., Hayashi S. (1994). Diploidy of Drosophila imaginal cells is maintained by a transcriptional repressor encoded by escargot. *Genes Dev.*, 8(19):2270-2281.

Galceran J., Sustmann C., Hsu S.C., *et al.* (2004). LEF1- mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis. *Genes Dev.*, 18:2718–2723.

Galli R., Borello U., Gritti A., *et al.* (2000). Skeletal myogenic potential of human and mouse neural stem cells. *Nat Neurol.*, 3:986-991.

Gang, E.J., Bosnakovski D., Figueiredo C.A., *et al.* (2007). SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood.,* 109:1743–1751.

Garcia-Moruja C., Alonso-Lobo J.M., Rueda P., et al. (2005). Functional characterization of SDF-1 proximal promoter. *J Mol Biol.*, 348:43-62.

Gaur T., Lengner C.J., Hovhannisyan H. et al. (2005). Canonical WNT signaling promotes osteogenesis by directly stimulating RUNX2 gene expression. *J Biol Chem.*, 280:33132–33140.

Gentry T., Foster S., Winstead L., *et al.* (2007). Simultaneous isolation of human BM hematopoietic, endothelial and mesenchymal progenitor cells by flow sorting based on aldehyde dehydrogenase activity: implications for cell therapy. *Cytotherapy.,* 9:259–274.

Giordano J., Prior H.M., Bamforth J.S., *et al.* (2001). Genetic study of Sox9 in a case of campomelic dysplasia. *Am J Med Genet.*, 98:176-181.

Glass DAII., Bialek P., Ahn J.D., *et al.* (2005) Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell.,* 8:751–764.

Glavic A., Silva F., Aybar M.J., *et al.* (2004). Interplay between Notch signaling and the homeoprotein Xiro1 is required for neural crest induction in Xenopus embryos. *Development.*, 131(2):347-359.

Glennie S., Soeiro I., Dyson P.J., *et al.* (2005). Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cell. *Blood.*, 105:2821-2827.

Goldring M.B., Tsuchimochi K., Ijiri K. (2006). The control of chondrogenesis. *J Cell Biochem.*, 97:33-44.

Gong Y., Slee R.B., Fukai N., *et al.* (2002). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell.*, 107:513-525.

Gould T.D., Manji H.K. (2002). The Wnt signaling pathway in bipolar disorder. *Neuroscientist.*, 8:497-511.

Grassel S and Ahmed N. (2007). Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation. *Front Biosci.*, 12:4946-4956.

Grau Y., Carteret C., Simpson P. (1984). Mutations and chromosomal rearrangements affecting the expression of Snail, a gene involved in embryonic patterning in Drosophila melanogaster. *Genetics.*, 108:347-360.

Gregory C.A., Gunn W.G., Reyes E., *et al.* (2005). How Wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow. *Ann N Y Acad Sci.*, 1049:97-106.

Gregory C.A., Singh H., Perry A.S., *et al.* (2003). The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow. *J Biol Chem.*, 278:28067–28078.

Greider C.W. Telomerase activity, cell proliferation, and cancer. (1998). *Proc Natl Acad Sci USA.*, 95:90 –92.

Griffith C.M., Wiley M.J., Sanders E.J. (1992). The vertebrate tail bud: three germ layers from one tissue. *Anat Embryol.*, 185(2):101-113.

Grimes H.L., Gilks C.B., Chan T.O., *et al.* (1996). The Gfi-1 proto-oncoprotein represses Bax expression and inhibits T-cell death. *Proc Natl Acad Sci U S A.*, 93(25):14569-14573.

Grosshans J., Wieschaus E. (2000). A genetic link between morphogenesis and cell division during formation of the ventral furrow in *Drosophila. Cell.*, 101:523–531.

Guicheux J., Lemonnier J., Ghayor C., *et al.* (2003). Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *J Bone Miner Res.*, 18:2060-2068.

Guillot P.V., De Bari C., Dell'Accio F., *et al.* (2008). Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. *Differentiation.*, 76(9):946-957.

Gundberg C.M. (2000). Biochemical markers of bone formation. *Clin Lab Med.*, 20(3):489-501.

Guo Y., Zi X., Koontz Z., *et al.* (2007). Blocking Wnt/LRP5 signaling by a soluble receptor modulates the epithelial to mesenchymal transition and suppresses met and metalloproteinases in osteosarcoma Saos-2 cells. *J Orthop Res.*, 25(7):964-971.

Habas R., Dawid I.B., He X. (2003). Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev.,*17(2):295-309.

Hajra K.M., Chen D.Y., Fearon E.R. (2002). The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res.*, 62(6):1613-1618.

Hamrick M.W., Ding K.H., Pennington C., *et al.* (2006). Age-related loss of muscle mass and bone strength in mice is associated with a decline in physical activity and serum leptin. *Bone.*,39(4):845-53.

Han K., Manley J.L. (1993). Functional domains of the Drosophila Engrailed protein. *EMBO J.*, 12(7):2723-2733.

Han Y., Lefebvre V. (2008). L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. *Mol Cell Biol.*, 28:4999-5013.

Hanada K., Dennis J.E., Caplan A.I. (1997). Stimulatory effects of basic fibroblast growth factor and bone morphogenic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchynal stem cells. *J Bone Miner Res.*, 12:1606-1614.

Hartmann C. (2009). Transcriptional networks controlling skeletal development. *Curr Opin Genet Develop.*, 19:437–443

Hartmann C.A. (2006). Wnt canon orchestrating osteoblastogenesis. *Trends Cell Biol.,* 16(3):151-158.

Hartmann T.N., Grabovsky V., Pasvolsky R., *et al.* (2008). A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. *J Leukoc Biol.*, 84:1130-1140.

Hassan M.Q., Tare R., Lee S.H., *et al.* (2007). HOXA10 controls osteoblastogenesis by directly activating bone regulatory and phenotypic genes. *Mol Cell Biol.*, 27(9):3337–3352.

He T.C., Chan T.A., Vogelstein B., *et al.* (1999). PPAR delta is an APC-regulated target of non steroidal anti-inflammatory drugs. *Cell.,* 99(3):335-345.

Heary R.F., Schlenk R.P., Sacchieri T.A:, *et al.* (2002). Persistent iliac crest donor site pain: independent outcome assessment. *Neurosurgery.*, 50:510-516.

Hemavathy K., Guru S.C., Harris J., *et al.* (2000). Human Slug is a repressor that localizes to sites of active transcription. *Mol Cell Biol.*, 20(14):5087–5095.

Hemavathy K., Hu X., Ashraf S.I. (2004). The repressor function of snail is required for Drosophila gastrulation and is not replaceable by Escargot or Worniu. *Dev Biol.*, 269(2):411-420.

Hemavathy K., Meng X., Ip Y.T. (1997). Differential regulation of gastrulation and neuroectodermal gene expression by Snail in the Drosophila embryo. *Development.*, 124(19):3683-3691.

Hentthorn P.S. (2002). Alkaline phosphatase. In *Principles of Bone Biology*. Academic Press: New York.

Hill T.P., Spa¨ter D., Taketo M.M., et al. (2005) Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev Cell.*, 8:727–738.

Hogan B.L. (1996). Bone morphogenic proteins: multifunctional regulators of vertebrate development. *Genes Dev.*, 10:1580-1594.

Holmen S.L., Zylstra C.R., Mukherjee A., *et al.* (2005). Essential role of β-catenin in postnatal bone acquisition. *J Biol Chem.*, 280:21162–21168.

Hong C.F., Chou Y.T., Lin Y.S., *et al.* (2009). MAD2B, a novel TCF4-binding protein, modulates TCF4-mediated epithelial-mesenchymal transdifferentiation. *J Biol Chem.*, 284(29):19613-19622.

Hong J.H., Hwang E.S., McManus M.T., *et al.* (2005). TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. *Science.*, 309(5737):1074–1078.

Honoré S.M., Aybar M.J., Mayor R. (2003). Sox10 is required for the early development of the prospective neural crest in Xenopus embryos. *Dev Biol.*, 260(1):79-96.

Horwitz E.M., Gordon P.L., Koo W.K., *et al.* (2002). Isolated allogeneic bonemarrow derived mesenchymal stem cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Nat Acad Sci USA.,* 99:8932–8937.

Horwitz E.M., Prockop D.J., Fitzpatrick L.A., *et al.* (1999). Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Medicine.,* 5:262–264.

Hosogane N., Huang Z., Rawlins B.A., (2010). Stromal derived factor-1 regulates bone morphogenic protein-2-induced osteogenic differentiation of primary mesenchynal stem cells. *Int J Biochem Cell Biol.*, 42(7):1132-1141.

Hou T., Xu J., Xie Z., *et al.* (2009). Umbilical cord Wharton's Jelly: a new potential cell source of mesenchymal stromal cells for bone tissue engineering. *Tissue Eng Part A.*, 15(9):2325-2334.

Hristov M., Erl W., Weber P.C. (2003), Endothelial progenitor cells mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol.*, 23(7):1185-1189.

Hsieh J.Y., Fu Y.S., Chang S.J., *et al.* (2010). Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton's jelly of umbilical cord. *Stem Cells Dev.*, 19:1895-1910.

Hu H., Hilton M.J., Tu X., et al. (2005). Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Developmen.,t* 132:49–60.

Hu Y., Liao L., Wang Q., *et al.* (2003). Isolation and identification of mesenchymal stem cells from human fetal pancreas. *J Lab Clin Med.*, 141:342-349.

Huang Q.Y., Li G.H., Kung A.W. (2009). The -9247 T/C polymorphism in the SOST upstream regulatory region that potentially affects C/EBPalpha and FOXA1 binding is associated with osteoporosis. *Bone.,* 45(2):289-294.

Huang W., Yang S., Shao J., *et al.* (2007). Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci.*, 12:3068-3092.

Hudson L.G., Newkirk K.M., Chandler H.L., *et al.* (2009). Cutaneous wound reepithelialization is compromised in mice lacking functional Slug (Snai2). *J Dermatol Sci.*, 56(1):19-26.

Hurlbut G.D., Kankel M.W., Lake R.J., *et al.* (2007). Crossing paths with Notch in the hyper-network. *Curr Opin Cell Biol.*, 19:166-175.

Ibbotson K.J., Harrod J., Gowen M., *et al.* (1986). Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. *Proc Natl Acad Sci USA.*, 83:2228-2232.

Ikeda T., Kamekura S., Mabuchi A., *et al.* (2004). The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum.*, 50, 3561-3573.

Ikeya M., Lee S.M., Johnson J.E., *et al.* (1997). Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature.*, 389(6654):966-970.

Ikuta T., Kawajiri K. (2006). Zinc finger transcription factor Slug is a novel target gene of aryl hydrocarbon receptor. *Exp Cell Res.*, 312(18):3585-3594.

Inoue A., Seidel M.G., Wu W., *et al.* (2002). Slug, a highly conserved zinc finger transcriptional repressor, protects hematopoietic progenitor cells from radiation-induced apoptosis in vivo. *Cancer Cell.*, 2(4):279-288.

In't Anker P.S., Noort W.A., Scherjon S.A., *et al.* (2003). Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica.,* 88:845–852.

In't Anker P.S., Scherjon S.A., Kleijburg-van der Keur C., *et al.* (2004). Isolation of mesenchymal stem cells of fetal and maternal origin from human placenta. *Stem Cells.,* 22:1338–1345.

Inukai T., Inoue A., Kurosawa H., *et al.* (1999). SLUG, a ces-1-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF oncoprotein. *Mol Cell.*, 4(3):343-352.

Ip Y.T., Park R.E., Kosman D., *et al.* (1992). dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. *Genes Dev.*, 6(8):1518-1530.

Isaac A., Cohna M.J., Ashby P., *et al.* (2000). FGF and genes encoding transcription factors in early limb specification. *Mech Develop.,* 93:41-48.

Ishimi Y., Miyaura C., Jin C.H., *et al.* (1990). IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol.*, 145:3297-3303.

Iwamoto T., Okamoto H., Toyama Y., *et al.* (2008). Molecular aspects of rheumatoid arthritis: chemokines in the joints of patients. *FEBS J.,* 275(18):4448-4455.

Jackson R.A., Nurcombe V., Cool S.M. (2006). Coordinated fibroblast growth factor and heparin sulfate regulation of osteogenesis. *Gene.*, 379:79-81.

Jaiswal N., Haynessworth S.E., Caplan AI., *et al.* (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J Cell Biochem.,* 64:295–312.

Javed A., Bae J.S., Afzal F., *et al.* (2008). Structural coupling of Smad and Runx2 for execution of the BMP2 osteogenic signal. *J Biol Chem.*, 283(23):8412-8422.

Javed A., Chen H., Farah Y.G. (2010). Genetic and transcriptional control of bone formation. *Oral Maxillofacial Surg Clin N Am.*, 22:283-293.

Javed A., Guo B., Hiebert S., *et al.* (2000). Grouncho/TLE/R-esp proteins associate with the nuclear matrix and repress RUNX (CBF(alpha)/AML/PEBP2(alpha)) dependent activation of tissue-specific gene transcription. *J Cell Sci.*, 113(Pt 12):2221-2231.

Jethwa P., Naqvi M., Hardy R.G., *et al.* (2008). Overexpression of Slug is associated with malignant progression of esophageal adenocarcinoma. *World J Gastroenterol.*, 14(7):1044-1052.

Jiang Y., Jahagirdar B.N., Reinhardt R.L., *et al.* (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature.,* 418:41–49.

Jiao W., Miyazaki K., Kitajima Y. (2002). Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines in vitro and in vivo. *Br J Cancer.*, 86(1):98-101.

Jin T., George Fantus I., Sun J. (2008). Wnt and beyond Wnt: multiple mechanisms control the transcriptional property of beta-catenin. *Cell Signal.*, 20(10):1697-1704.

Johnstone B., Hering T.M., Caplan A.I., *et al.* (1998). *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res.,* 238:265–272.

Jones D.C., Wein M.N., Oukka M., *et al.* (2006). Regulation of adult bone mass by the zinc finger adapter protein Schnurri-3. *Science.*, 312(5777):1223–1227.

Kadiyala S., Young R.G., Thiede M.A., *et al.* (1997). Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential *in vivo* and *in vitro*. *Cell Transpl.,* 6:125–134.

Kadner A., Hoerstrup S.P., Tracy J., *et al.* (2002). Human umbilical cord cells: a new cell source for cardiovascular tissue engineering. *Ann Thorac Surg.,* 74:S1422–1428.

Kahler R.A., Galindo M., Lian J., *et al.* (2006). Lymphocyte enhancer-binding factor 1 (Lef1) inhibits terminal differentiation of osteoblasts. *J Cell Biochem.*, 97:969-983.

Kahler R.A., Westendorf J.J. (2003). Lymphoid enhancer factor-1 and betacatenin inhibit Runx2-dependent transcriptional activation of the osteocalcin promoter. *J Biol Chem.*, 278:11937–11944.

Kahler R.A., Yingst S.M., Hoeppner L.H., *et al.* (2008). Collagen 11a1 is indirectly activated by lymphocyte enhancer-binding factor 1 (Lef1) and negatively regulates osteoblast maturation. *Matrix Biol.*, 27:330-338.

Kalluri R., Weinberg R.A. (2009). The basics of epithelial-mesenchymal transition. *J Clin Invest.*, 119(6):1420-1428.

Kanatani N., Fujita T., Fukuyama R., *et al.* (2006). Cbf beta regulates Runx2 function isoform-dependently in postnatal bone development. *Dev Biol.*, 296(1):48–61.

Kang P., Svoboda K.K. (2005). Epithelial-mesenchymal transformation during craniofacial development. *J Dent Res.*, 84(8):678-690.

Kang S., Bennett C.N., Gerin I., *et al.* (2007). Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancerbinding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem.,* 282:14515–14524.

Kang Y., Massagué J. (2004). Epithelial-Mesenchymal Transitions. *Cell.*, 118(3):277-279.

Karahuseyinoglu S., Cinar O., Kilic E., *et al.* (2007). Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. *Stem Cells.*, 25(2):319-31.

Karsenty G. (2008). Transcriptional control of skeletogenesis. *Annu Rev Genomics Hum Genet.*, 9:183-196.

Karsenty G., Wagner E.F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev Cell.*, 2(4):389-406.

Karystinou A., Dell'Accio F., Kurth T.B., *et al.* (2009). Distinct mesenchymal progenitor cell subsets in the adult human synovium. *Rheumatology* (Oxford)., 48:1057-1064.

Kataoka H., Murayama T., Yokode M. (2000). A novel Snail-related transcription factor Smuc regulates basic helix–loop–helix transcription factor activities via specific E-box motifs. *Nucleic Acids Res.*, 28(2):626–633.

Kato M., Patel M.S., Levasseur R., *et al.* (2002). Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J. Cell Biol.,* 157(2):303-314.

Katoh M. (2006). Notch ligand, JAG1, is evolutionarily conserved target of canonical WNT signaling pathway in progenitor cells. *Int J Mol Med.*, 17:681–685.

Katoh M., Katoh M. (2003). Identification and characterization of human SNAIL3 (SNAI3) gene in silico. *Int J Mol Med.*, 11(3):383-388.

Katoh M., Katoh M. (2005). Comparative genomics on SNAI1, SNAI2, and SNAI3 orthologs. *Oncol Rep.*,14(4):1083-1086.

Katoh S., Fukushima K., Matsumoto N., *et al.* (2005). Accumulation of CXCR3-expressing eosinophils and increased concentration of its ligands (IP10 and Mig) in bronchoalveolar lavage fluid of patients with chronic eosinophilic pneumonia. *Int Arch Allergy Immunol.*, 137(3):229-235.

Kaviani A., Guleserian K., Perry T.E., *et al.* (2003). Fetal tissue engineering from amniotic fluid. *J Am Coll Surg.,* 196:592–597.

Kaviani A., Perry T.E., Barnes C.M., *et al.* (2002). The placenta as a cell source in fetal tissue engineering. *J Pediatr Surg.,* 37:995–999

Kawaguchi J. (2006). Generation of osteoblasts and chondrocytes from embryonic stem cells. *Methods Mol Biol.*, 330:135-148.

Kern S., Eichler H., Stoeve J., *et al.* (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells.*, 24:1294-1301.

Kim I.S., Otto F., Zabel B., *et al.* (1999). Regulation of chondrocyte differentiation by Cbfa1. *Mech Dev.*, 80:159-170.

Kim K.J., Kim H.H., Kim J.H., *et al.* (2007). Chemokine stromal cell-derived factor-1 induction by C/EBPbeta activation is associated with all-trans-retinoic acid-induced leukemic cell differentiation. *J Leukoc Biol.*, 82:1332-1339.

Kim S., Koga T., Isobe M., *et al.* (2003). Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. *Genes Dev.*, 17(16):1979–1991.

Kirouac D.C., Zandstra P.W. (2008). The systematic production of cells for cell therapies. *Cell Stem Cell* 3:369-381.

Kitaori T., Ito H., Schwarz E.M., et al. (2009). Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum.*, 60:813-823.

Kokubu C., Heinzmann U., Kokubu T., *et al.* (2004). Skeletal defects in ringelschwanz mutant mice reveal that Lrp6 is required for proper somitogenesis and osteogenesis. *Development.,* 131(21):5469-5480.

Komori T. (2006). Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem.*, 99(5):1233–1239.

Komori T. (2008). Regulation of bone development and maintenance by Runx2. *Front Biosci.*, 13:898-903.

Komori T. (2009). Regulation of bone development and extracellular matrix protein genes by RUNX2. *Cell Tissue Res.*, doi 10.1007/s00441-009-0832-8.

Komori T., Yagi H., Nomura S., *et al.* (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell.*, 89 (5):755–764.

Kon E., Muraglia A., Corsi A., *et al.* (2000). Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res.*, 49:328-337.

Kosman D., Ip Y.T., Levine M., *et al.* (1991). Establishment of the mesoderm-neuroectoderm boundary in the Drosophila embryo. *Science.*, 254(5028):118-122.

Krampera M., Marconi S., Pasini A., *et al.* (2007). Induction of neural-like differentiation in human mesenchymal stem cellsderived from bone marrow, fat, spleen and thymus. *Bone.*, 40:382-390.

Krishnan V., Bryant H.U., Macdougald O.A. (2006). Regulation of bone mass by Wnt signaling. *J. Clin. Invest.,*116(5):1202-1209.

Kubota T., Michigami T., Ozono K. (2009). Wnt signaling in bone metabolism. *J. Bone Miner. Metab.,* 27(3):265-271.

Kugimiya F., Kawaguchi H., Ohba S., *et al.* (2007). GSK-3beta controls osteogenesis through regulating Runx2 activity. *PLoS One.,* 2(9):e837.

Kuhl M. (2004). The WNT/calcium pathway: biochemical mediators, tools and future requirements. *Front. Biosci.,* 9:967-974.

Kulkarni N.H., Onyia J.E., Zeng Q., *et al.* (2006). Orally bio-available GSK 3alpha/beta dual inhibitor increases markers of cellular differentiation *in vitro* and bone mass *in vivo*. *J Bone Miner Res.,* 21(6):910-920.

Kundu M., Javed A., Jeon J.P., *et al.* (2002). Cbfbeta interacts with Runx2 and has a critica role in bone development. *Nat Genet.*, 32(4):639-644.

Kuznetsov S.A., Mankani M.H., Gronthos S., *et al.* (2001). Circulating skeletal stem cells. *J Cell Biol.,* 153:1133–1140.

Kwan M.D., Slater B.J., Wan D.C., *et al.* (2008). Cell-based therapies for skeletal regenerative medicine. *Hum Mol Genet.*, 17:93-98.

LaBonne C., Bronner-Fraser M. (1998a). Neural crest induction in Xenopus: evidence for a two-signal model. *Development.*, 125(13):2403-2414.

LaBonne C., Bronner-Fraser M. (1998b). Induction and patterning of the neural crest, a stem cell-like precursor population. *J Neurobiol.*, 36(2):175-189.

LaBonne C., Bronner-Fraser M. (2000). Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev Biol.*, 221(1):195-205.

Lai C.F., Cheng S.L. (2002). Signal transductions induced by bone morphogenic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. *J Biol Chem.*, 277:15514-15522.

Lanotte M., Allen T.D., Dexter T.M. (1981). Histochemical and ultrastructural characteristics of a cell line from human bone-marrow stroma. *J Cell Sci.,* 50:281-297.

Lambertini E., Penolazzi L., Tavanti E., *et al.* (2007). Human estrogen receptor alpha gene is a target of Runx2 transcription factor in osteoblasts. *Exp Cell Res.*, 313:1548-1560.

Lambertini E., Tavanti E., Torreggiani E., *et al.* (2008). ERα and AP-1 interact in vivo with a specific sequence of F promoter of human ERα gene in osteoblasts. *J Cell Physiol.*, 216(1): 101-110.

Lammi L., Arte S., Somer M., *et al.* (2004). Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. *Am. J. Hum. Genet.,*74(5):1043-1050.

Larriba M.J., Martín-Villar E., García J.M., *et al.* (2009). Snail2 cooperates with Snail1 in the repression of vitamin D receptor in colon cancer. *Carcinogenesis.*, 30(8):1459-1468.

Lataillade J.J., Clay D., Dupuy C., *et al.* (2000). Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. *Blood.*, 95:756-768.

Lauffenburger D.A., Schaffer D.V. (1999). The matrix delivers. *Nat Med.,* 5:733-734.

Le Douarin N.M., Dupin E., Ziller C. (1994). Genetic and epigenetic control in neural crest development. *Curr Opin Genet Dev.*, 4(5):685-695.

Lee B., Thirunavukkarasu K., Zhou L., *et al.* (1997). Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet.*, 16(3):307–310.

Lee K.S., Kim H.J., Li Q.L., *et al.* (2000). Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol.*, 20(23):8783–8792.

Lee M.H., Kim Y.J., Kim H.J., *et al.* (2003). BMP-2–induced Runx2 expression is mediated by Dlx5, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. *J Biol Chem.*, 278:34387-34394.

Lee O.K., Kuo T.K., Chen W.M., *et al.* (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood.*, 103(5):1669-1675.

Lee R.H., Kim B., Choi I., *et al.* (2004). Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem.,* 14:311–324.

Lefebvre V. (1998). Toward understanding SOX9 function in chondrocyte differentiation. *Matrix Biol.*, 16:529-540.

Lefebvre V., Huang W., Harley V.R., *et al.* (1997). SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. *Mol Cell Biol.,* 17:2336–2346.

Lefebvre V., Smits P. (2005). Transcriptional control of chondrocyte fate and differentiation. Birth *Defects Res C Embryo Today.*, 75:200-212.

Lennon D.P., Caplan I. (2006). Isolation of human marrow-.derived mesenchymal stem cells. *Exp Hematol.*, 34:1604-1605.

Lennon D.P., Caplan I. (2006). Isolation of rat marrow-.derived mesenchymal stem cells. *Exp Hematol.*, 34:1606-1607.

Leptin M. (1991). twist and snail as positive and negative regulators during Drosophila mesoderm development. *Genes Dev.*, 5(9):1568-1576.

Lerner U.H. (2006). Bone remodeling in post-menopausal osteoporosis. *J Dent Res.*, 85(7):584-595.

Leucht P., Minears S., Ten Berge D., *et al.* (2008). Translating insights from development into regenerative medicine: the function of Wnts in bone biology. *Semin Cell Dev Biol.*, 19(5):434-443.

Levoye A., Balabanian K., Baleux F *et al.* (2009). CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood.*, 113:6085-6093.

Li J., Sarosi I., Cattley R.C., *et al.* (2006) Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. *Bone* 39:754–766.

Li X., Huang M., Zheng H., *et al.* (2008). CHIP promotes Runx2 degradation and negatively regulates osteoblast differentiation. *J Cell Biol.*, 181(6):959-972.

Li X., Ominsky M.S., Niu Q.T., *et al.* (2008). Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J. Bone Miner. Res.,* 23(6):860-869.

Li X., Zhang Y., Kang H., *et al.* (2005) Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem.*, 280:19883–19887.

Lian J.B., Javed A., Zaidi S.K., *et al.* (2004). Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. *Crit Rev Eukaryot Gene Expr.*, 14(1–2):1–41.

Liem K.F. Jr., Tremml G., Roelink H., *et al.* (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell.*, 82(6):969-979.

Lin C., Jiang X., Dai Z., *et al.* (2009). Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. *J. Bone Miner. Res.,* 24(10):1651-1661.

Lin L., Chen L., Wang H., *et al.* (2006). Adenovirus-mediated transfer of siRNA against Runx2/Cbfa1 inhibits the formation of heterotopic ossification in animal model. *Biochem Biophys Res Commun.*, 349:564-572.

Ling L., Nurcombe V., Cool S.M. (2009). Wnt signaling controls the fate of mesenchymal stem cells. *Gene.*, 433:1-7.

Lisignoli G., Codeluppi K., Todoerti K., *et al.* (2009). Gene array profile identifies collagen type XV as a novel human osteoblast-secreted matrix protein. *J Cell Physiol.*, 220(2):401-409.

Lisignoli G., Toneguzzi S., Piacentini A., *et al.* (2006). CXCL12 (SDF-1) and CXCL13 (BCA-1) chemokines significantly induce proliferation and collagen type I expression in osteoblasts from osteoarthritis patients. *J Cell Physiol.*, 206:78-85.

Liu W., Toyosawa S., Furuichi T., *et al.* (2001). Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J Cell Biol.*, 155(1):157–166.

Logan C.Y., Nusse R. (2004). The Wnt signaling pathway in development and disease. *Ann Rev Cell Dev Biol.*, 20:781-810.

Long F., Chung U.I., Ohba S., *et al.* (2004). Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development.*, 131:1309-1318.

Lorget F., Kamel S., Mentaverri R., *et al.* (2000). High extracellular calcium concentrations directly stimulate osteoclast apoptosis. *Biochem Biophys Res Commu.*, 268(3):899-903.

Loughlin J., Dowling B., Chapman K., *et al.* (2004). Functional variants within the secreted frizzled-related protein 3 gene are associated with hip osteoarthritis in females. *Proc Natl Acad Sci USA.,* 101(26):9757-9762.

Lu L.L., Liu Y.J., Yang S.G., *et al.* (2006). Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Hematologica.*, 91:1017-1026.

Luan Y., Yu X.P., Xu K., *et al.* (2007). The retinoblastoma protein is an essential mediator of osteogenesis that links the p204 protein to the Cbfa1 transcription factor thereby increasing its activity. *J Biol Chem.*, 282(23):16860–16870.

Lund R.D., Wang S., Lu B., *et al.* (2007). Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. Stem Cells., 25:1384-1392.

Luo W., Peterson A., Garcia B.A., *et al*. (2007). Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex. *EMBO J*., 26(6):1511-1521.

Luu H.H., Song W.X., Luo X., *et al*. (2007). Distinct roles of bone morphogenic proteins in osteogenic differentiation of mesenchymal stem cells. *J Orthop Res*., 25:665-677.

Mackay A.M., Beck S.C., Murphy J.M., *et al*. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.,* 4:415–428.

Mackay D.L., Tesar P.J., Liang L.N., *et al*. (2006). Characterizing medullary and human mesenchymal stem cell-derived adipocytes. *J Cell Physiol*., 207:722–728.

Mackay J.P., Crossley M., (1998). Zinc fingers are sticking together. *Trends Biochem Sci*., 23(1):1-4.

Majka M., Ratajczak J., Lee B., *et al*. (2000). The role of HIV-related chemokine receptors and chemokines in human erythropoiesis in vitro. *Stem Cells*., 18:128-138.

Mani A., Radhakrishnan J., Wang H., *et al*. (2007). LRP6 mutation in a family with early coronary disease and metabolic risk factors. *Science.,* 315(5816):1278-1282.

Mannello F., Tonti G.A.(2007). Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! *Stem Cells*., 25:1603-1609.

Manton K.J., Haupt L.M., Vengadasalam K., *et al*. (2007a). Glycosaminoglycan and growth factor mediated murine calvarial cell proliferation. *J Mol Histol*., 38:415-424.

Manzanares M., Locascio A., Nieto M.A. (2001). The increasing complexity of the Snail gene superfamily in metazoan evolution. *Trends in Genetics*.,17(4): 178-181.

Marchant L., Linker C., Ruiz P., *et al*. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev Biol*., 198(2):319-329.

Marie P.J. (2008). Transcription factor controlling osteoblastogenesis. *Archives of Biochemistry and Biophysics* 473:98–105).

Markov V., Kusumi K., Tadesse M.G., *et al*. (2007). Identification of cord blood-derived mesenchymal stem/stromal cell populations with distinct growth kinetics, differentiation potentials, and gene expression profiles. *Stem Cells Dev.,* 16:53-73.

Marks S.C., Hermey D.C. (1996). The structure and development of bone. In: Bilezikian J.P., Raisz L.G., Rodan G.A., *eds. Principles of Bone Biology*. San Diego, Calif: Academic Press:3-14.

Martin G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured inmedium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA.,* 78:7634–7638.

Martínez-Alvarez C., Blanco M.J., Pérez R., *et al.* (2004). Snail family members and cell survival in physiological and pathological cleft palates. *Dev Biol.*, 265(1):207-218.

Martínez-Estrada O.M., Cullerés A., Soriano F.X., *et al.* (2006). The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells. *Biochem J.*, 394(Pt 2): 449–457.

Maruyama Z., Yoshida C.A., Furuichi T., *et al.* (2007). Runx2 determines bone maturity and turnover rate in postnatal bone development and is involved in bone loss in estrogen deficiency. *Dev Dyn.*, 236(7):1876–1890.

Matsuo K., Irie N. (2008). Osteoblast-osteoclast communication. *Arch Biochem Biophys.*, 473(2):201-219.

Matsuzaki E., Takahashi-Yanaga F., Miwa Y., *et al.* (2006). Differentiation-Inducing Factor-1 alters canonical Wnt signaling and suppresses alkaline phosphatase expression in osteoblast-like cell lines. J Bone Miner Res., 21:1307-1316.

Mayor R., Morgan R. Sargent M.G. (1995). Induction of the prospective neural crest of Xenopus. *Development.*, 121:767-777.

Mbalaviele G., Sheikh S., Stains J., *et al.* (2005). Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. *J Cell Biochem.*, 94(2):403-418.

McCabe L.R., Banerjee C., Kundu R., *et al.* (1996). Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. *Endocrinology.*,137(10):4398-4408.

McCarthy T.L., Chang W.Z., Liu Y., *et al.* (2003). Runx2 integrates estrogen activity in osteoblasts. *J Biol Chem.*, 278:43121-43129.

Medici D., Hay E.D., Olsen B.R. (2008). Snail and Slug promote epithelial-mesenchymal transition through β-Catenin–T-Cell Factor-4-dependent expression of transforming growth factor-β3. *Mol Biol Cell.*, 19(11):4875-4887.

Metzstein M.M., Horvitz H.R. (1999). The *C. elegans* cell death specification gene ces-1 encodes a snail family zinc finger protein. Mol Cell., 4:309-319.

Mezek E., Key S., Vogelsang G., *et al.* (2000). Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci USA.*, 100:1364-1369.

Miller J., Horner A., Stacy T., *et al.* (2002). The core-binding factor beta subunit is required for bone formation and hematopoietic maturation. *Nat Genet.* 32(4):645–649.

Mimura J., Yamashita K., Nakamura K., *et al.* (1997). Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells., 2(10):645-654.

Minguell J.J., Erices A., Conget P. (2001). Mesenchymal stem cells. *Exp Biol Med* (Maywood)., 226(6):507-20.

Miraoui H., Severe N., Vaudin P., *et al.* (2010). Molecular silencing of Twist1 enhances osteogenic differentiation of murine mesenchymal stem cells: implication of FGFR2 signaling. *J Cell Biochem.*, 110:1147-1154.

Mitchell K.E., Weiss M.L., Mitchell B.M., *et al.* (2003). Matrix cells from Wharton's jelly form neurons and glia. Stem Cells., 21:50-60.

Mitka M. (2001). Amniotic cells show promise for fetal tissue engineering. *J Am Med Assoc.,* 286: 2083.

Mittal M.M., Myers J.N., Misra S., *et al.* (2008). In vivo binding to and functional repression of the VDR gene promoter by SLUG in human breast cells. *Biochem Biophys Res Com.*, 372:30–34.

Miyama K., Yamada G., Yamamoto T.S., *et al.* (1999). A BMP-inducible gene, dlx5, regulates osteoblast differentiation and mesoderm induction. *Dev Biol.*, 208:123-133.

Moggs J.G., Murphy T.C., Lim F.L., *et al.* (2005). Anti-proliferative effect of estrogen in breast cancer cells that re-express ERalpha is mediated by aberrant regulation of cell cycle genes. *J Mol Endocrinol.*, 34(2):535-551.

Montero J.A., Gañan Y., Macias D., *et al.* (2001). Role of FGFs in the control of programmed cell death during limb development. *Development.,* 128:2075-2084.

Montjovent M.O., Burri N., Mark S., *et al.* (2004). Fetal bone cells for tissue engineering. *Bone.,* 35:1323–1333.

Moretti P., Hatlapatka T., Marten D., *et al.* (2010). Mesenchymal stromal cells derived from human umbilical cord tissues: primitive cells with potential for clinical and tissue engineering applications. *Adv Biochem Eng Biotechnol.*, 123:29-54.

Morgan M.J., Woltering J.M., In der Rieden P.M.J., *et al.* (2004). YY1 Regulates the Neural Crest-associated *slug* Gene in *Xenopus laevis. J Biol Chem.*, 279(45):46826–46834.

Morvan F., Boulukos K., Cle´ment-Lacroix P., *et al.* (2006). Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. *J Bone Miner Res.,* 21:934–945.

Mosna F., Senseb L., Krampera M. (2010). Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide. *Stem Cells Dev.*, 19:1449-1470.

Moustafa A., Sugiyama T., Saxon L.K., et al. (2009). The mouse fibula as a suitable bone for the study of functional adaptation to mechanical loading. *Bone.,* 44(5):930-935.

Mukhopadhyay M., Shtrom S., Rodriguez-Esteban C., *et al.* (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev Cell.*, 1:423–434.

Mundy G.R., Elefteriou F. (2006). Boning up on ephrin signaling. *Cell.*, 126:441-443.

Muraglia A., Martin I., Cancedda R., *et al.* (1998). A nude mouse model for human bone formation in unloaded conditions. *Bone.*, 22:131S-134S.

Murakami T., Fukai K., Oiso N., *et al.* (2004). New KIT mutations in patients with piebaldism. *J Dermatol Sci.*, 35(1):29-33.

Murray S.A., Gridley T. (2006). Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice. *Proc Natl Acad Sci U S A.*, 103(27):10300-10304.

Murray S.A., Oram K.F., Gridley T. (2007). Multiple functions of Snail family genes during palate development in mice. *Development.*, 134(9):1789-97.

Nakamura T., Imai Y., Matsumoto T., *et al.* (2007). Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell.*, 130:811-823.

Nakayama H., Scott I.C., Cross J.C. (1998). The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor. *Dev Biol.*, 199:150–163.

Nibu Y., Zhang H., Bajor E., *et al.* (1998). dCtBP mediates transcriptional repression by Knirps, Krüppel and Snail in the Drosophila embryo. *EMBO J.*, 17(23):7009-7020.

Nielsen R.H., Karsdal M.A., Sorensen M.G., *et al.* (2007). Dissolution of the inorganic phase of bone leading to release of calcium regulates osteoclast survival. *Biochem Biophys Res Commun.*, 360(4):834-839.

Nieto M.A., Sargent M.G., Wilkinson D.G., Cooke J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science.*, 264(5160):835-839.

Nieto M.A. (2002). The Snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol.*, 3:155-166.

Nilsson S.K., Dooner M.S., Weier H.U., *et al.* (1999). Cells capable of bone production engraft from whole bone marrow transplant in non-ablated mice. *J Exp Med.,* 189:729–734.

Nobta M., Tsukasaki T., Shibata T., *et al.* (2005). Critical regulation of bone morphogenic protein-induced osteoblastic differentiation by Delta1/Jagged1-activated Notch1 signaling. *J Biol Chem.*, 280:15842-15484.

Noël D., Caton D., Roche S., *et al.* (2008). Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Exp Cell Res.*, 314:1575-1584.

Noh T., Gabet Y., Cogan J., *et al.* (2009). Lef1 haploinsufficient mice display a low turnover and low bone mass phenotype in a gender- and age-specific manner. *PLoS One.*, 4(5):e5438.

Nusse R. (1999). WNT targets. Repression and activation. *Trends Genet.*, 15(1):1-3.

Nusslein-Volhard *et al.* (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. I. Zygotic loci on the second chromosom. *Wilhelm Roux's Arch Dev Biol.*, 193:267-282.

Ogden S.K., Ascano M. Jr., Stegman M.A, *et al.* (2004). Regulation of Hedgehog signaling: A complex story. *Biochem Pharmacol.,* 67:805–814.

Ohgushi H., Okumura M. (1990). Osteogenic capacity of rat and human marrow cells in porous ceramics. Experiments in athymic (nude) mice. *Acta Orthop Scand.*, 61:431-434.

Ohno S. (1999). Gene duplication and the uniqueness of vertebrate genomes circa 1970-1999.*Semin Cell Dev Biol.*, 10(5):517-22.

Okazaki K., Li J., Yu H., *et al.* (2002). CCAAT/ enhancer-binding proteins beta and delta mediate the repression of gene transcription of cartilage-derived retinoic acid-sensitive protein induced by interleukin-1 beta. *J Biol Chem.,* 277:31526–31533.

Olesnicky Killian E.C., Birkholz D.A., Artinger K.B. (2009). A role for chemokine signaling in neural crest cell migration and craniofacial development. *Dev Biol.* 333:161-172.

Olsen B.R., Reginato A.M., Wang W. (2000). Bone development. *Ann Rev Cell Dev Biol.*, 16:191-220.

Ornitz D.M. (2005). FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev.*, 16:205-213.

Ornitz D.M., Marie P.J. (2002). FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.* 16:1446-1465.

Otsuru S., Tamai K., Yamazaki T., et al. (2008). Circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site by the CXCR4/stromal cell-derived factor-1 pathway. *Stem Cells.*, 26: 223-234.

Otto F., Thornell A.P., Crompton T., *et al.* (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell.*, 89(5):765–771.

Owen M., Friedestein A.J. (1988). Stromal cells: marrow-derived osteogenic precursors. *Ciba Found Symp.*, 136:42-60.

Panetta N.J., Gupta D.M., Quarto N., *et al.* (2009). Mesenchymal cells for skeletal tissue engineering. *Panminerva Med.*, 51:25-41.

Parekkadan B., Sethu P., van Poll D., *et al.* (2007).Osmotic selection of human mesenchymal stem/progenitor cells from umbilical cord blood. *Tissue Eng.*, 13:2465-2473.

Park S.H., Cheung L.W., Wong A.S., *et al.* (2008). Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. *Mol Endocrinol.*, 22(9):2085-2098.

Patel S., Doble B., Woodgett J.R. (2004). Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged word?. *Biochem Soc Trans.*, 32(Pt 5):803-808.

Peinado H., Quintanilla M., Cano A. (2003). Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem.*, 278(23):21113-21123.

Peng L., Jia Z., Yin X., *et al.* (2008). Comparative analysis of mesenchymal stem cells from bone marrow, cartilage and adipose tissue. *Stem Cells Dev.*, 17:761-772.

Pennati G. (2001). Biomechanical properties of the human umbilical cord. *Biorheology.*, 38(5-6):355-66.

Penolazzi L., Lambertini E., Tavanti E., *et al.* (2008). Evaluation of chemokine and cytokine profiles in osteoblast progenitors from umbilical cord blood stem cells by BIO-PLEX technology. *Cell Biol Int.*, 32(2):320-325.

Penolazzi L., Tavanti E., Vecchiatini R., *et al.* (2010). Encapsulation of mesenchymal stem cells from Wharton's jelly in alginate microbeads. *Tissue Eng Part C Methods.*, 16(1):141-155.

Penolazzi L., Zennaro M., Lambertini E., *et al.* (2007). Induction of estrogen receptor alpha expression with decoy oligonucleotide targeted to NFATc1 binding sites in osteoblasts. *Mol Pharmacol.*,71(6): 1457-1462.

Pereira R.F., Halford K.W., O'Hara M.D., *et al.* (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc Nat Acad Sci USA.*, 92:4857–4861.

Pérez-Losada J., Sánchez-Martín M., Rodríguez-García A., *et al.* (2002). Zinc-finger transcription factor Slug contributes to the function of the stem cell factor c-kit signaling pathway. *Blood.*, 100(4):1274-1286.

Perez-Moreno M.A., Locascio A., Rodrigo I., *et al.* (2001). A new role for E12/E47 in the repression of *E-cadherin* expression and epithelial-mesenchymal transitions. J Biol Chem., 276(29):27424–27431.

Perry C.R. (1999). Bone repair techniques, bone graft, and bone graft substitutes. *Clin Orthop Relat Res.*, 360:71-86.

Petersen B.E., Bowen W.C., Patrene K.D., *et al.* (1999). Bone marrow as a potential source of hepatic oval cells. *Science.,* 284:1168–1170.

Phimphilai M., Zhao Z., Boules H., *et al.* (2006). BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *Bone Miner Res.*, 21(4):637–646.

Phinney D.G. (2002). Building a consensus regarding the nature and origin of mesenchymal stem cells. *J Cell Biochem Suppl.*, 38:7-12.

Phinney D.G., Kopen W., Righter W., *et al.* (1999). Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J Cell Biochem.*, 75:424-436.

Piersanti S., Sacchetti B., Funari A., *et al.* (2006). Lentiviral transduction of human postnatal skeletal (stromal, mesenchymal) stem cells: *in vivo* transplantation and gene silencing. *Calcif Tissue Int.*, 78:372-384.

Pinson K.I., Brennan J., Monkley S., *et al.* (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature.,* 407(6803):535-538.

Piters E., Boudin E., Van Hul W. (2008). Wnt signaling: a win for bone. *Arch. Biochem. Biophys.*, 473(2):112-116.

Pittenger M.F., Mackay A.M., Beck S.C., *et al.* (1999). Multilineage potential of adult mesenchymal stem cells. *Science.,* 284:143–147.

Polak J.M., Bishop A.E. (2006). Stem cells and tissue engineering: past, present and future. *Ann N Y Acad Sci.*, 1068:352-366.

Polakis P. (2000). Wnt signaling and cancer. *Genes Dev.*, 14:1837-1851.

Ponomaryov T., Peled A., Petit I., *et al.* (2000). Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest.*, 106:1331-1339.

Porter W., Saville B., Hoivik D., *et al.* (1997). Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Mol Endocrinol.*, 11(11):1569-1580.

Poser I., Domınguez D., de Herreros A.G., *et al*. (2001). Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. *J Biol Chem*., 276(27): 24661–24666.

Prasad C.P., Rath G., Mathur S., *et al*. (2009). Expression analysis of E-cadherin, Slug and GSK3beta in invasive ductal carcinoma of breast. *BMC Cancer*., 9:325.

Pratap J., Galindo M., Zaidi S.K., *et al*. (2003). Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. *Cancer Res*., 63(17):5357–5362.

Preston S.L., Alison M.R., Forbes S.J., *et al*. (2003). The new stem cell biology: something for everyone. *Mol Pathol.,* 56:86–96.

Qiu W., Andersen T.E., Bollerslev J., *et al*. (2007). Patients with high bone mass phenotype exhibit enhanced osteoblast differentiation and inhibition of adipogenesis of human mesenchymal stem cells. *J Bone Miner Res*., 22:1720–1731.

Quarto N., Behr B., Longaker M.T. (2010). Opposite spectrum of activity of canonical Wnt signaling in the osteogenic context of undifferentiated and differentiated mesenchymal cells: implications for tissue engineering. *Tissue Eng Part A*., 16(10):3185-3197.

Quarto R., Mastrogiacomo M., Cancedda R., *et al*. (2001). Repair of large bone defects with the use of autologous bone marrow stromal cells. *N Engl J Med*., 344:385-386.

Quirici, N., Soligo D., Bossolasco P., *et al*. (2002). Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp. Hematol.,* 30:783–791.

Raio L., Ghezzi F., Di Naro E., *et al*. (1999). Sonographic measurement of the umbilical cord and fetal antrophometric parameters. *Eur J Ostet Gynecol Reprod Biol.,* 83:131-135.

Rao T.P., Kuhl M. (2010). An updated overview on Wnt signaling pathways: a prelude for more. *Circ. Res.,* 106(12):1798-1806.

Razzaque M.S., Soegiarto D.W., Chang D., *et al.* (2005). Conditional deletion of Indian hedgehog from collagen type 2alpha 1-expressing cells results in abnormal endochondral bone formation. *J Pathol*., 207:453-461.

Reubinoff B.E., Pera M.F., Fong C.Y., *et al*. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat Biotechnol.,* 18:399–404.

Reya T., Clevers H. (2005). Wnt signaling in stem cells and cancer. *Nature*., 434:843-850.
Robey P.G., Termine J.D. (1985). Human bone cells in vitro. *Calcif Tissue Int*., 37: 453-460.

Romano L.A., Runyan R.B. (1999). Slug is a mediator of epithelial-mesenchymal cell transformation in the developing chicken heart. *Dev Biol*., 212(1):243-254.

Romano L.A., Runyan R.B. (2000). Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. *Dev Biol.*, 223(1):91-102.

Roose J., Clevers H. (1999). TCF transcription factors: molecular switches in carcinogenesis. *Biochim Biophys Acta.*, 1424(2-3):M23-37.

Ros M.A., Sefton M., Nieto M.A. (1997). Slug, a zinc finger gene previously implicated in the early patterning of the mesoderm and the neural crest, is also involved in chick limb development. *Development.*, 124:1821-1829.

Rosser A.E., Dunnett S.B. (2003). Neural transplantation in patients with huntington's disease. *CNS Drugs.,* 17:853–867.

Rossi D., Zlotnik A. (2000). The biology of chemokines and their receptors. *Annu Rev Immunol.*, 18:217-242.

Roufosse C.A., Direkze N.C., Otto W.R., *et al.* (2004). Circulating mesenchymal stem cells. *Int J Biochem Cell Biol.,* 36:585–597.

Rubinfeld B., Souza B., Albert I., *et al.* (1993). Association of the APC gene product with beta-catenin. *Science.*, 262(5140): 1731-1734.

Sabbah M., Courilleau D., Mester J., *et al.* (1999). Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. Proc Natl Acad Sci USA., *Proc Natl Acad Sci USA.*, 96(20):11217-11222.

Sadler T.W. (2004). Second week of development: Bilaminar germ disc. In: *Langmann's Medical Embryology*. Philadelphia: Williams and Wilkins., 25:319-331.

Saegusa M., Hashimura M., Kuwata T., *et al.* (2009). Requirement of the Akt/β-catenin pathway for uterine carcinosarcoma genesis, modulating E-cadherin expression through the transactivation of SLUG. *Am J Pathol.* 174(6):2107-2115.

Sakai D., Suzuki T., Osumi N., *et al.* (2006). Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development. *Development.*, 133(7):1323-1333.

Sakai D., Tanaka Y., Endo Y., *et al.* (2005). Regulation of *Slug* transcription in embryonic ectoderm by β-catenin-Lef/Tcf and BMP-Smad signaling *Develop Growth Differ* 47:471–482.

Salgado A.J., Coutinho O.P., Reis R.L. (2004). Bone tissue engineering: state of the art and future trends. *Macromol Biosci.*, 4(8):743-765.

Salgado A.J., Oliveira J.T., Pedro A.J., *et al.* (2006). Adult stem cells in bone and cartilage tissue engineering. *Curr Stem Cell Res Ther.*, 1:345-364.

Salingcarnboriboon R., Yoshitake H., Tsuji K., *et al.* (2003). Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. *Exp Cell Res.*, 287:289–300.

Sánchez A., Alvarez A.M., Benito M., *et al.* Apoptosis induced by transforming growth factor-beta in fetal hepatocyte primary cultures: involvement of reactive oxygen intermediates. *J Biol Chem.*, 271(13):7416-7422.

Sánchez-Martín M., Rodríguez-García A., Pérez-Losada J., *et al.* (2002). SLUG (SNAI2) deletions in patients with Waardenburg disease. *Hum Mol Genet.*, 11(25):3231-3236.

Sandberg M.M. (1991). Matrix in cartilage and bone development: current views on the function and regulation of major organic components. *Ann Med.*, 23:207-217.

Sarnat H.B, Flores-Sarnat L. (2005). Embryology of the neural crest: its inductive role in the neurocutaneous syndromes. *J Child Neurol.*, 20(8):637-643.

Sarugaser R., Lickorish D., Baksh D., *et al.* (2005). Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells., 23:220-229.

Satija N.K., Gurudutta G.U., Sharma S., *et al.* (2007). Mesenchymal stem cells: molecular targets for tissue engineering. *Stem Cells Dev.*, 16:7-23.

Sato K., Ozaki K., Oh I., *et al.* (2007). Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood.*, 109:228-234.

Sato M., Nakashima A., Nashimoto M., *et al.* (2009). Bone morphogenic protein-2 enhances Wnt/beta-catenin signaling-induced osteoprotegerin expression. *Genes Cells.*, 14(2): 141-153.

Savagner P. (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays.*, 23(10):912-923.

Savagner P., Kusewitt D.F., Carver E.A., *et al.* (2005). Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes. *J Cell Physiol.*, 202(3):858-866.

Savagner P., Yamada K.M., Thiery J.P. (1997). The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J Cell Biol.*, 137(6):1403-1419.

Savitz S.I., Dinsmore J.H., Wechsler L.R., *et al.* (2004). Cell therapy for stroke. *Neuro Res.*, 1:406–414.

Schnabel M., Fichtel I., Gotzen I., *et al.* (2002). Differential expression of Notch genes in human osteoblastic cells. *Int J Mol Med.*, 9:229-232.

Sciaudone M., Gazzerro E., Priest L., *et al.* (2003). Notch 1 impairs osteoblastic cell differentiation. *Endocrinology.*, 144:5631–5639.

Secco M., Zucconi E., Vieira N.M., *et al.* (2008). Multipotent stem cells from umbilical cord:cord is richer than blood!. *Stem Cells.*, 26:146-150.

Seki K., Fujimori T., Savagner P., *et al.* (2003). Mouse Snail family transcription repressors regulate chondrocyte, extracellular matrix, type II collagen, and aggrecan. *J Biol Chem.*, 278(43):41862-41870.

Sekiya I., Larson B.L., Smith J.R., *et al.* (2002). Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evacuate their quality. *Stem Cells.,* 20:530–541.

Sekiya I., Tsuji K., Koopman P., *et al.* (2000). Sox9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line TC6. *J Biol Chem.*, 275(15):10738-10744.

Semenov M., Tamai K., He X. (2005). SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J. Biol. Chem.,* 280(29):26770-26775.

Sethe S., Scutt A., Stolzing A. (2006). Aging of mesenchymal stem cells. *Ageing Res Rev.,* 5:91–116.

Shahdadfar A., Fronsdal K., Haug T., *et al.* (2005). In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptosome stability. *Stem Cells.,* 23:1357–1366.

Shalitin N., Schlesinger H., Levy M.J., *et al.* (2003). Expression of procollagen C-proteinase enhancer in cultured rat heart fibroblast: evidence for coregulation with type I collagen. *J Cell Biochem.*, 90:397-407.

Shi Y, Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell.*, 113:685-700.

Shih D.T., Lee D.C., Chen S.C., *et al.* (2005). Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. *Stem Cells.,* 23:1012–1020.

Shih J.Y., Tsai M.F., Chang T.H., *et al.* (2005). Transcription repressor *Slug* promotes carcinoma invasion and predicts outcome of patients with lung adenocarcinoma. *Clin Cancer Res* 11:8070-8078.

Shimizu T., Kagawa T., Inoue T., *et al.* (2008). Stabilized beta-catenin functions through TCF/LEF proteins and the Notch/RBP-Jkappa complex to promote proliferation and suppress differentiation of neural precursor cells. *Mol Cell Biol.*, 28(24):7427-7441.

Shirozu M., Nakano T., Inazawa J., *et al.* (1995). Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1). Gene. *Genomics.*, 28:495-500.

Shook D., Keller R. (2003). Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. *Mech Dev.*, 120(11):1351-1383.

Simmons, P.J., Torok-Storb B. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood.,* 78:55–62.

Smith D.E., Franco del Amo F., Gridley T. (1992). Isolation of Sna, a mouse gene homologous to the Drosophila genes snail and escargot: its expression pattern suggests multiple roles during postimplantation development. *Development.*, 116(4):1033-1039.

Smith J.R., Pochampally R., Perry A., *et al.* (2004). Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. *Stem Cells.,* 22:823–831.

Smits P., Li P., Mandel J., *et al.* (2001). The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev Cell.*, 1(2):277-290.

Sobolewski K., Bańkowski E., Chyczewski L., *et al.* (1997). Collagen and glycosaminoglycans of Wharton's jelly. *Biol Neonate.*, 71(1):11-21.

Sock E., Pagon R.A., Keymolen K., *et al.* (2003). Loss of DNA-dependent dimerization of the transcription factor Sox9 as a cause for campomelic dysplasia. *Hum Mol Genet.*, 12:1439-1447.

Soltanoff C.S., Yang S., Chen W., *et al.* (2009). Signaling networks that control the lineage commitment and differentiation of bone cells. *Crit Rev Eukaryot Gene Expr.*, 19:1–46.

Sotiropoulou, P.A., Perez S.A., Salagianni M., *et al.* (2006). Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells.,* 24:462–471.

Spencer G.J., Utting J.C., Etheridge S.L., *et al.* (2006). Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis in vitro. *J Cell Sci.,* 119:1283–1296.

Spritz R.A., Lee S.T., Fukai K., *et al.* (1997). Novel mutations of the P gene in type II oculocutaneous albinism (OCA2). *Hum Mutat.*, 10(2):175-177.

Stegmann K., Boecker J., Kosan C., *et al.* (1999). Human transcription factor SLUG: mutation analysis in patients with neural tube defects and identification of a missense mutation (D119E) in the Slug subfamily-defining region. *Mutat Res.*, 406(2-4):63-69.

Stein G.S., Lian J.B., van Wijnen A.J., *et al.* (2004). Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. *Oncogene.*, 23(24):4315–4329.

Stewart T.L., Ralston S.H. (2000). Role of genetic factors in the pathogenesis of osteoporosis. *J Endocrinol.*, 166(2):235-245.

St-Jacques B., Hammerschmidt M., McMahon A.P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev*. 13:2072-2086.

Su L.K., Vogelstein B., Kinzler K.W. (1993). Association of the APC tumor suppressor protein with catenins. *Science*., 262(5140): 1734-1737.

Sugimachi K., Tanaka S., Kameyama T., *et al*. (2003). Transcriptional repressor snail and progression of human hepatocellular carcinoma. *Clin Cancer Res*., 9(7):2657-2664.

Tajima K., Takaishi H., Takito J., *et al*. (2010). Inhibition of STAT1 accelerates bone fracture healing. *J Orthop Res*., 28:937-941.

Takada I., Mihara M., Suzawa M., *et al*. (2007) A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat Cell Biol.,* 9:1273–1285.

Takemaru K., Yamaguchi S., Lee Y.S., *et al*. (2003). Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. *Nature.,* 422(6934):905-909.

Tanno B., Sesti F., Cesi V., *et al*. (2010). Expression of Slug is regulated by c-Myb and is required for invasion and bone marrow homing of cancer cells of different origin. *J Biol Chem*., 285(38): 29434–29445.

Tateno M., Fukunishi Y., Komatsu S., *et al*. (2001). Identification of a novel member of the *Snail/Gfi-1* repressor family, *mlt 1,* which is methylated and silenced in liver tumors of SV40 T antigen transgenic mice. *Cancer Res.,* 61:1144-1153.

ten Dijke P., Krause C., de Gorter D.J., *et al.* (2008). Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenetic protein and Wnt signaling. *J. Bone Joint Surg. Am.,* 90(Suppl. 1):31-35.

Tetsu O., McCormick F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature.,* 398(6726):422-426.

Tezuka K., Yasuda M., Watanabe N., *et al*. (2002). Stimulation of osteoblastic cell differentiation by Notch. *J Bone Miner Res*., 17:231-239.

Theoleyre S., Wittrant Y., Tat S.K., *et al*. (2004). The molecular triad OPG/RANK/RANKL: Involvement in the orchestration of pathophysiological bone remodeling. *Cytokine Growth Factor Rev*., 15:457-475.

Thiery J.P. (2003). Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol*., 15(6):740-746.

Thomson J.A., Itskovitz J., Shapiro S.S., *et al*. (1998). Embryonic stem cell lines derived from human blastocysts. *Science.,* 282:1145–1147.

Titorencu I., Jinga V.V., Constantinescu E., *et al.* (2007). Proliferation, differentiation and characterization of osteoblasts from human BM mesenchymal cells. *Cytopatology.*, 9(7):682-696.

Toh Y., Oki E., Oda S., *et al.* (1997). Overexpression of the MTA1 gene in gastrointestinal carcinomas: correlation with invasion and metastasis. *Int J Cancer.*, 74(4):459-463.

Tominaga H., Maeda S., Miyoshi H., *et al.* (2009). Expression of osterix inhibits bone morphogenetic protein-induced chondrogenic differentiation of mesenchymal progenitor cells. *J Bone Miner Metab.*, 27:36-45.

Tonnarelli B., Manferdini C., Piacentini A., *et al.* (2008) Surface-dependent modulation of proliferation, bone matrix molecules, and inflammatory factors in human osteoblasts. *J Biomed Mater Res.*, 89:687–696.

Torres M.A., Yang-Snyder J.A., Purcell S.M., *et al.* (1996). Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus Development. *J. Cell Biol.,* 133(5):1123-1137.

Tribioli C., Lufkin T., (1999). The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen. *Development.*, 126(24):5699–5711.

Tripathi M.K., Misra S., Khedkar S.V. (2005). Regulation of *BRCA2* gene expression by the SLUG repressor protein in human breast cells. *J Biol Chem.*, 280(17):17163–17171.

Troyer D.L., Weiss M. (2008). Concise review: Wharton's Jelly-derived cells are a primitive stromal cell population. *Stem Cells.*, 26:591-599.

Trubiani O., Di Primio R., Traini T., *et al.* (2005). Morphological and cytofluorimetric analysis of adult mesenchymal stemcells expanded *ex vivo* from periodontal ligament. *Int J Immunopathol Pharmacol.,* 18:213–221.

Tu X., Joeng K.S., Nakayama K.I., *et al.* (2007) Noncanonical Wnt signaling through G protein-linked PKC-delta activation promotes bone formation. *Dev Cell.,* 12:113–127.

Tuan R.S., Boland G., Tuli R. (2003). Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther.*, 5(1):32-45.

Turner F.E., Broad S., Khanim F.L., et al. (2006). Slug regulates integrin expression and cell proliferation in human epidermal keratinocytes. *J Biol Chem.*, 281(30):21321-32131.

Uccelli A., Moretta L., Pistoia V. (2008). Mesenchymal stem cells in health and disease. *Nat Rev Immunol.*, 8(9):726-36.

Vallin J., Thuret R., Giacomello E., et al. (2001). Cloning and characterization of three Xenopus slug promoters reveal direct regulation by Lef/beta-catenin signaling. *J Biol Chem.*, 276(32):30350-30358.

van Bezooijen R.L., Roelen B.A.J., Visser A., *et al.* (2004). Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J Exp Med.*, 199(6):805-14.

van Bezooijen R.L., ten Dijke P., Papapoulos S.E., *et al.* (2005). SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. *Cytokine Growth Factor Rev.,* 16(3):319-327.

Vega S., Morales A.V., Ocaña O.H., *et al.* (2004). Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev.*, 18(10):1131-1143.

Vernon A.E., LaBonne C. (2006). Slug stability is dynamically regulated during neural crest development by the F-box protein Ppa. *Development.*, 133(17):3359-3370.

Wade C., Kronenberg F., Kelly A., *et al.* (1999). Hormone-modulating herbs: implications for women's health. *J Am Med Womens Assoc.*, 54(4):181-183.

Wagner E.R., Zhu G., Zhang B.Q., *et al.* (2011). The therapeutic potential of the Wnt signaling pathway in bone disorders. *Curr Mol Pharmacol.*, 4(1):14-25.

Wakeyama H., Akiyama T., Takahashi K., *et al.* (2007). Negative feedback loop in the Bim-caspase-3 axis regulating apoptosis and activity of osteoclasts. *J Bone Miner Res.*; 22(10):1631-9.

Wan C., Shao J., Gilbert S.R., *et al.* (2010). Role of HIF-1alpha in skeletal development. *Ann N Y Acad Sci.*, 1192:322-326.

Wang H.S., Hung S.C., Peng S.T., *et al.* (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells.*, 22:1330-1337.

Wang J., Wynshaw-Boris A. (2004). The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. *Curr Opin Genet Dev.*, 14(5):533-539.

Wang Z., Ma Q., Liu Q., *et al.* (2008). Blockade of SDF-1/CXCR4 signalling inhibits pancreatic cancer progression in vitro via inactivation of canonical Wnt pathway. *Br J Cancer.*, 99:1695-1703.

Wang Z., Wade P., Mandell K.J., *et al.* (2007). Raf 1 represses expression of the tight junction protein occludin via activation of the zinc-finger transcription factor slug. *Oncogene.*, 26(8):1222-1230.

Watt F.M., Hogan B.L. (2000). Out of Eden: stem cells and their niches. *Science.*, 287(5457):1427-1430.

Webb P., Lopez G.N., Uht R.M., *et al.* (1995). Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol.*, 9(4):443-456.

Weiss M.L., Medicetty S., Bledsoe A.R. *et al.* (2006). Human umbilical cord matrix stem cells: Preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells.*, 24:781–792.

Westendorf J.J., (2006). Transcriptional co-repressors of Runx2. *J Cell Biochem.*, 98(1):54–64.

Westendorf J.J., Kahler R.A., Schroeder T.M. (2004). Wnt signaling in osteoblasts and bone diseases. *Gene.*, 341:19-39.

Whang P.G., Lieberman J.R. (2003). Clinical issues in the development of cellular systems for use as bone graft substitutes. In *Bone Graft Substitutes*. ASTM International: USA.

Williams B.O., Insogna K.L. (2009). Where Wnts went: the exploding field of Lrp5 and Lrp6 signaling in bone. *J. Bone Miner. Res.,* 24(2):171-178.

Wodarz A., Nusse R. (1998). Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol.*, 14:59-88

Woei Ng K., Speicher T., Dombrowski C., *et al.* (2007). Osteogenic differentiation of murine embryonic stem cells is mediated by fibroblast growth factor receptors. *Stem Cells Dev.*, 16:305-318.

Woodbury D., Schwarz E.J., Prockop D.J., *et al.* (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res.*, 61(4):364-370.

Wu W.S., Heinrichs S., Xu D., *et al.* (2005). Slug antagonizes p53-mediated apoptosis of hematopoietic progenitors by repressing puma. *Cell.*, 123(4):641-653.

Xiao G., Jiang D., Thomas P., *et al.* (2000). MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem.*, 275(6):4453–4459.

Xiao L., Naganawa T., Obugunde E., *et al.* (2004). Stat1 controls postnatal bone formation by regulating fibroblast growth factor signaling in osteoblasts. *J Biol Chem.*, 279:27743-27752.

Xue Y., Wong J., Moreno G.T., *et al.* (1998). NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell.*, 2(6):851-861.

Yamashita S., Andoh M., Ueno-Kudoh H., *et al.* (2009). Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Exp Cell Res.*, 315:2231-2240.

Yan Y., Tang D., Chen M., *et al.* (2009). Axin2 controls bone remodeling through the beta-catenin-BMP signaling pathway in adult mice. *J. Cell Sci.,* 122(Pt 19):3566-3578.

Yang J., Mani S.A., Donaher J.L., et al. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell.*, 117(7):927-939.

Yang X., Matsuda K., Bialek P., *et al.* (2004). ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell.*, 117:387-398.

Ye Y., Xiao Y., Wang W., *et al.* (2008). ERalpha suppresses slug expression directly by transcriptional repression. *Biochem J.*, 416(2):179-187.

Yen B.L., Huang H.I., Chien C.C., *et al.* (2005). Isolation of multipotent cells from human term placenta. *Stem Cells.*, 23:3-10.

Yook J.I., Li X.Y., Ota I., *et al.* (2005). Wnt-dependent regulation of the E-cadherin repressor snail. *J Biol Chem.*, 280(12):11740-11748.

Yook J.I., Li X.Y., Ota I., *et al.* (2006). A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. *Nat Cell Biol.*, 8(12):1398-1406.

Young R.G., Butler D.L., Weber W., *et al.* (1998). Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J Orthop Res.*, 16:406–413.

Yu C., Wang F., Jin C., *et al.* (2003). Role of fibroblast growyh factor type 1 and 2 in carbon tetrachloride-induced hepatic injury and fibrogenesis. *Am J Pathol.*, 163:1653-1662.

Yu H.M., Jerchow, B., Sheu T.J., *et al.* (2005). The role of Axin2 in calvarial morphogenesis and craniosynostosis. *Development.,* 132(8):1995-2005.

Yu L., Cecil J., Peng S.B., et al. (2006). Identification and expression of novel isoforms of human stromal cell-derived factor 1. *Gene.*, 374:174-179.

Zallone A. (2006). Direct and indirect estrogen actions on osteoblasts and osteoclasts. *Ann N.Y Acad Sci.*, 1068:173-179.

Zhang W., Ou G., Hamrick M., *et al.* (2008). Age-related changes in the osteogenic differentiation potential of mouse bone marrow stromal cells. *J Bone Miner Res.*, 23(7):1118-28.

Zhang Y.W., Yasui N., Ito K., *et al.* (2000). A RUNX2/PEBP2alpha A/CBFA1 mutation displaying impaired transactivation and Smad interaction in cleidocranial dysplasia. *Proc Natl Acad Sci USA.*, 97(19):10549–10554.

Zhang Z.Y., Teoh S.H., Chong M.S., *et al.* (2009). Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. *Stem Cells.*, 27:126-137.

Zhao C., Irie N., Takada Y., *et al.* (2006). Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab.*, 4:111-121.

Zhao L.R., Duran W.M., Reyes M., *et al*. (2002). Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischaemic brain of rats. *Exp Neurol*., 174:11-20.

Zhao M., Qiao M., Harris S.E., *et al*. (2004). Smurf1 inhibits osteoblast differentiation and bone formation in vitro and in vivo. *J Biol Chem*., 279(13):12854–12859.

Zhao Q., Eberspaecher H., Lefebvre., *et al*. (1997). Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. *Dev Dyn*., 209(4):377-386.

Zhou B.P., Deng J., Xia W., *et al*. (2004). Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol*., 6(10):931-940.

Zhou G., Zheng Q., Engin F., *et al*. (2006). Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proc Natl Acad Sci USA.,* 103(50):19004–19009.

Zhu W., Boachie-Adjei O., Rawlins B.A., et al. (2007). A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells. *J Biol Chem*., 282:18676-18685.

Zlotnik A., Yoshie O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity*., 12:121-127.

Zur Nieden N.I., Kempka G., Ahr H.J. (2003). In vitro differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation*., 71(1):18-27.

APPENDIX: ABSTRACTS AND PUBBLICATIONS

Abstracts 2008-2010

Penolazzi L, Lambertini E, Tavanti E, Torreggiani E, Franceschetti T, Pocaterra B, Gambari R, Piva R. "Human primary osteoclasts and osteoblasts for bone disease drug screening. Biotecnologie cellulari e molecolari applicate alla medicina". 20-22 February 2008: Congress *"Biotecnologie Cellulari e Molecolari applicate alla medicina"*, Santa Margherita Ligure (Ge), Italy.

Lambertini E, Torreggiani E, Tavanti E, Franceschetti T, Penolazzi L, Piva R. "Runx2 is involved in the regulation of Human Estrogen Receptor alpha gene". ORAL PRESENTATION. 18-20 June 2008: PhD School *"Silenziamento genico"* University of Ferrara, Italy.

Lambertini E, Franceschetti T, Torreggiani E, Penolazzi L, Tavanti E, Vecchiatini R, Pastore A, Pelucchi S, Gambari R, Piva R. "Slug regulation by Wnt signaling in human osteoblasts". 17-19 September 2008: 10th Biotechnology National Congress CNB10, University of Perugia, Italy.

Lambertini E, Penolazzi L, Torreggiani E, Franceschetti T, Vecchiatini R, Tavanti E, Piva R. "Slug gene expression and regulation in human osteoblasts and human mesenchymal stem cells". 26-28 November 2008: Congress AICC: "La cellula: dalla regolazione trascrizionale e post-trascrizionale alla terapia cellulare" Bologna, Italy.

Vecchiatini R, Penolazzi L, Lambertini E, Torreggiani E, Franceschetti T, Nastruzzi C, Piva R, Trombelli L, Calura G. "Caratterizzazione di cellule mesenchimali indotte al differenziamento in sistemi di coltura tridimensionali". 22-24 April 2009: Congresso Nazionale dei Docenti di Discipline Odontostomatologiche e Maxillo-Faciali, Roma, Italy.

Trombelli L, Penolazzi L, Farina R, Lambertini E, Torreggiani E, Vecchiatini R, Piva R. "In vitro Analysis of the behaviour of human osteoblasts cultured in different hydroxyapatite-based biomaterials". Europerio 6, 2-4 June 2009, Stockholm, Sweden.

Torreggiani E, Penolazzi L, Vecchiatini R, Bignardi S, Lambertini E, Franceschetti T, Vesce F, Piva R. 15-17 June 2009: EMBO Conference: "Advances in stem cell research: stem cells, systems and synthetic biology", Cambridge, UK.

Penolazzi L, Lambertini E, Vecchiatini R, Torreggiani E, Bignardi S, Vesce F, Nastruzzi C, Piva R. "Mesenchymal stem cells from Wharton's jelly: encapsulation in alginate microbeads and osteogenic differentiation". 15-17 June 2009: EMBO Conference: "Advances in stem cell research: stem cells, systems and synthetic biology", Cambridge, UK.

Torreggiani E. "Mesenchymal stem cells from Wharton's jelly: encapsulation in alginate microbeads and osteogenic differentiation". ORAL PRESENTATION. 7-10 October 2009: "Bone Stem Cells", Bertinoro (Forlì), Italy.

Torreggiani E. "New potential targets for therapeutical approach in bone tissue engineering". ORAL PRSENTATION. 30 March 2010. Journal Club, Department of Reconstructive Sciences, School of Dental Medicine, University of Connecticut Health Center, Farmington, CT, USA.

Torreggiani E. "The osteogenic potential of mesenchymal stem cells from Wharton's jelly: molecular and tissue engineering implications". ORAL PRESENTATION. 18 May 2010: "I Meeting Stem Cell Research", Certosa di Pontignano (Siena), Italy.

Bianchini C, Pastore A, Torreggiani E, Lambertini E, Piva R. "Osteogenic potential of cells derived from nasal septum". September 2010: AAO-HNSF Annual Meeting & OTO Expo, Boston, MA.

Vecchiatini R, Penolazzi L, Torreggiani E, Nastruzzi C, Calura G, Piva R. "Periodontal Ligament-Derived Stem Cells in Different Culture Systems: Achieving Regenerative Potential". SIO International Congress , Bologna, 11-12 February 2011, Bologna, Italy.

Colina M, Torreggiani E, Lambertini E, Penolazzi L, Ciancio G, Vecchaitini R, Trotta F, Govoni M, Piva R. "Wnt related proteins and osteblast differentiation genes in ankylosing spondylitis: preliminary results". EULAR Congress May 2011, London, UK.

Pubblications 2008-2010

Evaluation of chemokine and cytokine profiles in osteoblast progenitors from umbilical cord blood stem cells by BIO-PLEX technology. Penolazzi L, Lambertini E, Tavanti E, Torreggiani E, Vesce F, Gambari R, Piva R. Cell Biol Int. 2008 Feb; 32(2): 320-325.

Sex hormone receptor levels in laryngeal carcinoma: a comparison between protein and RNA evaluation. Bianchini C, Pastore A, Pelucchi S, Torreggiani E, Lambertini E, Marchesi E, Magri E, Frasson C, Querzoli P, Piva R. Eur Arch Otorhinolaryngol. 2008 Jan; 265: 1089-1094.

ERα and AP-1 interact in vivo with a specific sequence of F promoter of human ERα gene in osteoblasts. Lambertini E, Tavanti E, Torreggiani E, Penolazzi L, Gambari R, Piva R. J Cell Physiol. 2008 July; 216(1): 101-110.

Modulation of expression of specific transcription factors involved in the bone microenvironment. Lambertini E, Penolazzi L, Tavanti E, Pocaterra B, Schincaglia GP, Torreggiani E, Franceschetti T, Vecchiatini R, Gambari R, Piva R. Minerva Biotecnologica 2008; 2(20): 69-77.

Apoptosis of human primary osteoclasts treated with molecules targeting nuclear factor kappaB. Piva R, Penolazzi L, Borgatti M, Lampronti I, Lambertini E, Torreggiani E, Gambari R. Ann N Y Acad Sci. 2009 Aug; 1171: 448-456.

Slug gene expression supports human osteoblast maturation. Lambertini E, Lisignoli G, Torreggiani E, Manferdini C, Gabusi E, Franceschetti T, Penolazzi L, Gambari R, Facchini A, Piva R. Cell Mol Life Sci. 2009 Nov; 66(22): 3642-3653.

Influence of obstetric factors on osteogenic potential of umbilical cord-derived mesenchymal stem cells. Penolazzi L, Vecchiatini R, Bignardi S, Lambertini E, Torreggiani E, Canella A, Franceschetti T, Calura G, Vesce F, Piva R. Reprod Biol Endocrinol. 2009 Oct 5; 7: 106

SLUG: a new target of lymphoid enhancer factor-1 in human osteoblasts. Lambertini E, Franceschetti T, Torreggiani E, Penolazzi L, Pastore A, Pelucchi S, Gambari R, Piva R. BMC Molecular Biology. 2010 Feb 3; 11:13.

Effect of hydroxyapatite-based biomaterials on human osteoblast phenotype. Trombelli L, Penolazzi L, Torreggiani E, Farina R, Lambertini E, Vecchiatini R, Piva R. Minerva Stomatol. 2010 Mar; 59(3):103-115.

Osteogenic potential of cells derived from nasal septum. E. Torreggiani, C. Bianchini, L. Penolazzi, E. Lambertini, R. Vecchiatini, A. Cannella, R. Gambari, E. Magri, S. Pelucchi, A. Pastore, R. Piva. Rhinology, ACCEPTED.

Slug contributes to the regulation of CXCL12 expression in human osteoblasts. Piva R, Manferdini C, Lambertini E, Torreggiani E, Penolazzi L, Gambari R, Pastore A, Pelucchi A, Gabusi E, Piacentini A, Filardo G, Facchini A, Lisignoli G. Experimental Cell Research. ACCEPTED.

Transcription factor decoy against NFATc1 in human primary osteoblsts. Penolazzi L, Lisignoli G, Lambertini E, Torreggiani E, Manferdini C, Vecchiatini R, Ciardo F, Lolli A, Gabusi E, Facchini A, Gambari R, Piva R. International Journal of Molecular Medicine. ACCEPTED.

Role of Slug transcription factor in human mesenchymal stem cells. Torreggiani E, Lisignoli G, Manferdini C, Lambertini E, Penolazzi L, Vecchiatini R, Gabusi E, Chieco P, Facchini A, Gambari R, Piva R. Cell Research. SUBMITTED.

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Cell Biology International 32 (2008) 320–325

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Short communication

Evaluation of chemokine and cytokine profiles in osteoblast progenitors from umbilical cord blood stem cells by BIO-PLEX technology

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Received 10 July 2007; revised 22 August 2007; accepted 29 August 2007

Abstract

We have used cytokine protein array to analyze the secretion of cytokines from an osteoblastic clone derived from human umbilical cord blood mesenchymal stem cells (MSCs) cultured in an osteogenic differentiation medium. The analysis demonstrated the unexpected ability of osteoblast committed cells and their early progenitors to produce significant amounts of a range of soluble immune mediators without in vitro exposure to clinically relevant bacterial pathogens. The cells were expanded and their osteogenic potential analyzed over 45 days of culture was revealed by the expression of osteoblast-specific markers (alkaline phosphatase and Runx2), and by matrix mineralization. Over this culture period, the cells secreted particularly high levels of IL-8, MCP-1 and VEGF, but did not express IL-2, IL-7, IL-17, eotaxin, G-CSF and IFN-γ. These findings should encourage the use of human umbilical cord blood as a potential stem cells source for bone regeneration.

Keywords: Osteoblasts; Mesenchymal stem cells; Cytokines; Chemokines; Bio-plex analysis

1. Introduction

There is evidence that umbilical cord blood (UCB) can be used not only as an alternative source for hematopoietic cells, but also for mesenchymal stem cells (MSCs). These cells are of interest because they can differentiate into osteogenic, chondrogenic, adipogenic, myogenic and neuronal lineages under controlled in vitro conditions (Kang et al., 2006; Rogers and Casper, 2004; Erices et al., 2000). However, the frequency of non-hematopoietic precursors in UCB is low and our evidence, together with several other observations (Kang et al., 2006; Rogers and Casper, 2004; Erices et al., 2000; Chang et al., 2006; Gang et al., 2004; Rosada et al., 2003), suggests that their isolation, growth, expansion and differentiation are difficult. Concerning the development of bone-forming osteoblastic cells from MSCs, several morphologic, biochemical and molecular techniques for the identification and characterization of osteogenic commitment are now available. These include the identification of expression of bone-related markers, such as Runx2, type I collagen, alkaline phosphatase, osteopontin, osteonectin, osteocalcin (Rosada et al., 2003; Bieback et al., 2004; Hutson et al., 2005), and the sites of mineralization through von Kossa-positive bone nodules analysis (Erices et al., 2000).

Accumulating evidence indicates that both bone-forming osteoblastic cells (OBs) and osteoclasts (OCs), the unique bone resorptive cells, may release and respond to chemokines (Kim et al., 2005; Dimitriou et al., 2005; Liu and Hwang,

Abbreviations: OBs, osteoblasts; MSC, mesenchymal stem cell; UCB, umbilical cord blood; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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1065-6995/$ - see front matter © 2007 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2007.08.030

2005; Takayanagi, 2007). However, little is known regarding which chemokines or corresponding receptors play important roles in the development and function of bone cells under normal or pathological conditions. To date, the investigation of cytokines and chemokines production by cells from bone tissue has been restricted to inflammatory conditions with a dysregulated bone remodeling, including bacterial infections and osteoarthritis (Wright and Friedland, 2002; Marriott et al., 2005; Abramson and Yazici, 2006). On the contrary, we are interested in understanding whether the secretion of OBs precursors from MSC soluble factors can promote the induction of osteogenesis and OB clonal expansion. This information is of great interest from both the theoretical and practical point of view, since it may be exploited to improve osteogenic cell culture medium and facilitate isolation procedures together with expansion efficacy.

2. Materials and methods

2.1. Isolation of mononuclear umbilical cord blood cells

UCB cells were separated into a Hystopaque-1077 (Sigma) and mononuclear cells were washed, suspended in culture medium (D-MEM High-glucose containing 10% FBS) (Euroclone) at a concentration of 1×10^6 cells/cm^2. Cultures were maintained at 37 °C in a humidified atmosphere, 5% CO_2 in air, with a change of culture medium every 7 days. UCB derived mesenchymal stem cells (UCB-derived MSCs) were cultured in osteogenic medium consisting in DMEM High-glucose supplemented with 10% FBS, 10 mM β-glycerophophate, 0.1 μM dexamethasone and 50 μM ascorbate up to 45 days. In the committed cells, the osteogenic medium was changed every three days. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) staining, calcium deposition and immunocytochemistry analysis.

For alkaline phophatase staining, prefixed mono-layered cells were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (Alkaline Phosphatase Leukocyte kit, Sigma). The presence of sites of ALP activity appeared as blue cytoplasmatic staining. The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma). Briefly, cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature, washed five times with deionized water and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnification using a Leitz microscope.

Immunocytochemistry analysis was performed employing the streptavidin-biotin method using Ultraystain Polyvalent-HRP Immunostaining Kit (Ylem). Cells grown in chamber slides were fixed in cold 100% methanol, and permeabilized with 0.2% (vol/vol) Triton X-100 (Sigma) in TBS (Tris-buffered saline). Cells were incubated in 3% H_2O_2 and the endogenous peroxidase was blocked with Super Block reagent. A polyclonal antibody for Runx2 (clone M-70, rabbit anti-human, 1:1000 dilution—Santa Cruz Biotec), was applied and incubated at 4 °C overnight. Cells were then incubated at room temperature with anti-polyvalent Biotinylated Antibody. After rinsing in TBS, Streptavidin HRP was applied, and followed by the addition of Substrate-chromogen mix (AEC Cromogeno kit-Ylem). After washing, cells were mounted in glycerol/PBS 9:1 and observed using a Leitz microscope.

2.2. Bio-Plex analysis

UCB-derived MSCs secretion samples were collected at different days (Fig. 1) and were assayed in duplicate. Concentrations of IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, FGF-basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, PDGF, VEGF and TNF-α were simultaneously evaluated using commercially available multiplex bead-based sandwich immunoassay kits (Human 27-plex, Bio-Rad Laboratories). Assays were performed following the manufacturer's instructions. Briefly, 27 distinct sets of fluorescently dyed beads loaded with capture monoclonal antibodies specific for each cytokine to be tested, were used. Secretion samples (50 μl/well) or standards (50 μl/well) were incubated with 50 μl of pre-mixed bead sets into the wells of a pre-wet 96 well microtitre plate. After incubation and washing, 25 μl of fluorescent detection antibody mixture were added for 30 min and then the samples were washed and resuspended in assay buffer. High standard curves for each soluble factor were used, ranging from 2.00 to 32,000.00 pg/ml and the minimum detectable dose was <10 pg/ml. The formation of different sandwich immunocomplexes on distinct bead sets was measured and quantified using the Bio-Plex Protein Array System (Bio-Rad Laboratories). A 50 μl volume was sampled from each well and the fluorescent signal of a minimum of 100 beads per region (chemokine/cytokine) was evaluated and recorded. Values presenting a coefficient of variation beyond 10% were discarded before the final data analysis.

All values were normalized with respect to total protein amount of each secretion sample. The protein recovery was determined with Bradford method.

2.3. Data analysis and statistics

Data were analysed using the Bio-Plex Manager software version 3.0 (Bio-Rad Laboratories). Standard levels between 80 and 120% of the expected values were considered to be accurate and were used. In general, at least 5 standards were accepted and used to establish standard curves following a 5-parameter logistic regression model (5PL). Samples concentrations were immediately interpolated from the standard curves. Values were expressed as pg/ml and presented as mean ± SD.

3. Results

3.1. Isolation and differentiation of an osteogenic clone from hUCB

We are routinely generating heterogeneous adherent cells from the mononuclear fraction of human UCB under exposure to osteogenic medium. The osteogenic potential of MSCs and the progression of osteogenesis are considerably variable and, as also reported by other researchers, spindle-shaped MSCs yield varies with the age and the condition of the donor (Chang et al., 2006; Gang et al., 2004; Rosada et al., 2003).

In order to characterize OBs and their progenitor cells throughout the period of clonal expansion in vitro in respect of their chemokines secretion, the levels of 27 cytokines were simultaneously measured using the multiplex Bio-Plex technology (Vignali, 2000). The soluble factors analyzed include those belonging to the inflammatory panel (GM-CSF, IL-1β, IL-1RA, IL-6, IL-8 and TNFα), the chemokine panel (eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES), the Th1/Th2 panel (IFN-γ, IL-2, IL-4, IL-5, IL-9, IL-10), the cytokine II panel (IL-7, IL-12p70, IL-13, IL-15, IL-17) and VEGF (Vascular Endothelial Growth Factor), b-FGF (basic Fibroblast Growth Factor), G-CSF (Granulocyte Colony Stimulating Factor) and PDGF-BB growth factors.

We chose to consider a cellular clone that was expanded from mesenchymal-like cells isolated from the Ficoll layer of an UCB sample (a 28 year old donor at the 39th gestational week) and plated in a 24 multi-well plate. This clone was spontaneously selected in one well out of 24 after 14 days of osteogenic culture medium exposure. This behaviour was observed in the majority (10 out of 11) of samples subjected to

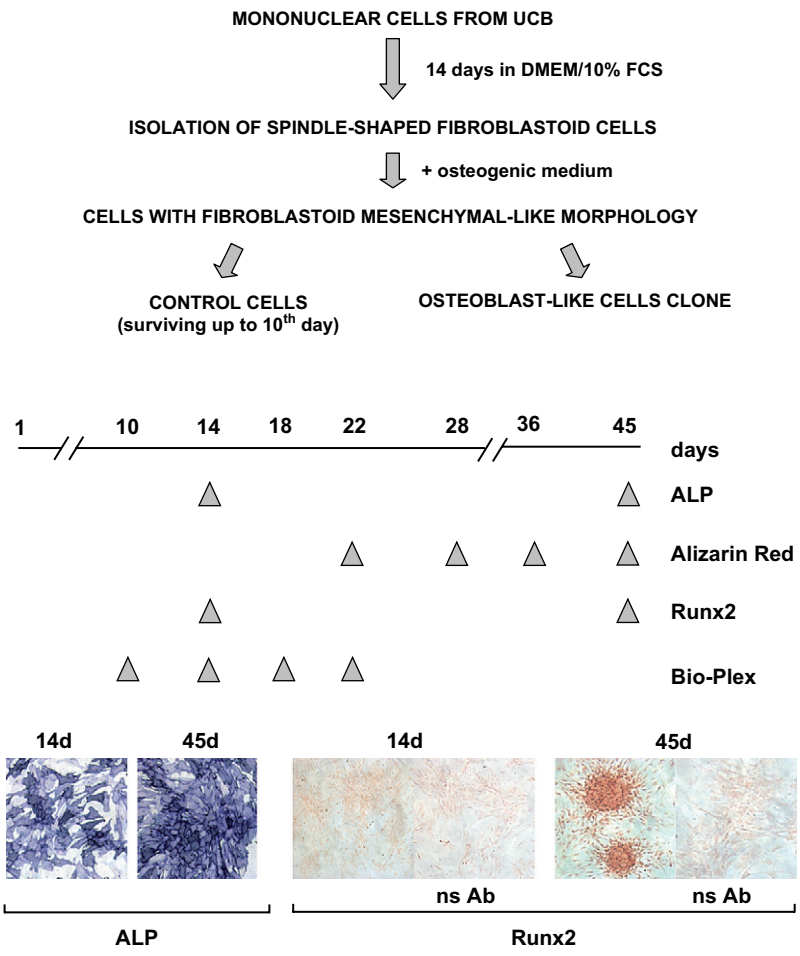

Fig. 1. Schematic representation of the experimental strategy for the analysis of osteoblastic differentiation potential of a cellular clone expanded from mesenchymal-like cells isolated from UCB. Cells were exposed to differentiation medium and subjected to the indicated analysis at the reported days. ALP staining for Alkaline Phosphatase activity and immunocytochemical analysis for determination of Runx2 expression levels with specific anti Runx2 antibody were performed. ns = staining with non-specific antibody.

the same procedure. The clone was able to proliferate actively for another 7 days up to confluence, when it was expanded and replated in a 6 multi-well plate for the biological analysis. The initial adherent fibroblastoid morphology changed to a cuboidal shape during the osteogenic medium exposure that extended to 45 days (Fig. 1).

Time-dependent differentiation of the foci from MSCs to OBs was demonstrated by immunocytochemical analysis of ALP and Runx2 osteogenic markers levels (Fig. 1) and staining the secreted calcified matrices with alizarin red (Fig. 2). Approximately 80–90% of these osteogenic-medium treated cells were differentiated into OBs and were homogeneous for the OB-like phenotype evaluation.

The osteogenic potential greatly increased during the process of expansion, suggesting that UCB-derived MSCs effectively differentiated into OBs. It should be emphasised that Runx2 is a transcription factor critical for commitment to an osteogenic lineage (Komori, 2002; Lambertini et al., 2007), and therefore its increase is a marker of OB differentiation. In contrast, the cells used as controls and derived from mesenchymal-like cells isolated from the same UCB sample were unable to differentiate in OBs and survived only up to 10th day, even when cultured in osteogenic medium. Overall the data reported in Figs. 1 and 2 suggest that we can consider these cells homogeneous for the OB like phenotype starting from day 14.

3.2. Cytokine and growth factor profile

At the various time-points corresponding to days 10, 14, 18 and 22, the adherent cells were subjected to Bio-Plex analysis. The secretion profile (Table 1) was compared with that obtained from mesenchymal-like cells immediately before the beginning of the differentiation process (C, control cells) cultured in osteogenic medium up to 10th day, without ALP expression and unable to differentiate in OBs. IL-2, IL-7, IL-17, eotaxin, G-CSF and IFN-γ were undetectable both in induced OBs and the control cells. IL-1RA and PDGF were undetectable in induced OBs but detectable at appreciable levels in control cells. Low levels of IL-1β, IL-2, IL-4, IL-5, IL-13, IL-15, RANTES and MIP-1α, were detected. Appreciable levels (from 153.32 to 1518 pg/ml) of IL-6, IL-9, IL-10, L-12(p70), FGF-basic, GM-CSF, IP-10,

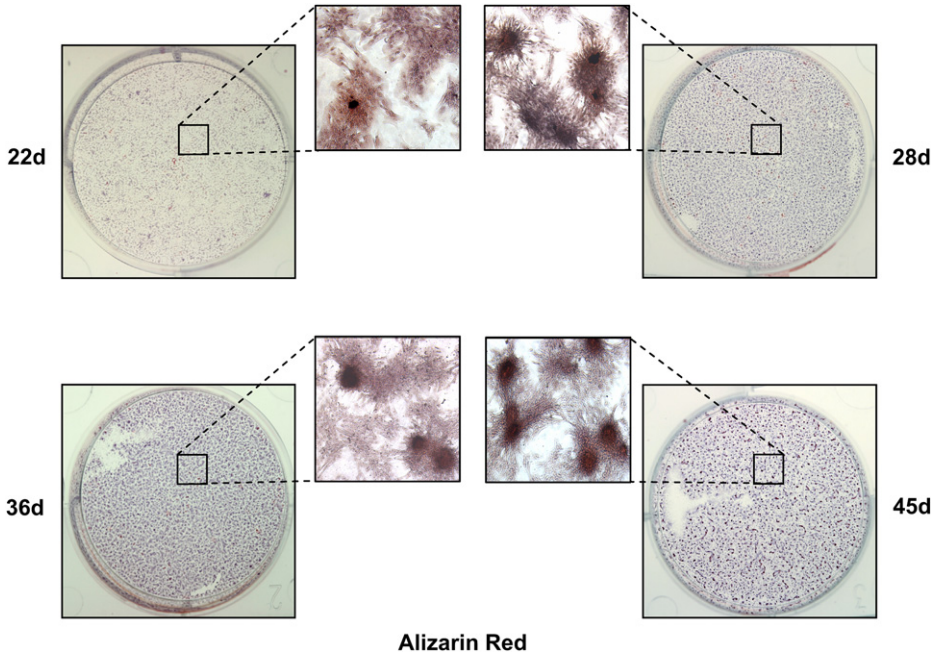

Fig. 2. Osteoblastic cell differentiation analysis. Osteogenic differentiation was also assessed by Alizarin Red staining that showed the presence of matrix mineralization.

MIP-1β and TNFα were detected in induced OBs cells. Particularly high levels (from 1160 to 7163 pg/ml) were detected for IL-8, MCP-1 and VEGF. In the control cells, the levels of IL-8 and MCP-1 were high, the levels of IL-10, IL-12(p70), FGF-basic and GM-CSF low, IL-6, and VEGF undetectable and very low, respectively. Considering that IL-1 beta is the most important factor that induces the MSCs to produce IL-6 (Le and Vilcek, 1989), the very low levels of IL-1 beta in our control cells is consistent with the lack of IL-6.

It is important to note that the control cells used in this study – even if ALP negative – cannot be considered pluripotent MSCs, since they grew for 7 days in osteogenic medium. In agreement with this observation, their cytokines profile differs from that reported by Liu and Hwang (2005) who, using pluripotent cord blood-derived mesenchymal stem cells, demonstrated that IL-6, IL-8, TIMP-1 and TIMP-2 were the most abundant interleukins expressed.

3.3. Time-dependent decrease of IL-6, MCP-1 and MIP-1β

Interestingly, IL-6, MCP-1 and MIP-1β levels decreased significatively during the OB differentiation process. IL-6 is a multifunctional cytokine that has important physiological effects on a wide range of functions, such as promoting B cell differentiation, T cell activation, and inducing acute phase proteins (Akira et al., 1993). As for bone metabolism, IL-6 has been shown to stimulate bone resorption and promote OCs formation (Le and Vilcek, 1989; Kwan Tat et al., 2004). Another important chemokine, monocyte chemoattractant protein-1 (MCP-1), is produced by OBs in initiating inflammation in progressive bone destruction (Kim et al., 2006). It is an immediate early stress responsive chemokine involved in OCs fusion and osseous inflammation. In particular, it has been reported that OBs were the principal cells expressing MCP-1 in inflamed bone (Rahimi et al., 1995). With regard to macrophage inflammatory protein 1β (MIP-1β), its role in the development of lytic bone lesions and in the suppression of bone formation, in particular in multiple myeloma has been demonstrated by Hashimoto et al. (2004).

Therefore, together these observations suggest that decreases in IL-6, MCP-1 and MIP-1β expression correlates with osteoblastic trait and may be proposed as putative osteogenic marker.

3.4. Time-dependent increase of IL-10, IL-12, FGF-basic and VEGF

Unlike IL-6, MCP-1 and MIP-1β, the levels of some soluble factors including IL-10, IL-12, FGF-basic and especially VEGF increased during differentiation to osteogenic lineage, with a comparable profile peaking at 18 day (Table 1). The levels of these factors were very low in control cells. Therefore, we assume that these molecules may play a potential osteogenic role. In fact, IL-10 and IL-12 are two cytokines that inhibit OCs formation (Xu et al., 1995; Kitaura et al., 2002). The skeleton is also an important target tissue for FGFs since these factors are involved in bone development, growth, remodeling, and repair (Hurley et al., 2001). In particular, FGF basic is produced by cultured OBs as a constituent of the bone matrix, stimulates proliferation of OBs and can increase the amount of bone formation (Ornitz and Marie, 2002; Tang et al., 2007). With regard to VEGF, it is well known that bone formation is closely related to the formation of blood vessels (Tang et al., 2007), and that OBs produce this

Table 1
Secretion profile obtained by BIOPLEX analysis

			DAYS			
		C	10	14	18	22
VERY LOW	IL-1RA	1116	0	0	0	0
	IL-7	0	0	0	0	0
	IL-17	0	0	0	0	0
	EOTAXIN	0	0	0	0	0
	G-CSF	0	0	0	0	0
	IFN-γ	0	0	0	0	0
	PDGF	216	0	0	0	0
LOW	IL-1β	20	17	21	24	21
	IL-2	0	0	0	9	9
	IL-4	59	19	44	60	15
	IL-5	48	44	46	48	48
	IL-13	14	18	29	37	30
	IL-15	134	135	136	133	129
	RANTES	588	137	134	131	125
	MIP-1α	210	189	110	109	101
MEDIUM	IL-6	0	1518	541	319	210
	IL-9	840	866	839	766	678
	IL-10	163	274	494	622	466
	IL-12(p70)	104	263	486	735	495
	β-FGF	298	423	530	568	506
	GM-CSF	169	195	230	180	153
	IP-10	271	226	253	264	245
	MIP-1β	2069	972	441	279	172
	TNFα	368	377	436	420	318
HIGH	IL-8	4925	7163	4071	4579	5744
	MCP-1	4698	4234	2759	1840	1160
	VEGF	34	1832	4310	5905	4140

The reported values of secretion levels are means of duplicate samples (expressed as pg/ml).

factor maximally at the late phase of osteogenic differentiation, when extracellular matrix is mineralized (Harada et al., 1995). Of the various angiogenic factors, VEGF is perhaps the most critical driver of vascular formation during osteogenesis (Hu et al., 2003). Several studies have also shown that VEGF stimulates the proliferation, migration and differentiation of OBs (Mayer et al., 2005; Decker et al., 2000).

4. Discussion

During "in vitro" osteogenic differentiation from UCB mesenchimal cells several cytokines, chemokines and growth factors are produced in different ways. Reduction of IL-6, MCP-1 and MIP-1β levels occurred as osteoblastic differentiation procedes. In contrast, increase of IL-10, IL-12, FGF-basic and VEGF was observed in association with high expression of OB markers.

Regarding IL-8, its levels are particularly high both in the MSC control and OB committed cells, with an oscillatory profile during all the cell culture period. IL-8 assumes a contradictory role; nevertheless it can be considered an immediate early stress-responsive chemotactic cytokine important for the activation and chemotaxis of neutrophils and macrophages. It was also shown that OBs produce IL-8, with the secretion being regulated by different hormones and cytokines (Dovio et al., 2004). Even if IL-8, together with other inflammatory mediators, seems to be correlated with chronic inflammatory rheumatic diseases, including osteoarthritis and rheumatoid arthritis (Sarkar and Fisher, 2006), our data nevertheless do not allow us to suggest a functional role for IL-8 in OB differentiation.

In conclusion, our data confirm that human MSCs from UCB are promising tools for bone regeneration. In addition, the experiments here reported demonstrated the surprising ability of osteoblasts and their early progenitors to produce significant amounts of an array of soluble immune mediators without in vitro exposure to clinically relevant bacterial pathogens. Therefore, decrease of IL-6, MCP-1 and MIP-1β levels, and increase of IL-10, IL-12, FGF-basic and VEGF should be considered markers of OB differentiation. Future studies based on the current data will look at the functional role of the specific cytokines or chemokines in different stages of the OB differentiation and in the cross-talk with osteoblasts and osteoclasts. Therefore, our results can be useful both for elucidating biochemical properties of osteoblast progenitors and facilitating the development of novel therapeutic tools for both bone-forming and bone loss diseases.

Acknowledgments

This work was supported in part by grants from MIUR FIRB-2005, MIUR COFIN-2005, the Associazione Italiana Ricerca contro il Cancro (AIRC), STAMINA, Fondazione CaRiPaRo and Fondazione Cassa di Risparmio di Ferrara. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

References

Abramson SB, Yazici Y. Biologics in development for rheumatoid arthritis: relevance to osteoarthritis. Adv Drug Deliv Rev 2006;58:212–25.

Akira S, Taga T, Kishimoto T. Interleukin-6 in biology and medicine. Adv Immunol 1993;54:1–78.

Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 2004; 22:625–34.

Chang YJ, Tseng CP, Hsu LF, Hsieh TB, Hwang SM. Characterization of two populations of mesenchymal progenitor cells in umbilical cord blood. Cell Biol Intern 2006;30:495–9.

Decker MM, Karperien M, Van der bent C, Yamashita T, Papapoulos SE, Lowik CW. Expression of vascular endothelial growth factors and their receptors during osteoblastic differentiation. Endocrinology 2000;141: 1667–74.

Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing. Injury 2005;36:1392–404.

Dovio A, Sartori ML, Masera RG, Peretti L, Perotti L, Angeli A. Effects of physiological concentrations of steroid hormones and interleukin-11 on basal and stimulated production of interleukin-8 by human osteoblast-like cells with different functional profiles. Clin Exp Rheumatol 2004; 22:79–84.

Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 2000;109:235–42.

Gang EJ, Hong SH, Jeong JA, Hwang SH, Kim SW, Yang IH, et al. In vitro mesengenic potential of human umbilical cord blood-derived mesenchymal stem cells. Biochem Biophys Res Comm 2004;321:102–8.

Harada S, Rodan SB, Rodan GA. Expression and regulation of vascular endothelial growth factor in osteoblasts. Clin Orthop Relat Res 1995;313: 76–80.

Hashimoto T, Abe M, Oshima T, Shibata H, Ozaki S, Inoue D, et al. Ability of myeloma cells to secrete macrophage inflammatory protein (MIP)- 1alpha and MIP-1beta correlates with lytic bone lesions in patients with multiple myeloma. Br J Haematol 2004;125:38–41.

Hu J, Zou S, Li J, Chen Y, Wang D, Gao Z. Temporospatial expression of vascular endothelial growth factor and basic fibroblast growth factor during mandibular distraction osteogenesis. J Craniomaxillofac Surg 2003;31: 238–43.

Hurley MM, Marie PJ, Florkiewicz RZ. Fibroblast growth factor (FGF) and FGF receptor families in bone. In: Principles of bone biology. 2nd ed., Vol. 1. USA: Academic Press; 2001. pp. 825–851.

Hutson EL, Boyer S, Genever PG. Rapid isolation, expansion, and differentiation of osteoprogenitors from full-term umbilical cord blood. Tissue Eng 2005;11:1407–20.

Kang XQ, Zang WJ, Bao LJ, Li DL, Xu XL, Yu XJ. Differentiating characterization of human umbilical cord blood-derived mesenchymal stem cells in vitro. Cell Biol Int 2006;30:569–75.

Kim DH, Yoo KH, Choi KS, Choi J, Choi SY, Yang SE, et al. Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. Cytokine 2005;31:119–26.

Kim MS, Day CJ, Selinger CI, Magno CL, Stephens SR, Morrison NA. MCP-1-induced human osteoclast-like cells are tartrate-resistant acid phosphatase, NFATc1, and calcitonin receptor-positive but require receptor activator of NFkappaB ligand for bone resorption. J Biol Chem 2006; 281:1274–85.

Kitaura H, Nagata N, Fujimura Y, Hotokezaka H, Yoshida N, Nakayama K. Effect of IL-12 on TNF-alpha-mediated osteoclast formation in bone marrow cells: apoptosis mediated by Fas/Fas ligand interaction. J Immunol 2002;169:4732–8.

Komori T. Runx2, a multifunctional transcription factor in skeletal development. J Cell Biochem 2002;87:1–8.

Kwan Tat S, Padrines M, Theoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev 2004;15:49–60.

Lambertini E, Penolazzi L, Tavanti E, Schincaglia GP, Zennaro M, Gambari R, et al. Human estrogen receptor alpha gene is a target of Runx2 transcription factor in osteoblasts. Exp Cell Res 2007;313:1548–60.

Le J, Vilcek J. Interleukin 6: a multifunctional cytokine regulating immune reactions and the acute phase protein response. Lab Invest 1989;61:588–602.

Liu CH, Hwang SM. Cytokine interactions in mesenchymal stem cells from cord blood. Cytokine 2005;32:270–9.

Marriott I, Gray DL, Rati DM, Fowler Jr VG, Stryjewski ME, Levin LS, et al. Osteoblasts produce monocyte chemoattractant protein-1 in a murine model of *Staphylococcus aureus* osteomyelitis and infected human bone tissue. Bone 2005;37:504–12.

Mayer H, Bertram H, Lindenmaier W, Korff T, Weber H, Weich H. Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. J Cell Biochem 2005;95:827–39.

Ornitz DM, Marie PJ. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev 2002; 16:1446–65.

Rahimi P, Wang CY, Stashenko P, Lee SK, Lorenzo JA, Graves DT. Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse. Endocrinology 1995;136:2752–9.

Rogers I, Casper RF. Umbilical cord blood stem cells. Best Pract Res Clin Obstet Gynaecol 2004;18:893–908.

Rosada C, Justesen J, Melsvik D, Ebbesen P, Kassem M. The human umbilical cord blood: a potential source for osteoblast progenitor cells. Calcif Tissue Int 2003;72:135–42.

Sarkar D, Fisher PB. Molecular mechanisms of aging-associated inflammation. Cancer Lett 2006;236:13–23.

Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev 2007;7:292–304.

Tang CH, Yang RS, Chen YF, Fu WM. Basic fibroblast growth factor stimulates fibronectin expression through phospholipase C gamma, protein kinase C alpha, c-Src, NF-kappaB, and p300 pathway in osteoblasts. J Cell Physiol 2007;211:45–55.

Vignali DA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000;243:243–55.

Wright KM, Friedland JS. Differential regulation of chemokine secretion in tuberculous and staphylococcal osteomyelitis. J Bone Miner Res 2002;17: 1680–90.

Xu LX, Kukita T, Kukita A, Otsuka T, Niho Y, Iijima T. Interleukin-10 selectively inhibits osteoclastogenesis by inhibiting differentiation of osteoclast progenitors into preosteoclast-like cells in rat bone marrow culture system. J Cell Physiol 1995;165:624–9.

Eur Arch Otorhinolaryngol
DOI 10.1007/s00405-008-0589-9

LARYNGOLOGY

Sex hormone receptor levels in laryngeal carcinoma: a comparison between protein and RNA evaluations

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Received: 25 June 2007 / Accepted: 16 January 2008

Abstract The larynx is a secondary sex organ, and the hormone dependence of laryngeal carcinomas is considered an interesting matter of speculation. However, while tumors of other secondary sex organs, including the prostate, breast, and endometrium, have been recognized as hormone-dependent cancers, the laryngeal carcinomas are still subject to controversy. In this study, samples from 15 laryngeal carcinomas obtained at the time of surgery were assayed for specific estrogen alpha, progesterone, and androgen receptor expression, both at mRNA and protein levels. Detectable levels of specific estrogen and progesterone receptors, 53.3 and 73.3%, respectively, were found in the tumors. This positive detection by immunohistochemical analysis was higher in tumors than in normal mucosa adjacent to the tumor areas and was correlated with the absence of metastatic lymph nodes. No androgen receptor protein was detected in any sample analyzed, even if quantitative RT-PCR revealed high mRNA levels specific for this receptor. A strict correspondence between protein and mRNA hormone receptor levels was not found. This is in agreement with the transcriptional and protein synthesis mechanisms, and it is also compatible with the complex larynx tumorigenesis.

Keywords Estrogen receptor · Progesterone receptor · Androgen receptor · Larynx carcinoma · Quantitative RT-PCR

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Introduction

Larynx cancer in Europe accounts for 2–5% of all cancers with about 45,900 new cases in 2006: 42,100 men and 3,800 women. It still represents the second most frequent neoplasm of the respiratory apparatus, secondary to pulmonary carcinoma. Its incidence has increased in the last few decades, and its peak incidence occurs among people in their sixties and seventies. Major risk factors for the development of this tumor are tobacco and alcohol consumption. Relative 5-year survival is about 63% but prognosis varies depending on the anatomical site (glottic cancer has a better prognosis) and the stage. Nearly 95% of all larynx cancers are squamous cell carcinomas.

This cancer has a male to female sex ratio of 11:1 in Europe, one of the highest among all cancer sites [1, 2]. It should be noted that the difference in susceptibility to larynx cancer based on gender has remained unchanged through the years in spite of the increasing tobacco and alcohol consumption among women [3–5].

The larynx is considered a secondary sexual organ. It is influenced by sexual hormones, not only during puberty but also during adulthood as it is subject to laryngeal epithelial layer modifications, cartilage metaplasia, and morphostructural changes [1, 5–7]. These considerations, in association with the peculiar epidemiological connotations, imply that

endocrine factors can be involved in the carcinogenesis process, in particular the receptor status for sex hormones including estrogen, androgen, and progesterone receptors [7]. Even though a number of researchers have provided evidence in the last 20 years that sex hormone receptors are expressed in laryngeal carcinomas, the presently performed overall studies about the presence and the functional role of these receptors are, nevertheless, controversial.

In order to contribute to the gathered information about this issue, we present a perspective study on 15 patients with larynx cancer. The expression of estrogen receptor alpha (ERα), progesterone receptor (PR), and androgen receptor (AR) was analyzed at both protein and mRNA levels by immunocytochemistry and quantitative RT-PCR, respectively. The data obtained from neoplastic tissues were compared with those obtained from normal mucosa adjacent to the tumor areas. In addition, a correlation between the expression levels of these receptors and lymph node status was performed.

Materials and methods

Tissue specimens

Laryngeal cancer tissues were collected from 15 patients according to protocols approved by the Committee of Ethics in Research of the University. Surgical samples were collected from laryngeal tumor tissue and control regions (normal laryngeal tissue) of the same patient. Every sample was immediately divided into two parts. One part was fixed in 4–8% buffered formaldehyde and subjected to immunohistochemical analysis. The other part was immediately frozen in liquid nitrogen for RNA analysis.

The clinical data of the patients are reported in Tables 1 and 2.

Table 1 Clinical data of SCC of the larynx patients

Number of patients	15
Age [mean ± SD, range (years)]	64.1 ± 11.8 (34–77)
Sex (M/F)	14/1
T stage	
1	0/15 (0%)
2	8/15 (53.3%)
3	7/15 (46.7%)
4	0/15 (0%)
N stage	
0	10/15 (66.7%)
+	5/15 (33.3%)
M stage	
0	15/15 (100%)
+	0/15 (0%)
Grade	
1	3/15 (20%)
2	7/15 (46.7%)
3	4/15 (26.7%)

Table 2 Tumor localization

One site	
Supraglottic	8/15 (53.3%)
Transglottic	2/15 (13.3%)
Two sites	
Cord–ventricular	2/15 (13.3%)
Glottic–hypoglottic	3/15 (20%)

Quantitative RT-PCR

Total RNA from fragments (0.5 mm) of laryngeal tumor and its normal counterpart tissue of the same patient was extracted using Total RNA Isolation System (Promega). Two micrograms of the total RNA was then used for double-stranded cDNA synthesis. It was reverse transcribed with the ImProm-II RT System (Promega). The mRNA of target genes was quantified by real-time PCR using ABI Prism 7700 system and the following TaqMan probes (Applied Biosystems):

5′ FAM-ATGATGAAAGGTGGGATACGAAAAG-TAMRA 3′ for ERα
5′ FAM-ATCATTGCCAGGTTTTCGAAACTTA-TAMRA 3′ for PR
5′ FAM-GGATGACTCTGGGAGCCCGGAAGCT-TAMRA 3′ for AR.

PCR was performed in a final volume of 25 µl. After a 10 min pre-incubation at 95°C, runs corresponded to 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/elongation). The mRNA levels of target genes were corrected for GAPDH mRNA levels (reference gene) and normalized to a calibrator sample as previously described [8].

Immunohistochemistry

Staining for ERα (clone 6F11, Ventana) and PR (clone 1A6, Ventana) was done on paraffin-embedded sections; immunohistochemical procedures were done with an automatic immunostaining device (Ventana XT Medical System, Tucson, AZ) and Ventana Kits (Strasbourg, France).

Immunohistochemistry of AR was performed as follows: sections of paraffin-tissues were deparaffinized and microwaved for antigen retrieval in Tris–EDTA–citrate pH 7.8. Subsequently, sections were incubated with mouse

monoclonal primary antibody (AR clone AR411, DIAPATH), ready to use, at room temperature. Kit EN-VISION (DAKO) was then used for the detection of primary antibody. Staining was completed by incubation with 3,3′-diaminobenzidine as chromogen. The sections were counterstained with ematossilin for 2 min and washed again in PBS 1× (5 min).

Immunostaining was quantified with a Computerized Image Analysis System (Eureka-Menarini), and only cancer cells with distinct nuclear immunostaining for ERα, PR, and AR were recorded as positive.

The specificity of immunolabeling was verified in all experiments by controls in which the specific primary antibody was omitted.

Results

Characterization of the patients

A series of 15 patients was selected (14 men and one woman, average age 64 years, range 34–77 years). Thirteen patients were affected by primary laryngeal carcinoma while two were affected by recurrent laryngeal carcinoma (first treated by radiotherapy). The patients were selected so that we had access to sufficient tumor material for histopathological and biochemical analysis. Ten patients underwent a total laryngectomy and five a partial laryngectomy. Thirteen neck dissections were also performed.

Histological diagnosis of all analyzed samples was squamous cell carcinoma (SCC), predominantly of Grade 2 (moderately differentiated tumor) as reported in Table 1. Four subgroups (Table 2) were defined on the basis of tissue localization: about half of the cases were in the supraglottic area.

Sex hormone receptor expression

All paraffin-embedded tumors and their normal counterparts were tested for the presence of ERα, PR, and AR by immunocytochemical analysis. In all tumors and all normal tissues, we did not detect any specific nuclear positive staining for AR. On the contrary, ERα and PR proteins were detected at substantial levels. Immunohistochemistry demonstrated a particular nuclear ERα and PR positivity (Fig. 1), although cytoplasmic reaction was also detected at a lower frequency. Notably, the normal counterparts of SCC, obtained during surgery at the periphery of the resections, showed less sex hormone receptor reactivity by immunocytochemical analysis than the tumorous tissue. In fact, as reported in Table 3, the frequency of ERα and PR expression were higher in tumors compared to normal mucosa adjacent to the tumor areas (53.3 vs. 26.7%, and 73.3 vs. 60%, respectively).

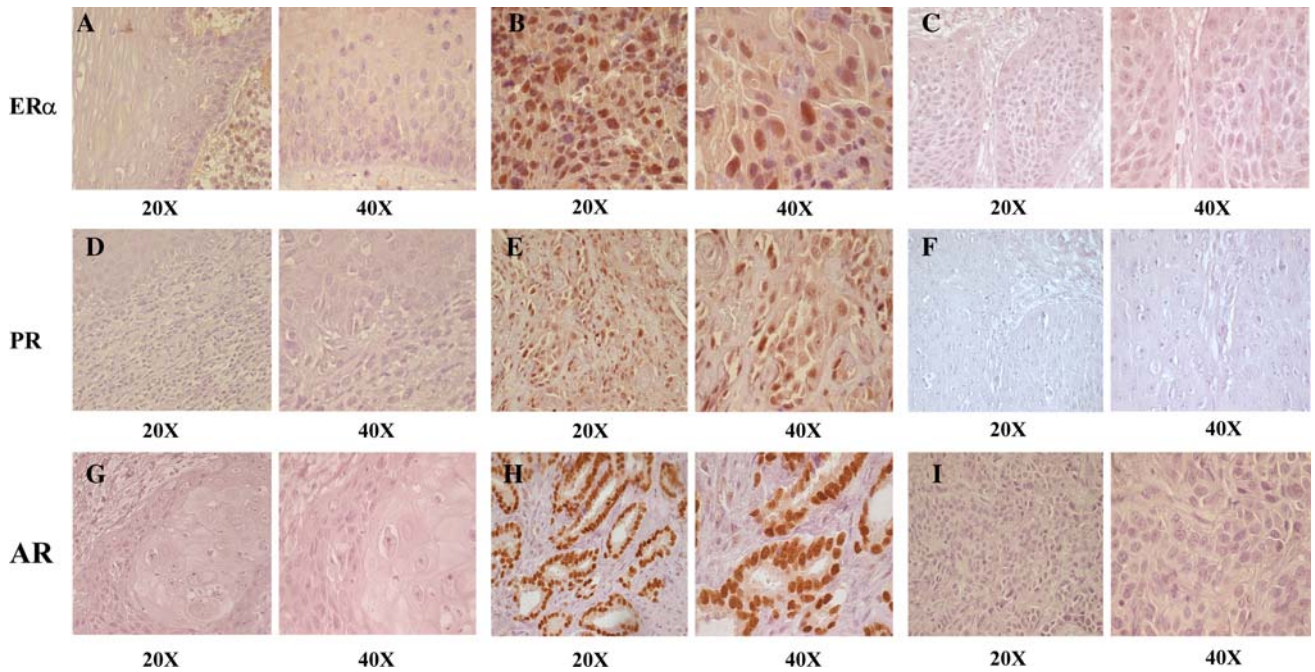


Fig. 1 Immunohistochemistry analysis of ERα, PR (**D**, **E**, **F**), and AR (**G**, **H**, **I**) steroid hormone receptors. A sample with predominant expression of ERα and PR in the tumor cells is shown. **A**, **D** Normal areas; **B**, **E** tumorous areas. No expression of AR is seen in any analyzed samples. In **G** a tumorous area is reported. The positive control tissue (prostate carcinoma) shows extensive immunostaining for AR (**H**). Original magnifications: ×20 and ×40. Control experiments carried out with omission of the primary antibody are also reported (**C**, **F**, **I** negative staining controls)

Table 3 Sex hormone receptor status of SCC of the larynx (% protein)

	ERα	PR	AR
N	4/15 (26.7%)	9/15 (60%)	0/15 (0%)
T	8/15 (53.3%)	11/15 (73.3%)	0/15 (0%)

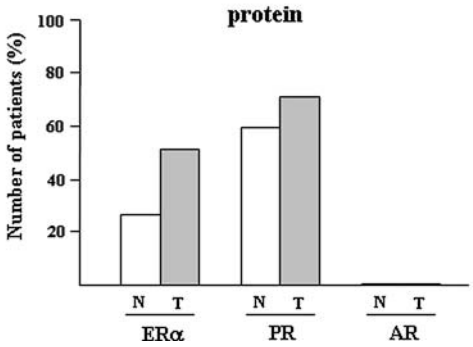
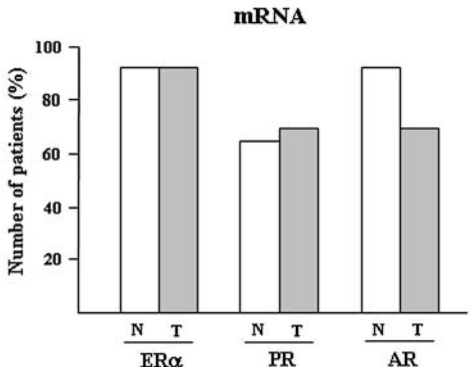


Table 4 Sex hormone receptor status of SCC of the larynx (% mRNA)

	ERα	PR	AR
N	14/15 (93.3%)	10/15 (66.7%)	14/15 (93.3%)
T	12/13 (93.3%)	9/13 (69.2%)	9/13 (69.2%)



The results of protein expression were then correlated with the levels of mRNA for ERα, PR, and AR. Quantitative real time RT-PCR analysis was performed on the mRNA extracted from samples of fresh frozen normal and neoplastic tissues by using the TaqMan probes specific for ERα, PR, and AR transcripts as reported in the "Materials and methods" section. The sensitivity of the method was such that a very high percentage of transcription was detected in all cases, particularly for ERα and AR (Table 4). As expected, this indicates that a portion of mRNA is not translated into the corresponding protein. Concerning AR, the presence of such elevated mRNA levels without corresponding protein detection is very surprising. The same results were obtained in all five of the repeated experiments, and this is probably due to the altered characteristics of the tumor microenvironment that escape the normal control mechanisms of many molecular events.

In addition, as reported in Table 4, it is of note that in all three cases there were no significant differences at the transcription levels between tumors and their normal counterparts (see a representative plot in Fig. 2), suggesting that in the normal cells surrounding the tumor area, a tumorous behavior may be recognized at molecular levels.

When hormone receptor positivity was correlated with lymph node status, a significant correlation was detected between pN0 status and the expression of ERα and PR proteins (Fig. 3). In fact, in the ERα-positive or PR-positive cancer groups, the pN0 status was overrepresented.

Discussion

Currently, prognostic evaluation regarding head and neck squamous cell carcinomas is based mainly on tumor site, clinical stage (including lymph node involvement and the presence of distant metastasis), and histopathologic grade. Considering that laryngeal cancer has different epidemio-

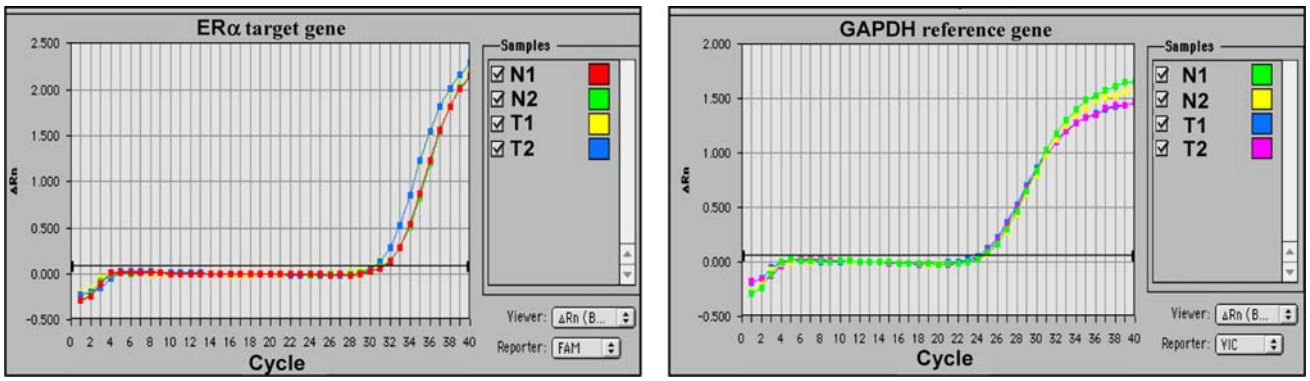


Fig. 2 Example of RT-PCR amplification plot monitored in real time. The threshold cycle (Ct) is the first cycle with detectable fluorescence in relation to an internal standard

N Status	ERα protein in tumoral cases (%)			PR protein in tumoral cases (%)		
		ERα +	ERα -		PR +	PR -
	pN0	7/14 (50%)	3/14 (21.4%)	pN0	9/14 (64.3%)	1/14 (7.14%)
	pN+	2/14 (14.3%)	2/14 (14.3%)	pN+	2/14 (14.3%)	2/14 (14.3%)
	total cases	9/14 (64.3%)	5/14 (35.7%)	total cases	11/14 (78.6%)	3/14 (21.4%)

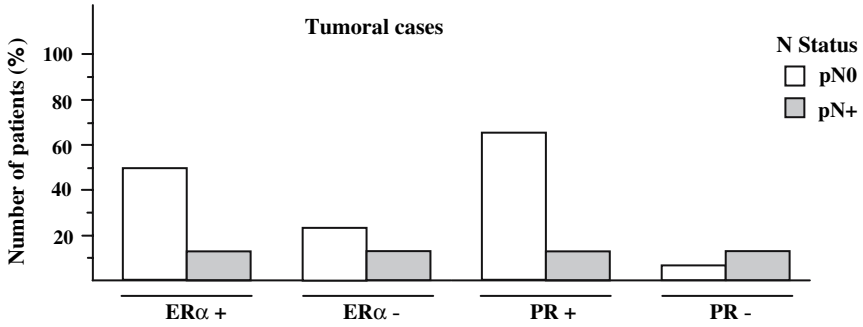


Fig. 3 Correlation between ERα and PR protein expression and lymph node status

logical connotations between men and women (like incidence and clinical presentation and features), several studies since 1980 have analyzed the role of sex hormone receptors in the complex process of larynx tumorogenesis. Nevertheless, as previously reported, there is still no consensus about the presence and the functional role of these receptors in larynx carcinomas.

Schuller et al. [9] reported the evidence of low concentration levels of androgen and estro-progesterone receptors in laryngeal neoplasms, concluding that larynx cancer may not be considered a hormone-dependent neoplasm. In contrast with this study, Mattox et al. [10] have found positivity for androgens in 91% of the laryngeal neoplastic specimens examined from 23 patients, also recovering high hormonal levels in the lymph node metastases. Moreover, ten patients with advanced disease have been treated with flutamide, a powerful anti-androgenic, without satisfactory results [10]. Virolainen et al. [11] demonstrated the presence of androgen receptors in 31% of the evaluated laryngeal tumoral samples, estrogen receptors in 69%, and progesterone receptors in 53% of the cases without substantial differences between the sexes. Inconclusive results were obtained by Ferguson et al. [12] in 1987 using immunohistochemical analysis and by Vecerina-Volic et al. [13] in the same year, who described high androgen receptor levels represented in women correlated to male receptor levels in laryngeal cancer. Also, Toral et al. [14] considered larynx as an androgen target organ and correlated the larynx carcinomas to the loss of androgen receptors. In 1989, Berg et al. [15] supported the hypothesis of a possible hormonal therapy in the ENT district, describing the presence of progesterone receptors in laryngeal cancers as having similar characteristics to those identified in breast cancer. Other evidence supporting the presence and functionality of steroid hormone receptors, and in agreement with our results, comes from the inhibitory effect of antiestrogens on the growth of squamous-cell carcinoma cell lines from head and neck cancers [16, 17]. This suggests that antihormones such as tamoxifen may have a therapeutic role in laryngeal cancer expressing ER and PR [16, 17]. On the contrary, other research groups obtained the opposite results. Ogretmenoglu and Ayas [18] in 1998 were unable to recognize the clinical importance of estrogen expression in larynx carcinoma. In a more recent paper, Hagedorn and Nerlich [19] have associated the immunohistochemical technique to immunoenzymatic (the EIA) analysis evaluating ER, PR, and AR expression in normal and neoplastic specimens coming from patients affected by larynx carcinoma, demonstrating a complete lack of sex hormone receptors in their analysis. All of these controversial results indicate that growth and differentiation of head and neck squamous cell carcinomas are regulated by several factors and receptors and that a useful unique predictor of tumor aggressiveness has not yet been demonstrated. Nevertheless, the presence of estrogen receptors in a high proportion of laryngeal carcinoma cells suggests that hormonal therapy may be a useful adjunctive therapy in selected patients with cancer of the larynx. Therefore, it becomes imperative for the diagnostic histopathologist to test routinely for the presence of steroid hormone receptors on tumor tissues received in the laboratory. In addition, the employment of quantitative methods, such as those described here, may contribute to the understanding of the molecular mechanisms that sustain a specific larynx cancer phenotype.

The data obtained from our experiments show specific staining for ER alpha and PR but not for AR, even if high levels of mRNA for AR were detected. This ERα and PR positivity was significantly associated with the absence of lymph node metastasis. Taken together, the experiments suggest that ERα and PR could mediate estrogen- and progesterone-dependent changes during tumor progression in the larynx, and that their effects may anticipate or replace the action of AR, probably acting in combination with other factors. It is important to emphasize that several investigators employing high-throughput technologies have suggested that specific factors including E-cadherin, focal adhesion kinase (FAK), p53 protein, Ki-67 antigen, stomatin-like protein 2, VEGF, PTTG, stratifin, S100 calcium-binding protein A9, p21-ARC, stathmin, and enolase may play a critical role in the biology of head and neck squamous cell carcinomas [20–25] and may improve our ability to predict the clinical course of these tumors. These new findings will certainly help to increase our understanding of the role of sex hormones in the beginning and in the progression of the larynx tumor by analyzing possible unexplored crosstalk between different signaling pathways.

Acknowledgments This research was supported by grants from Fondazione Cassa di Risparmio di Ferrara. English revision of the article was carried out by Rebecca K. Swartz.

References

1. Licitra L, Bernier J, Grandi C, Locati L, Merlano M, Gatta G, Lefebvre JL (2003) Cancer of the larynx. Crit Rev Oncol Hematol 47:65–80
2. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P (2007) Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 18:581–592
3. Andre K, Schraub S, Mercier M, Bontemps P (1995) Role of alcohol and tobacco in the aetiology of head and neck cancer: a case–control study in the Doubs region of France. Eur J Cancer B Oral Oncol 31:301–309
4. Jaber MA, Porter SR, Gilthorpe MS, Bedi R, Scully C (1999) Risk factors for oral epithelial dysplasia—the role of smoking and alcohol. Oral Oncol 35:151–156
5. Farshadpour F, Hordijk GJ, Koole R, Slootweg PJ (2007) Non-smoking and non-drinking patients with head and neck squamous cell carcinoma: a distinct population. Oral Dis 13(2):239–243
6. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK (1993) Head and neck cancer. N Engl J Med 328:184–194
7. Hagedorn HG, Nerlich AG (2002) Analysis of sex-hormone-receptor expression in laryngeal carcinoma. Eur Arch Otorhinolaryngol 259:205–210
8. Lambertini E, Penolazzi L, Magaldi S, Giordano S, del Senno L, Piva R (2005) Transcription factor decoy against promoter C of estrogen receptor alpha gene induces a functional ER alpha protein in breast cancer cells. Breast Cancer Res Treat 92(2):125–132
9. Schuller DE, Abou-Issa H, Parrish R (1984) Estrogen and progesterone receptors in head and neck cancer. Arch Otolaryngol 110:725–727
10. Mattox DE, Von Hoff DD, McGuire WL (1984) Androgen receptors and antiandrogen therapy for laryngeal carcinoma. Arch Otolaryngol 110:721–724
11. Virolainen E, Tuohimaa P, Aitasalo K, Kytta J, Vanharanta-Hiltunen R (1986) Steroid hormone receptors in laryngeal carcinoma. Otolaryngol Head Neck Surg 94:512–517
12. Ferguson BJ, Hudson WR, McCarty KS (1987) Sex steroid receptor distribution in the human larynx and laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 113:1311–1315
13. Vecerina-Volic S, Romic-Stojkovic R, Krajina Z, Gamulin S (1987) Androgen receptors in normal and neoplastic laryngeal tissue. Arch Otolaryngol Head Neck Surg 113:411–413
14. Toral I, Ciliv G, Gursel B, Ozdem C (1990) Androgen receptors in laryngeal carcinoma. Eur Arch Otorhinolaryngol 247:244–246
15. Berg NJ, Colvard DS, Neel HB 3rd, Weiland LH, Spelsberg TC (1989) Progesterone receptors in carcinomas of the upper aerodigestive tract. Otolaryngol Head Neck Surg 101:527–536
16. Shapira A, Virolainen E, Jameson JJ (1986) Growth inhibition of laryngeal UM-SCC cell lines by tamoxifen. Arch Otolaryngol Head Neck Surg 112:1151–1158
17. Grenman R, Virolainen E, Shapira A, Carey T (1987) In vitro effects of tamoxifen on UM-SCC head and neck cancer cell lines: correlation with the estrogen and progesterone receptor content. Int J Cancer 39:77–81
18. Ogretmenoglu O, Ayas K (1998) Laryngeal carcinoma and estrogen receptor analysis in patients after long-term follow-up. Eur Arch Otorhinolaryngol 255:457–461
19. Hagedorn HG, Nerlich AG (2002) Analysis of sex-hormone-receptor expression in laryngeal carcinoma. Eur Arch Otorhinolaryngol 259:205–210
20. Pastuszewski W, Dziegiel P, Krecicki T, Podhorska-Okolow M, Ciesielska U, Gorzynska E, Zabel M (2007) Prognostic significance of metallothionein, p53 protein and Ki-67 antigen expression in laryngeal cancer. Anticancer Res 27:335–342
21. Rodrigo JP, Dominguez F, Suarez V, Canel M, Secades P, Chiara MD (2007) Focal adhesion kinase and E-cadherin as markers for nodal metastasis in laryngeal cancer. Arch Otolaryngol Head Neck Surg 133(2):145–150
22. Cao WF, Zhang LY, Liu MB, Tang PZ, Liu ZH, Sun BC (2007) Prognostic significance of stomatin-like protein 2 overexpression in laryngeal squamous cell carcinoma: clinical, histologic, and immunohistochemistry analyses with tissue microarray. Hum Pathol 38(5):747–752
23. Onesto C, Hannoun-Levi JM, Chamorey E, Formento JL, Ramaioli A, Pages G (2006) Vascular endothelial growth factor-A and Poly(A) binding protein-interacting protein 2 expression in human head and neck carcinomas: correlation and prognostic significance. Br J Cancer 94(10):1516–1523
24. Solbach C, Roller M, Eckerdt F, Peters S, Knecht R (2006) Pituitary tumor-transforming gene expression is a prognostic marker for tumor recurrence in squamous cell carcinoma of the head and neck. BMC Cancer 6:242
25. Sewell DA, Yuan CX, Robertson E (2007) Proteomic signatures in laryngeal squamous cell carcinoma. ORL J Otorhinolaryngol Relat Spec 69(2):77–84

Journal of Cellular Physiology

ERα and AP-1 Interact In Vivo With a Specific Sequence of the F Promoter of the Human ERα Gene in Osteoblasts

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Estrogen-responsive genes often have an estrogen response element (ERE) positioned next to activator protein-1 (AP-1) binding sites. Considering that the interaction between ERE and AP-1 elements has been described for the modulation of bone-specific genes, we investigated the 17-β-estradiol responsiveness and the role of these cis-elements present in the F promoter of the human estrogen receptor alpha (ERα) gene. The F promoter, containing the sequence analyzed here, is one of the multiple promoters of the human ERα gene and is the only active promoter in bone tissue. Through electrophoretic mobility shift (EMSA), chromatin immunoprecipitation (ChIP), and re-ChIP assays, we investigated the binding of ERα and four members of the AP-1 family (c-Jun, c-fos, Fra-2, and ATF2) to a region located approximately 800 bp upstream of the transcriptional start site of exon F of the human ERα gene in SaOS-2 osteoblast-like cells. Reporter gene assay experiments in combination with DNA binding assays demonstrated that F promoter activity is under the control of upstream *cis*-acting elements which are recognized by specific combinations of ERα, c-Jun, c-fos, and ATF2 homo- and heterodimers. Moreover, ChIP and re-ChIP experiments showed that these nuclear factors bind the F promoter in vivo with a simultaneous occupancy stimulated by 17-β-estradiol. Taken together, our findings support a model in which ERα/AP-1 complexes modulate F promoter activity under conditions of 17-β-estradiol stimulation.

J. Cell. Physiol. 216: 101–110, 2008.

In bone metabolism, estrogen hormone is essential for sustaining bone mineral density in both males and females (Compston, 2001); accordingly, several 17-β-estradiol-induced effects on gene expression in bone cells have been described (Syed and Khosla, 2005). The 17-β-estradiol-mediated effects are achieved through the two isoforms of estrogen receptors, alpha and beta (ERα and ERβ) (Nilsson et al., 2001), whose expression levels are important in determining cell responses. In particular, a clear clinical role has been established for the ERα isoform, which is specifically involved in the regulation of factors associated with bone formation (Bodine et al., 1998). Therefore, studies on the regulation of the expression of the ERα gene are of great interest from the theoretical as well as the practical points of view. The transcription of the ERα gene has been reported as very complex. In fact, seven distinct promoter regions are dispersed within a more than 150 Kb sequence upstream of the +1 transcription initiation site (Kos et al., 2002). With regard to bone, we and others recently demonstrated that ERα transcription is only directed by the distal F promoter, located at −117140 (Denger et al., 2001a; Lambertini et al., 2003). The activity of the F promoter seems to be autoregulated by ERα (Denger et al., 2001b), even though specific evidence for a direct role of 17-β-estradiol in the regulation of its receptor is not fully provided.

It is well known that ERα can directly bind estrogen response elements (EREs) in the promoters of estrogen-regulated genes through a well described genomic mechanism involving specific molecular interactions (Klinge, 2001; Nilsson et al., 2001; Gruber et al., 2004). The consensus ERE is an inverted repeat of the sequence AGGTCA, separated by three base pairs. Its natural occurrence is rare, and many endogenous estrogen responsive genes contain non-palindromic EREs, imperfect EREs, or half-sites, in which unpaired AGGTCA sites are present (Hall et al., 2002; Tang et al., 2004). In addition to direct binding to specific EREs, ERα may transactivate target genes by interacting with other transcriptional factors (TFs) such as Sp1 (Safe, 2001), AP-1 (Petz and Nardulli, 2000; Cheung et al., 2005; Shur et al., 2007) and NF-kappa B (Kalaitzidis and Gilmore, 2005). Both processes result in the recruitment of coactivators and components of the RNA polymerase II transcription initiation complex that modulates target gene transcription in a cell type- and DNA context-dependent manner (Petz and Nardulli, 2000; Hall et al., 2002; McKenna and O'Malley, 2002). The AP-1 complex is one of the best characterized targets of non-classically acting ERα and regulates multiple genes in different tissues, including ovalbumin, collagenase, IGF-1, and MMP-13 (Kushner et al., 2000; Lu et al., 2006). In particular, a large number of "in vitro" and "in vivo" studies have

This article includes Supplementary Material available from the authors upon request or via the Internet at http://www.interscience.wiley.com/jpages/0021-9541/suppmat.

Abbreviations: AP1, activator protein 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen receptor element; Luc, luciferase; α-MEM, α-minimal essential medium.

Elisabetta Lambertini and Elisa Tavanti contributed equally to this work.

Contract grant sponsor: MIUR COFIN-2005;
Contract grant number: 2005038704005.

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Received 8 November 2007; Accepted 30 November 2007

DOI: 10.1002/jcp.21379

demonstrated that AP-1 proteins have important roles as key regulators of bone development (Karsenty and Wagner, 2002).

The F promoter sequence analyzed here extends from −117,884 to −117,140 bp and contains an ERE half-site (5′-GGTCA-3′) and potential binding sites for a number of TFs including several AP-1 motifs. It has been reported that this ERE half-site binds ERα and confers transcriptional activation to a heterologous promoter (Denger et al., 2001b), but a direct interaction of ERα with this regulatory element has not been described. Considering that the interaction between ERE and AP-1 elements has also been described for the modulation of bone-specific genes (McCabe et al., 1995; Schultz et al., 2005; Wagner and Eferl, 2005; Lu et al., 2006), we investigated the possibility that 17-β-estradiol responsiveness of the F promoter may also be mediated by indirect action of ERα through AP-1.

Here we used electrophoretic mobility-shift assays (EMSA) with nuclear extracts and recombinant proteins to test in particular the binding ability of an AP-1 site located near the ERE half site, and the ERE half site overlapping with an AP-1 half site. The "in vivo" dynamic status of these factors binding the F promoter was also examined using a chromatin immunoprecipitation (ChIP) assay and re-ChIP assays. Our results suggest that the functional cooperation between ERα and AP-1 proteins, including c-jun, c-fos and ATF-2, is estrogen-dependent and may contribute to the modulation of F promoter activity and, consequently, the expression of the ERα gene in different cells in vivo. Therefore, this study extends our knowledge of the transcriptional regulation of the human ERα gene in osteoblasts in addition to implicating for the first time CREB-AP1 protein complexes in F promoter activity.

Materials and Methods

Plasmid constructions

All promoter fragments were cloned in the promoterless pGL3-Basic vector (Promega, Madison, WI) upstream of the firefly luciferase (LUC) reporter gene. First, an 862 bp sequence (−117,884/−117,014) was cloned as a *BglII/MluI* fragment obtained by PCR on human genomic DNA using the following 5′ and 3′ primers, respectively (the restriction endonuclease recognition sequences are indicated in lower case): 5′-cgacgcgtACCTACTGCATAAACCACACC-3′ (forward) and 5′-gaagatctTTGAAGAGAAGATTATCACTC-3′ (reverse). The resulting PCR product was digested with *MluI* and *BglII* and inserted into a pGL3-Basic LUC reporter vector, digested at the same sites. This initial fragment was used to generate the 411 (−117,426/−117,014) truncation construct using an internal *HindIII* site. The 272 deletion fragment was generated by PCR using the oligonucleotide cgacgcgtAAGCAAGGCCAC as the 5′ primer and the same 3′ primer that was used for the 862 construct production. The 180 deletion fragment was generated by PCR using the oligonucleotide cgacgcgtTTCCTAATTTCATGGTCA as the 5′ primer and the same 3′ primer that was used for the 862 construct production.

The E/779 fragment was generated by PCR using the oligonucleotide cgacgcgtTAATATTCATGCATAAGAA as the 5′ primer and the oligonucleotide gaagatctGCTTGGAACCTTAGAA as the 3′ primer.

Cell cultures and transient transfections

SaOS-2 and MCF-7 cells were maintained in α-minimal essential medium (α-MEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 10% FBS (CELBIO EuroClone, Milan, Italy) in a humidified incubator at 37°C with 5% CO_2. For transfection experiments, the cells were plated in 24-well plates, were maintained in phenol-red free α-MEM + 10% charcoal stripped FBS, and were transiently transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) with 1 μg of promoter constructs in and out of the presence of 10 nM 17-β-estradiol. Following overnight incubation, the medium was removed and fresh medium was added. After another 24 h, cells were harvested, lysed, and assayed for luciferase activity. The LUC activity was normalized by total protein content and by β-galactosidase values resulting from cotransfection of 0.25 μg of pCMV-Sport-βgal (Invitrogen). β-galactosidase activity was measured by using Beta-Glo Assay System (Promega).

RT-PCR analysis

For mRNA analysis, all cellular RNA was extracted using Total RNA Isolation System (Promega). Two micrograms of the total RNA were reverse transcribed with the ImProm-II RT System (Promega). mRNA for ERα was quantified by Real-time PCR using TaqMan probe: 5′ FAM-ATGATGAAA-GGTGGGATACGAAAAG-TAMRA 3′ and the ABI Prism 7700 system (Applied Biosystems). After a 10′ pre-incubation at 95°C, runs corresponded to 40 cycles of 15″ at 95°C (denaturation) and 1′ at 60°C (annealing/elongation). The mRNA levels of target genes were corrected for GAPDH mRNA levels.

Electrophoretic mobility shift assays (EMSA) and supershift assays

Nuclear extracts were prepared as previously described (Dignam et al., 1983). Double-stranded oligonucleotides were end-labeled with (γ ^{32}P) ATP and T4 polynucleotide kinase. For gel shift assay 10 μg of nuclear proteins were preincubated for 5′ at room temperature with 1.2 μg of non-specific competitor DNA poly (dI-dC)-(dI-dC) (Pharmacia Corporation, Bridgewater, NJ) and then 10,000 cpm of labeled oligonucleotides were added and incubated for another 30′ at room temperature. For the competition experiments, a 25, 50, 100, and 250-fold excess of unlabeled oligonucleotides was incubated with the nuclear extracts for 15′ at room temperature and then incubated with the appropriate ^{32}P-labeled DNA probe. For supershift experiments, nuclear extracts were preincubated for 15′ at RT with 4 μg of antibodies against ERα, c-jun, c-fos or ATF-2 (Santa Cruz Biotechnology, Santa Cruz, CA) prior to their 30′ incubation with the labeled oligonucleotides at room temperature. In the experiments with purified proteins, 300 ng of ERα c-jun, c-fos, or ATF-2 were added to the reaction mixture for 15′ at 25°C. Reactions were run on 6% polyacrylamide gels and electrophoresed at 150 V for 2 h in 0.5 × TBE buffer. Gels were then dried and exposed to film at −80°C.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out using the ChIP kit from Upstate Biotechnology, Inc. (Lake Placid, NY) as previously described (Penolazzi et al., in press). The cells were cross-linked with 1% formaldehyde for 10 min at 37°C, washed in ice-cold PBS and resuspended in SDS lysis buffer for 10′ on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors, and precleared with 80 μl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 5 μg of anti-ERα, c-jun, c-fos, ATF-2 or Fra-2 (Santa Cruz Biotechnology) overnight at 4°C. Immunocomplexes were mixed with 80 μl of DNA-coated protein A-agarose followed by incubation for 1 h at 4°C. Beads were collected and sequentially washed five times with 1 ml each of the following buffers: low salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-pH 8.1) and TE buffer. The immunocomplexes were eluted two times by adding a 250 μl aliquot of a freshly prepared 1% SDS, 0.1 M

$NaHCO_3$ solution, and the cross-linking reactions were reversed by incubation at 65°C for 4 h. Further, the samples were digested with proteinase K (10 mg/ml) at 42°C for 1 h, DNA was purified by Qiaquick Spin Columns (Qiagen, Inc., Germantown, MD). For PCR analysis, aliquots of chromatin prior to immunoprecipitation were saved (input). Nested PCR was performed to analyze the presence of DNA precipitated by specific antibodies. Two primers were used to detect the DNA segment located at −117,254/−117,040 that encompasses the AP-1(1) and the ERE half site within the F promoter (Fw (5′-TGAGATTTTCCAATCCTAGT-3′) and Rev (5′-ACTGTCTTCTTATGCTATAGAA-3′)).

Each PCR reaction was performed with 2 μl of the bound DNA fraction or 1 μl of the input. The PCR was performed as follows: preincubation at 95°C for 10 min, 30 cycles of 1′ denaturation at 95°C, 1′ at the optimal annealing temperature (50°C), and 1 min at 72°C, with one final incubation at 72°C for 5′. For the re-ChIP assay the complexes were eluted from the primary immunoprecipitation by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:40 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1), followed by re-immunoprecipitation with a different relevant antibody. Subsequent steps of ChIP reimmunoprecipitations were the same as for the initial immunoprecipitations. No-antibody control was included in any experiment.

Statistical analysis

Data are presented as the mean ± SEM from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

The F promoter of the human ERα gene contains potential binding sites for ERα-mediated autoregulation

It was previously shown that a region located about 1 Kb upstream of the human ERα exon F retains putative regulatory elements involved in the control of ERα gene transcription in osteoblasts (Denger et al., 2001b). In order to better define the transcriptional activity of this promoter sequence in terms of 17-β-estradiol responsiveness, we initially performed a computational dissection of the region spanning from −117,884 to −117,140, based on the search for estrogen responsive elements (EREs) and for binding sites for transcription factors including AP-1 (Petz et al., 2002; Garcia-Arencibia et al., 2005) and Sp1 (Safe, 2001), that are potentially able to interact with ERα and influence ERα-mediated gene expression. Sequence analysis, schematically shown in Figure 1A, revealed the presence of several AP-1 like sites with 6, 5, or 4 of the 7 bases of the original 5′-TGAC/GTCA-3′ (Lee et al., 1987). In addition, the presence of only one Estrogen Responsive Element in the form of one-half ERE (5′-GGTCA-3′, at −117,181) and one-half AP-1 site (5′-GTCA-3′), (Bergman et al., 2003) was confirmed. Putative binding sites for Sp1 were not found.

Cell transfection experiments were initially employed to evaluate 17-β-estradiol responsiveness of the F promoter context in human osteoblast-like SaOS-2 cells. These cells display a well-differentiated phenotype with high ALP activity, low proliferation rate, and substantial ability to form mineralized nodules (Lin et al., 2004). To this end, we used four promoter-LUC constructs, including progressive 5'-deletions of the F promoter sequence (Fig. 1B), previously characterized in part by our group (Lambertini et al., 2007). As shown in the graph of Figure 1B, the entire F promoter sequence under investigation, corresponding to the 862 bp construct, is used by SaOS-2 but not by ERα-positive MCF-7 breast cancer cells. Deletion of the region from −117,884 to −117,426 generates the 411 bp construct (−117,426/−117,014), designed as the minimal promoter sequence containing the TATA-like motif localized upstream of the half ERE under investigation. This construct exhibited a higher level of Luc activity than the full-length 862 bp construct, whereas further deletions resulting in TATA less constructs (the 272 bp and the 180 constructs) completely abolished Luc activity. These results assigned maximal transcriptional activity to the region between nucleotides −117,426 and −117,014, indicating that (a) the −117,426/−117,286 region is required to sustain transcription, (b) the TATA-like sequence positively mediates the activity of this promoter, and (c) the deletion of the sequence from −117,884 to −117,426 removes a negative control region (Lambertini et al., 2007). Interestingly, all promoter-Luc constructs except the 180 bp construct significantly increased their activity after transfection in the presence of 10 nM 17-β-estradiol for 24 h. This time point corresponds to the major 17-β-estradiol-mediated induction of ERα mRNA, as measured by quantitative RT-PCR (see the insert of Fig. 1B). In particular, although luciferase activity was lower for the 272 bp construct, fold-inducibility by E2 was >5.6, suggesting that the ERE half-site and AP-1 sites present in the sequence of this construct may be critical cis-elements. The same E2 treatment had no effects on the 180 bp construct in which the AP-1 sites upstream of the ERE half site were deleted and the ERE half site was maintained. This suggests that the ERE half site alone is not sufficient to confer 17-β-estradiol responsiveness to the F promoter. As expected, the same constructs transfected into non osseous cell lines including MCF-7 (see Fig. 1B), HeLa, and MDA-MB-231 cells (data not shown) were 17-β-estradiol unresponsive. The empty pGL3 basic vector showed no induction of reporter transcription after 17-β-estradiol treatment.

Therefore, these results are compatible with the presence of functional elements in the F promoter that mediate the responsiveness to 17-β-estradiol in osteoblasts through an autoregulatory mechanism that may potentially be exerted by a combined action of ERE and AP-1 regulatory sequences.

In order to provide further evidence for the importance of the sequence −117,884/−117,140 of the F promoter in osteoblasts, its hormone responsiveness with respect to another regulatory region was analyzed. A promoter-LUC construct containing a sequence of 779 bp upstream of the E promoter (−151,786/−151,007) (Kos et al., 2002) and with an ERE half site was transfected into SaOS-2 and MCF7 cells, and, as reported in the insert of Figure 1B, it did not show any LUC activity or any response to 17-β-estradiol.

ERα and AP-1 are recruited to the F promoter "in vivo"

The role of these putative cis elements was then investigated analyzing the "in vivo" assembly of ERα and AP-1 family members on the F promoter by chromatin immunoprecipitation (ChIP) assay following 17-β-estradiol treatment of SaOS-2 cells (Fig. 2).

The experiments were assessed every 15′ for 90′ to determine the order and timing of complex recruitment and to check for their presence after 24 h of 17-β-estradiol treatment. After cross-linking with formaldehyde, nuclear extracts were obtained from the cells and sonicated to fragment the chromatin. Equal amounts of the same chromatin preparation were incubated with specific antibodies against ERα, c-Jun, c-fos, Fos-related antigen 2 (Fra-2), and activating transcription factor-2 (ATF-2) factors. These components of the AP-1 family were chosen because it is well documented that they are expressed in differentiated osteoblasts and have a significant effect on the developing or remodeling skeleton (McCabe et al., 1995; Wagner and Eferl, 2005). After immunoprecipitation, DNA eluted from the beads was used as a template to generate a 214 bp-F promoter specific PCR product. As shown in Figure 2A, in the absence of 17-β-estradiol (time 0), the

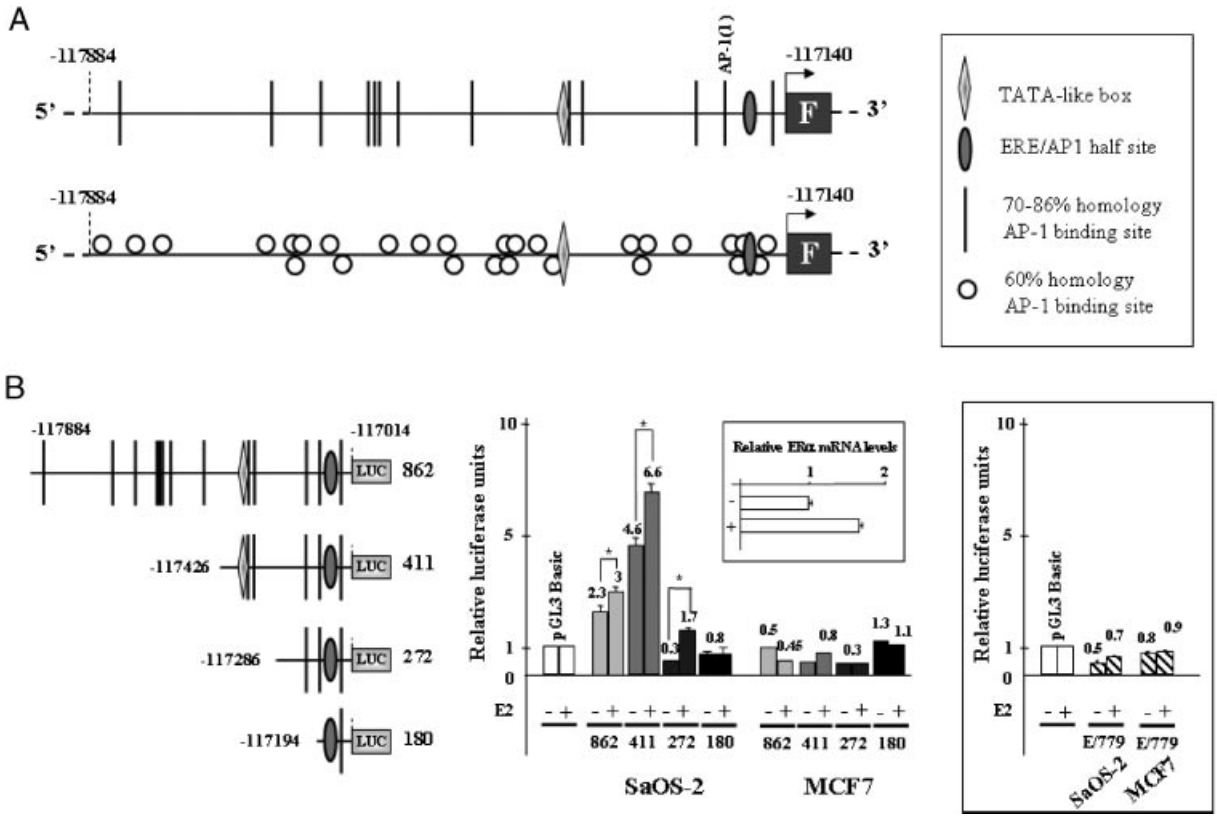

Fig. 1. A,B: Functionality of the F promoter of the human ERα gene. A: Schematic representation of the F promoter region between nucleotides −117,884 and −117,140. The nucleotides are numbered relative to the main transcription start site (+1). By using the Transfac database, several AP-1 like sites with 6, 5 (70–86% homology), or 4 (60% homology) of 7 bases of the original 5′-TGAC/GTCA-3′ were identified. In addition, one estrogen responsive element in the form of half ERE (5′-GGTCA-3′), one half AP-1 site (5′-GTCA-3′) and a TATA-like element were identified. Capital letter means exon F. B: Promoter activity of deletion constructs of the F promoter of the ERα gene. SaOS-2 and MCF7 cells were transiently transfected with the F promoter-luciferase (LUC) constructs (862, 411, 272, and 180 respectively) reported on the left, in the presence (+) and not (−) of 10 nM 17-β-estradiol (E2) for 24 h. The positions of potential transcription factor binding sites are reported. The position of AP-1 (1) is pointed out. Reporter luciferase activity was assayed 48 h after transfection. The results were normalized with protein concentration and β-gal activity for transfection efficiency and represented as the average of five independent experiments performed in duplicate. The data are expressed as the mean ± standard errors and indicated as fold inductions over the promoterless pGL3 Basic vector. Asterisk (*) indicates statistically significant differences between the transfectants indicated in the figure ($P < 0.05$). The level of ERα gene transcription in SaOS-2 cells is also reported. The cells were cultured in the presence of 10 nM E2 for 24 h (+) or remained untreated (−). The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for ERα transcript analysis. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate; ΔΔCt method was used to compare gene expression data; standard error of the mean (SEM) was calculated. In the insert to the right, the promoter activity of the E/779 construct of the E promoter (Kos et al., 2002) of the ERα gene is reported. SaOS-2 and MCF7 cells were transiently transfected with the E promoter-luciferase (LUC) construct (−151,786/−151,007), in the presence (+) or not (−) of 10 nM 17-β-estradiol (E2) for 24 h.

recruitment of transcription factors appeared low. After 17-β-estradiol treatment, ERα displayed an oscillating promoter occupancy, peaking at 30 and 60 min and then showing a decreasing level for the remainder of the time course. Within 15′ following the addition of 17-β-estradiol, promoter occupancy peaked when ATF-2 recruitment was analyzed. Together with ERα, ATF-2 represents the factor most engaged on the sequence under investigation, whereas Fra-2 does not appear to be associated with the F promoter at any time of the treatment. The recruitment of c-Jun and c-fos was present at lower levels, peaking at 24 h and 15 min, respectively.

As a control, genomic DNA not subjected to immunoprecipitation was amplified (input). As expected, no amplification was obtained in the samples collected with beads alone (NoAb).

These results support the conclusion that ERα and members of the AP-1 family, particularly ATF-2, interact "in vivo" with the F promoter region; this might involve the sequence containing the overlapping half ERE and the AP-1 half site and the AP-1 sites in osteoblastic SaOS-2 cells.

An additional test for the functionality of the F promoter in the chromatin context of SaOS-2 cells was performed by re-ChIP experiments in response to 0, 15, 30, 45, 60, 90 min, and 24 h treatment with 17-β-estradiol. Initial rounds of immunoprecipitation were performed with the ERα antibody, and then the precipitated material was challenged further with antibodies against c-jun, c-fos, ATF-2, and Fra-2. This approach was undertaken to enrich for chromatin templates that were associated with both ERα and AP-1 factor partner proteins at the same time. As shown in Figure 2B, all signals appeared stronger, and there was rather constant and rapid association of ERα with c-jun, c-fos, and ATF-2, even if the DNA protein interactions presented individual profiles for the different transcription factors.

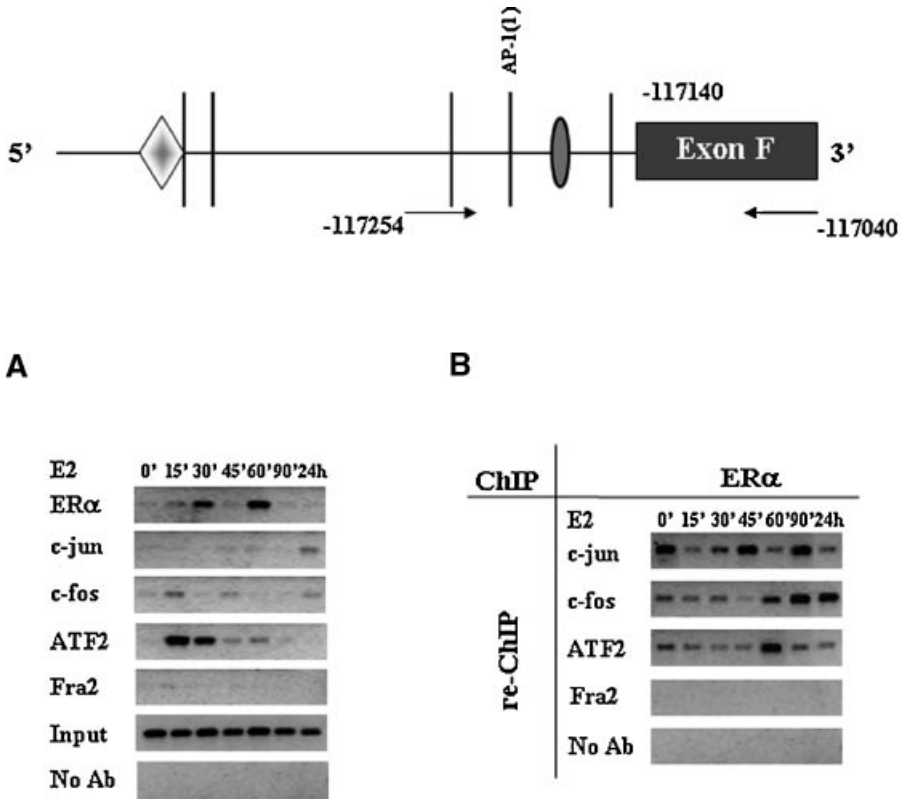

Fig. 2. A,B: "In vivo" recruitment of 17-β-estradiol-modulated ERα and AP-1 factors to the F promoter. Chromatin was extracted from SaOS-2 cells plated at a density of 1×10^6 and treated for indicated time periods with 10 nM 17-β-estradiol. A: ChIP assays using antibodies against ERα, c-jun, c-fos, ATF-2 and Fra-2 were performed. The association of the F promoter was monitored for the seven treatment times. Representative agarose gels of at least three independent cell treatments are shown. The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the F promoter from −117,254 to −117,040 bp. Aliquots of chromatin taken prior to immunoprecipitation were used as "Input" controls whereas chromatin eluted from immunoprecipitations lacking antibody were used as "no antibody" controls. B: Re-ChIPs were performed first with an immuno-precipitation with anti-ERα and second with a precipitation with c-jun, c-fos, ATF-2, and Fra-2 antibodies, as indicated. Representative agarose gels are shown.

Analysis by EMSA of DNA-protein interactions at AP-1 and ERE half sites as a whole

To delineate the promoter elements involved in the binding "in vivo" with ERα and AP-1 factors and the mediating of the regulatory effect of 17-β-estradiol, electrophoretic mobility-shift assays (EMSA) were carried out with various ^{32}P-labeled oligonucleotides (see Table 1). The labeled oligonucleotides had been incubated with nuclear extracts from 17-β-estradiol-treated SaOS-2 cells or ERα, c-jun, c-fos, and ATF-2 purified recombinant proteins and the corresponding specific antibodies. The data obtained from promoter activity analysis and experiments performed with oligonucleotides covering other AP-1 like sites present in the F promoter (see Fig. 1A of Supplemental Data), led us to focus our analysis on the sequence around the ERE half site and on the AP-1(1) site strictly associated to it (see panel A of Fig. 3 for oligonucleotide localization).

TABLE 1. Oligonucleotide sequences used in EMSA experiments

Oligonucleotide	Sequence
AP-1 consensus	5′-ttccggc**tgactca**tcaagcg-3′
CRE consensus	5′-agctcctagcctgacgtcagagagagagct-3′
ERE consensus	5′-aattcgtccaaagtcaggt*ca*cagt*ga*cctgatcaaagtt-3′
AP-1/ERE half site	5′-tttatttt**tgagtta**ctgcatttcctaatttcatggtcataacagcct-3′
ERE half site	5′-tgcatttcctaatttcatggtcataacagcct-3′
AP-1(1)	5′-ttatttt**tgagtta**ctgcat-3′
Oligo 2	5′-**ac**tgcatttcctaatttcat-3′
Oligo 3	5′-atttcatggtcataacagcc-3′
Oligo 3m	5′-atttcat*AA*tcataacagcc-3′
Oligo 4	5′-ggtcataacagcctcctgtc-3′

Initially, we used the AP-1/ERE half site oligonucleotide as a probe, which consisted of the sequence between nucleotides −117,215 and −117,159. As shown in Figure 3B, specific band shifts were obtained, generating five major DNA/protein complexes. Interestingly, the band c2 strongly decreased when antibodies against c-Fos and ATF-2 were added to the binding reactions, suggesting that these transcription factors were present in complex c2. On the contrary, in these experimental conditions, bands were not depleted by the addition of ERα and c-Jun antibodies. This lack of activity of MoAbs might be ascribed to the presence of highly structured heterogeneous complexes masking MoAb-binding sites.

In order to further characterize the sequence containing the ERE half site, we performed EMSA competition experiments on a labeled AP-1/ERE half site oligonucleotide using as a competitor the AP-1(1) and ERE half site shorter oligonucleotides encompassing the AP-1/ERE half site sequence. As shown in the insert of Figure 3B, the presence of 25-, 50-, 100-, or 250-fold molar excess of cold AP-1(1) oligonucleotide did not affect binding ability. On the contrary, cold ERE half site competitor significantly reduced formation of

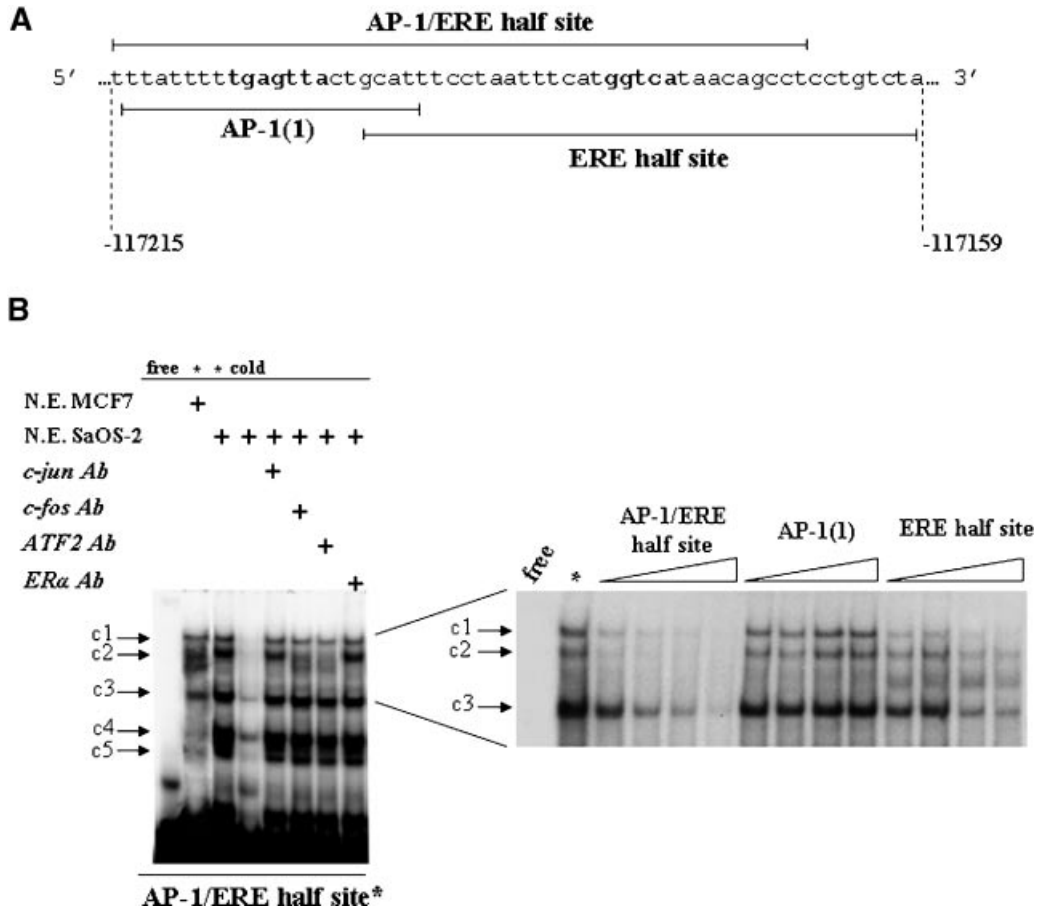

Fig. 3. A,B: In vitro analysis of DNA-protein interactions at the AP-1/ERE half site sequence in presence of nuclear extracts. A: Schematic representation of oligonucleotides used in this EMSA analysis. B: Gel shift experiments were performed with nuclear extracts from SaOS-2 cells in the presence of ^{32}P labeled AP-1/ERE half site oligonucleotide. Protein–DNA complexes were resolved from free probe through non-denaturing 6% polyacrylamide gels. A representative gel is shown. The ^{32}P labeled oligonucleotide was incubated alone (free), in combination with 10 μg of nuclear extracts (NE) (*), and in the presence of a 100-fold molar excess of specific unlabeled oligonucleotide (cold), respectively. Where indicated, antibodies against c-jun, c-fos, ATF-2, and ERα (Santa Cruz Biotechnology) were incorporated into the binding reactions. The five major complexes formed are marked as c_1–c_5. To the right, cross competitions with 25-, 50-, 100-, or 250-fold molar excess of AP-1/ERE half site, AP-1(1), and ERE half site cold oligonucleotides are reported.

the c1, c2, and c3 complexes, suggesting a prevalent role for the ERE half site with respect to the AP-1(1) sequence in the binding of proteins to the AP-1/ERE half site.

In addition, to determine whether any of the complexes binding to the AP-1/ERE half site were osteoblastic-specific, we compared the EMSA pattern obtained using nuclear extracts from MCF7 breast cancer cells. As shown in Figure 3B, all binding activities, in particular c3, c4 and c5, were stronger in SaOS-2 than in MCF7 cells. In addition, a complex with an intermediate mobility between c2 and c3 was present in MCF7, but not in SaOS-2.

To unequivocally characterize the binding abilities of transcription factors to this AP-1/ERE half site sequence, we then performed the EMSA assay with recombinant proteins, alone or in combination. First, purified c-Jun, c-fos, ATF-2 and ERα available recombinant proteins were confirmed for their specific efficacy with the oligonucleotides containing high-affinity AP-1, CRE and ERα binding sites (AP-1, CRE and ERα consensus, respectively) (Fig. 4A). In addition, a Western Blot analysis was performed on both nuclear extracts and total lysates of E2-treated SaOS-2 cells to quantify the presence of the transcription factors under investigation. With the exception of Fra2 (which is found mainly in the total extracts), substantial levels of ERα, c-Jun, c-fos, and ATF-2 were present in both fractions (insert of Fig. 4A).

Next, the experiments reported in Figure 4B were performed. The results showed supershifts with c-Jun and ATF-2 antibodies implying that c-Jun/c-Jun, c-Jun/ATF-2, and ATF-2/ATF-2 combinations occur. A supershift with c-fos antibody is present only in the c-jun/c-fos combination. Antibody specific for ERα interfered only partially with ERα recombinant protein binding but completely abolished the binding when ATF-2 was also added, providing convincing evidence that ERα/ATF-2 heterodimers form. On the contrary, the formation of the ERα/c-jun complex seems not to be involved. It is noteworthy that the failure to demonstrate a strong direct binding of ERα to DNA is in agreement with the observations of several other research groups. In fact, because of the formation of fragile protein–DNA complexes (Porter et al., 1996; Sun et al., 1998; Weinmann et al., 2001; Petz et al., 2002), the interaction between ERα and an identified ERE appears too weak in many cases for detection in EMSA when using either nuclear extracts, purified ERα, or in vitro translated ERα as the ERα protein source. The ability of the AP-1/ERE half

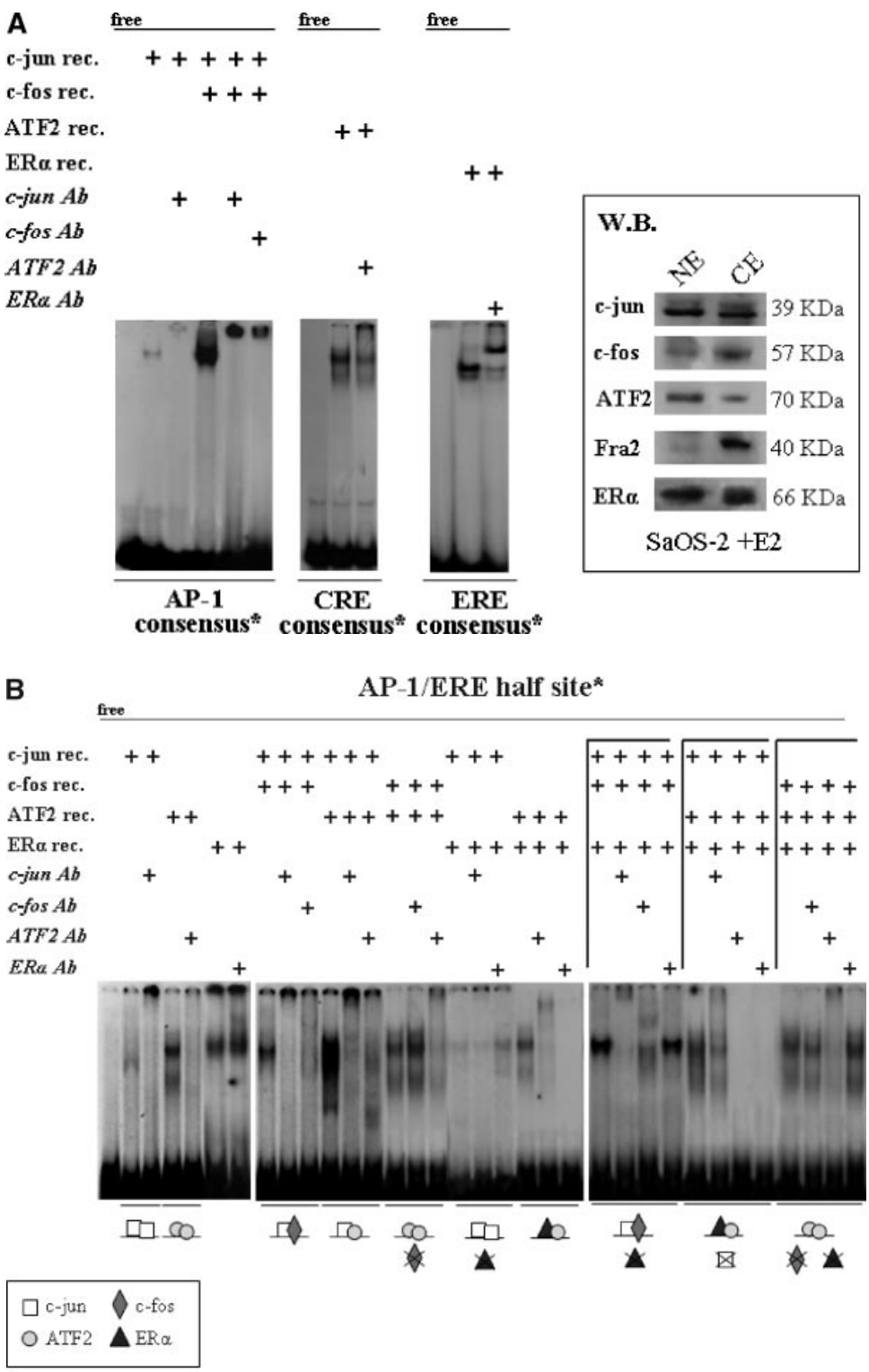

Fig. 4. A,B: In vitro analysis of DNA-protein interactions at the AP-1/ERE half site sequence in the presence of recombinant proteins. A: The efficiency of recombinant proteins and antibodies was tested on AP-1, CRE, and ERE consensus oligonucleotides. Western blots (WB) were also performed to detect the expression of c-jun, c-fos, ATF-2, Fra-2, and ERα in nuclear (NE) and total cellular (CE) extracts from SaOS-2 cells. B: Gel shift experiments were performed with recombinant c-jun, ATF-2, c-fos, and ERα proteins in the presence of ^{32}P labeled AP-1/ERE half site oligonucleotide. Protein–DNA complexes were resolved from free probe through non-denaturing 6% polyacrylamide gels. Representative gels are shown. Where indicated, antibodies against c-jun, c-fos, ATF-2, and ERα (Santa Cruz Biotechnology) were incorporated into the binding reactions. A simplified model of the favored protein complexes following each EMSA analysis are reported below.

site sequence to bind different heterodimers was then tested employing three different recombinant proteins at the same time in the EMSA reaction. This assay (Fig. 4B right side) confirmed that the protein complexes bound to the sequence may be composed of a mixture of dimers and revealed that c-Jun/c-fos, ATF-2/ATF-2, and ERα/ATF-2 are the preferred protein combinations. It is noteworthy that when ERα is present, ATF-2 preferentially forms homodimers or heterodimers with ERα over c-Jun or c-fos.

Analysis by EMSA of DNA-protein interactions at individual AP-1 and ERE half sites

When the above experiments with nuclear extracts and recombinant proteins were performed using the ERE half site oligonucleotide (see Fig. 5A), fainter DNA protein interactions were observed, suggesting that the larger sequence (AP-1/ERE half site) containing multiple cis elements has a greater binding ability than the shorter sequence. In particular, the shorter sequence was not able to bind recombinant ATF-2 protein as a homodimer. Nevertheless, comparable results were obtained concerning the c-Jun/c-Jun, c-Jun/c-fos, and c-Jun/ATF-2 complexes.

In order to understand the role of single putative cis elements, additional EMSA were carried out and the four short oligonucleotides reported in Figure 5B, covering the entire AP-1/ERE half site, were individually used. As shown in Figure 5B, we were only able to detect a faint interaction between the oligonucleotide 3 containing the ERE half site and the ERα recombinant protein. When mutant oligonucleotide 3 with 2-bp mutations in the ERE half site (3m) was used, no gel shifted bands were observed, indicating the potential ability of the 5′-GGTCA-3′ sequence in the specific recruitment of the ERα protein. Contrarily, we were unable to detect any protein-DNA complexes with the other oligonucleotides, suggesting that the direct recruitment of AP-1 members and the AP-1-mediated recruitment of ERα require the AP-1/ERE half site sequence as well as combined AP-1 sites and the ERE half site.

In addition, it should be noted that the molar ratios of recombinant proteins were held constant in all EMSA experiments and that the binding reactions were processed in parallel. Thus, the differences observed in the binding of recombinant proteins to the oligoprobes reflected differences in protein-DNA interactions and could not be attributed to differences in experimental conditions. Therefore, the overall data and, in particular, the major intensity of the gel shifted band detected with the AP-1/ERE half site suggest that while c-jun, c-fos, ATF2, and ERα may be individually important, it is only through their combination that they show maximum DNA affinity and regulatory effects on promoter activity. These conclusions are also supported by the analysis of the abilities of c-jun, c-fos, ATF2, and ERα to bind mutant AP-1(1) and ERE half sites. In fact, as reported in the Figure 1B of Supplemental Data, when the AP-1(1) site was mutated from TGAGTTA to CAAGTAC, ATF2 binding ability drastically decreased, whereas enhanced ERα binding was observed. On the contrary, when the ERE half site was mutated from GGTCA to AATCA, ERα binding was abrogated, but ATF2 binding strongly increased. This confirms a substantial flexibility of the analyzed sequence and a compensation of protein/protein and protein/DNA interactions.

Discussion

The primary conclusion of the results reported here is that ERα and c-jun, c-fos and ATF-2, but not Fra-2 AP-1 factors interact "in vivo" with specific estrogen-responsive regulatory sequences and AP-1 cis-elements within the F promoter of the human ERα gene in osteoblast-like SaOS-2 cells. Western blotting analysis (insert of Fig. 4A) indicates that the lack of Fra2 recruitment to the F promoter is probably due to a cytoplasmic compartimentalization of this protein in SaOS-2 cells, even when they are treated with 17-β-estradiol.

The recruitment of ERα and c-jun, c-fos and ATF-2 factors is oscillatory and is modulated by 17-β-estradiol. These transcription factors are co-associated with the F promoter region −117,884/−117,140 in different combinations through protein/protein and protein/DNA interactions, as suggested by EMSA experiments with recombinant proteins and variously sized oligoprobes. Additionally, to advance knowledge of the transcriptional pathways that regulate the activity of F promoter and, consequently, the expression of the ERα gene in bone, these findings provide new details about the ER/AP-1 pathway at target promoters. In particular, we focused on the sequence −117,214/−117,167 containing the AP-1(1) site and the ERE half site. We demonstrated that c-jun/c-fos, ATF-2/ATF-2, and ERα/ATF-2 are the preferred protein–protein combinations (Fig. 4B) and that the combined contribution of multiple cis elements, rather than single cis elements alone, is a key regulatory process of F promoter activity.

In addition, our results are consistent with recent studies showing that AP-1 family proteins and ERα sometimes form heterodimers and interact with Estrogen Responsive Elements located in the promoter, or upstream/downstream of the promoter of a number of genes, up- or downregulating their transcription (Weisz and Rosales, 1990; Petz et al., 2002; Garcia-Arencibia et al., 2005; Cascio et al., 2007). Conversely, the activity of AP-1 containing promoters may be regulated by ERα through direct interaction with Jun (Kushner et al., 2000; Cheung et al., 2005; Jeffy et al., 2005; Lu et al., 2006). For example, direct interactions between ERα and Jun were demonstrated for ovalbumin, c-fos, collagenase, IGF-1, progesterone receptor, and BRCA1 genes (Gaub et al., 1990; Umayahara et al., 1994; Kushner et al., 2000; Petz and Nardulli, 2000; Jeffy et al., 2005; Cascio et al., 2007). A mechanism has been also demonstrated through which ERα can regulate gene transcription through a promoter that lacks canonical ERE, such as cyclin D1 promoter in which the c-jun/ATF-2/ER complex is specifically recruited at the CRE-D1 element, mediating 17-β-estradiol control of the proliferation of target cells (Sabbah et al., 1999). The ability of ERα to stimulate activation domains of Jun and ATF-2 was demonstrated (Sabbah et al., 1999; Wang et al., 2004). In agreement with these considerations, ERα represents another gene whose expression may be influenced both by ERα and AP-1. Even if c-jun is considered the most potent transcriptional activator in the AP-1 protein family, we nevertheless hypothesize that in the promoter context analyzed here, its activity can be attenuated by ERα/ATF-2 heterodimerization. In fact, this combination together with ATF-2/ATF-2 homodimers seems to be particularly strong, as results from EMSA analysis. However, we do not exclude the possibility that other ERE-type elements and cooperative functions of different combinations of DNA binding proteins contribute to promoter responsiveness in the context of different and larger promoter fragments of the ERα gene.

The findings presented in this study, together with further investigations aimed at the elucidation of the ERα/AP-1 pathway involved in regulating ERα expression, may also have clinical relevance for bone diseases. The members of the Jun, Fos and ATF families, as well as ERα are differentially expressed during osteoblast maturation (McCabe et al., 1995; Wagner and Eferl, 2005). Being at the receiving end of several signal transduction cascades, AP-1 activity converts different extra- and intracellular signals, including estrogens, into changes in gene expression via expression of AP-1 responsive target genes, integrating estrogens activity and modulating the critical role of ERα in the regulation of bone cell function (McCabe et al., 1995; Schultz et al., 2005; Wagner and Eferl, 2005; Lu et al., 2006; Shur et al., 2007). Therefore, identification of ERα as a target of

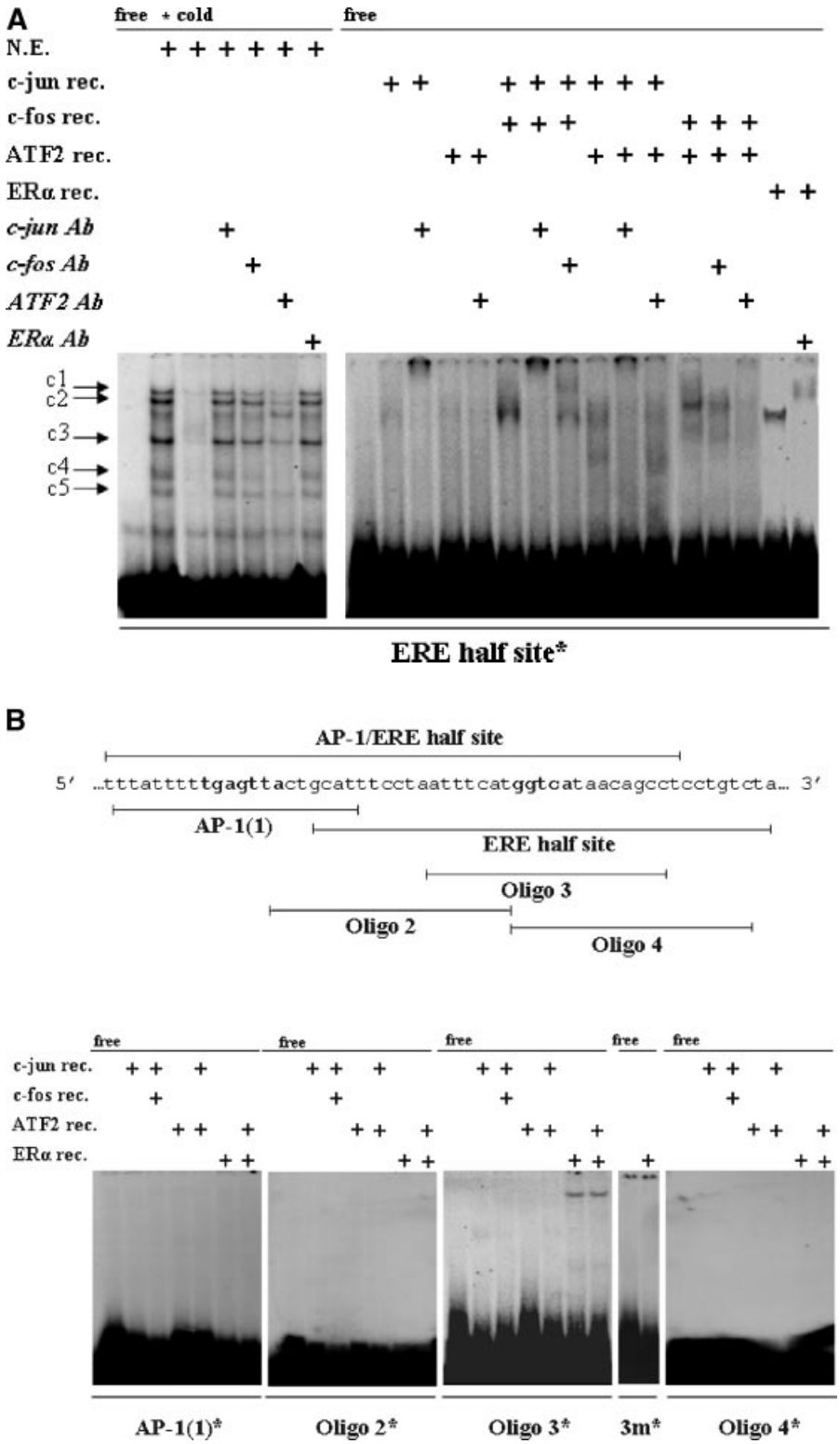

Fig. 5. A,B: In vitro analysis of DNA-protein interactions at individual AP-1 and ERE half sites. A: Gel shift experiments were performed with nuclear extracts from SaOS-2 cells and with recombinant c-jun, c-fos, ATF2, and ERα proteins in presence of ^{32}P labeled ERE half site oligonucleotide. Protein–DNA complexes were resolved from free probe through non-denaturing 6% polyacrylamide gels. Representative gels are shown. The ^{32}P labeled oligonucleotide was incubated alone (free), in combination with 10 μg of nuclear extracts (NE) (*), and in presence of a 100-fold molar excess of specific unlabeled oligonucleotide (cold), respectively. Where indicated, antibodies against c-jun, c-fos, ATF2, and ERα (Santa Cruz Biotechnology) were incorporated into the binding reactions. The five major complexes formed are marked as c_1–c_5. B: Gel shift experiments were performed with recombinant c-jun, c-fos, ATF2 and ERα proteins in the presence of ^{32}P labeled AP-1(1), 2, 3, and 4 oligonucleotides. 3m, mutant oligonucleotide 3 with 2-bp mutations in the ERE half site. The position of the oligoprobes used in this EMSA is reported above.

specific AP-1 factors may be very useful for understanding how these transcription factors control osteoblast differentiation, in both physiological and pathological conditions, including osteopenic diseases such as osteoporosis (Compston, 2001; Deroo and Korach, 2006; Penolazzi et al., in press), and for facilitating the development of novel therapeutic tools for bone diseases.

Acknowledgments

This research was supported by grants from AIRC, MIUR COFIN-2005, STAMINA, Fondazione CaRiFe and CaRiPaRo. E.L. is a recipient of a fellowship from "Fondazione CaRiCe". English revision of the article was carried out by Rebecca K. Swartz.

Literature Cited

Bergman MR, Cheng S, Honbo N, Piacentini L, Karliner JS, Lovett DH. 2003. A functional activating protein 1 (AP-1) site regulates matrix metalloproteinase 2 (MMP-2) transcription by cardiac cells through interactions with JunB-Fra1 and JunB-FosB heterodimers. Biochem J 369:485–496.

Bodine PV, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS. 1998. Estrogen receptor-alpha is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology 139:2048–2057.

Cascio S, Bartella V, Garofalo C, Russo A, Giordano A, Surmacz E. 2007. Insulin-like growth factor 1 differentially regulates estrogen receptor-dependent transcription at estrogen response element and AP-1 sites in breast cancer cells. J Biol Chem 282:3498–3506.

Cheung E, Acevedo ML, Cole PA, Kraus WL. 2005. Altered pharmacology and distinct coactivator usage for estrogen receptor-dependent transcription through activating protein-1. Proc Natl Acad Sci USA 102:559–564.

Compston JE. 2001. Sex steroids and bone. Physiol Rev 81:419–447.

Denger S, Reid G, Kos M, Flouriot G, Parsch D, Brand H, Korach KS, Sonntag-Buck V, Gannon F. 2001a. ER alpha gene expression in human primary osteoblasts: Evidence for the expression of two receptor proteins. Mol Endocrinol 15:2064–2077.

Denger S, Reid G, Brand H, Kos M, Gannon F. 2001b. Tissue-specific expression of human ERalpha and ERbeta in the male. Mol Cell Endocr 178:155–160.

Deroo BJ, Korach KS. 2006. Estrogen receptors and human disease. J Clin Invest 116:561–570.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Garcia-Arencibia M, Davila N, Campion J, Carmen Carranza M, Calle C. 2005. Identification of two functional estrogen response elements complexed with AP-1-like sites in the human insulin receptor gene promoter. J Steroid Biochem Mol Biol 94:1–14.

Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P. 1990. Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex. Cell 63:1267–1676.

Gruber CJ, Gruber DM, Gruber IML, Wieser F, Huber JC. 2004. Anatomy of the estrogen response element. Trends Endocr Metab 15:73–78.

Hall JM, McDonnel DP, Korach KS. 2002. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. Mol Endocrinol 16:469–486.

Jeffy BD, Hockings JK, Kemp MQ, Morgan SS, Hager JA, Beliakoff J, Whitesell LJ, Bowden GT, Romagnolo DF. 2005. An estrogen receptor-A/p300 complex activates the/BRCA-1 promoter at an AP-1 site that binds Jun/Fos transcription factors: Repressive effects of p53 on BRCA-1 transcription 1. Neoplasia 7:873–882.

Kalaitzidis D, Gilmore TD. 2005. Transcription factor cross-talk: The estrogen receptor and NF-kappaB. Trends Endocrinol Metab 16:46–52.

Karsenty G, Wagner EF. 2002. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2:389–406.

Klinge CM. 2001. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res 29:2905–2919.

Kos M, Denger S, Reid G, Gannon F. 2002. Upstream open reading frames regulate the translation of the multiple mRNA variants of the estrogen receptor alpha. J Biol Chem 277:37131–37138.

Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. 2000. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311–317.

Lambertini E, Penolazzi L, Giordano S, Del Senno L, Piva R. 2003. Expression of the human oestrogen receptor-alpha gene is regulated by promoter F in MG-63 osteoblastic cells. Biochem J 372:831–839.

Lambertini E, Penolazzi L, Tavanti E, Schincaglia GP, Zennaro M, Gambari R, Piva R. 2007. Human estrogen receptor alpha gene is a target of Runx2 transcription factor in osteoblasts. Exp Cell Res 313:1548–1560.

Lee W, Mitchell P, Tjian R. 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49:741–752.

Lin Y, Liu LJ, Murray T, Sodek J, Rao L. 2004. Effect of raloxifene and its interaction with human PTH on bone formation. J Endocrinol Invest 27:416–423.

Lu T, Achari Y, Sciore P, Hart DA. 2006. Estrogen receptor alpha regulates matrix metalloproteinase-13 promoter activity primarily through the AP-1 transcriptional regulatory site. Biochim Biophys Acta 1762:719–731.

McCabe LR, Kockx M, Lian J, Stein J, Stein G. 1995. Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. Exp Cell Res 218:255–262.

McKenna NJ, O'Malley BW. 2002. Minireview: Nuclear receptor coactivators—An update. Endocrinology 143:2461–2465.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson J. 2001. Mechanisms of estrogen action. Physiol Rev 81:1535–1565.

Penolazzi L, Zennaro M, Lambertini E, Tavanti E, Torreggiani E, Gambari R, Piva R. 2007. Induction of ER{alpha} expression with decoy oligonucleotide targeted to NFATc1 binding sites in osteoblasts. Mol Pharmacol 71:1457–1462.

Petz LN, Nardulli AM. 2000. Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Mol Endocrinol 14:972–985.

Petz LN, Ziegler YS, Loven MA, Nardulli AM. 2002. Estrogen receptor α and activating protein-1 mediate estrogen responsiveness of the progesterone receptor gene in MCF-7 breast cancer cells. Endocrinology 143:4583–4591.

Porter W, Wang F, Wang W, Duan R, Safe S. 1996. Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. Mol Endocrinol 10:1371–1378.

Sabbah M, Courilleau D, Mester J, Redeuilh G. 1999. Estrogen induction of the cyclin D1 promoter: Involvement of a cAMP response-like element. Proc Natl Acad Sci USA 96:11217–11222.

Safe S. 2001. Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor- Sp1 interactions. Vitam Horm 62:231–252.

Schultz JR, Petz LN, Nardulli AM. 2005. Cell- and ligand-specific regulation of promoters containing activator protein-1 and Sp1 sites by estrogen receptors alpha and beta. J Biol Chem 280:347–354.

Shur I, Zemer-Tov E, Socher R, Benayahu D. 2007. SVEP1 expression is regulated in estrogen-dependent manner. J Cell Physiol 210:732–739.

Sun G, Porter W, Safe S. 1998. Estrogen-induced retinoic acid receptor alpha 1 gene expression: Role of estrogen receptor-Sp1 complex. Mol Endocrinol 12:882–890.

Syed F, Khosla S. 2005. Mechanisms of sex steroid effects on bone. Biochem Biophys Res Commun 328:688–696.

Tang S, Tan SL, Ramadoss SK, Kumar AP, Tang MH, Bajic VB. 2004. Computational method for discovery of estrogen responsive genes. Nucleic Acids Res 32:6212–6217.

Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y, Kamada T. 1994. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 269:16433–16442.

Wagner EF, Eferl R. 2005. Fos/AP-1 proteins in bone and the immune system. Immunol Rev 208:126–140.

Wang MM, Traystman RJ, Hurn PD, Liu T. 2004. Non-classical regulation of estrogen receptor- alpha by ICI182, 780. J Steroid Biochem Mol Biol 92:51–62.

Weinmann AS, Bartley SM, Zhang MQ, Zhang T, Farnham PJ. 2001. Use of chromatin immunoprecipitation to clone novel E2F target promoters. Mol Cell Biol 21:6820–6832.

Weisz A, Rosales R. 1990. Identification of an estrogen response element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. Nucleic Acids Res 18:5097–5106.

Modulation of expression of specific transcription factors involved in the bone microenvironment

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The skeletal system functions and maintains itself thanks to the communication between cells of diverse origins such as osteoclasts (OCs) and osteoblasts (OBs). In bone remodelling, bone resorption by OCs is followed by osteoblastic bone formation, so that resorbed lacunae are filled to the original level by OBs. In this respect many growth factors and transcription factors are involved in both in OBs and in OCs differentiation. Here, the roles of NF-kB, NFATc1, estrogen receptor α, and Runx2, that are critical regulators of osteclastogenesis and OB differentiation, are discussed. In particular, the effects of "decoy" oligodeoxynucleotides (ODN) against NF-kB and NFATc1 on OCs activation and regulation both *in vitro* and *in vivo* are examined. The transcription factor (TF) "decoy" technology is based on the employment of synthetic double-stranded ODN containing a cis-element with high affinity for a target TF. In the cells transfected with these ODN the authentic cis-trans interactions are attenuated, the TF from the endogenous cis elements is removed and a specific modulation of gene expression is obtained. In addition, the siRNA approach was used to analyse the role of Runx2 transcription factor in the regulation of ERα gene expression which is a key regulator of bone homeostasis.

Key words: **Osteoclasts - NF-kappa B - NFATC transcription factors - Receptors, estrogen.**

Fundings.—This research was supported by grants from MIUR FIRB-2005, MIUR COFIN-2005, the Fondazione Cassa di Risparmio di Ferrara. E.L. is the recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento. R.G. has received a grant by Associazione Cassa di Risparmio di Padova e Rovigo.

Received on May 29, 2008.
Accepted for publication on June 3, 2008.

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The adult skeleton regenerates continuously with a periodic replacement of old bone with new one. This process, called remodelling, result from the intricately coupled actions of bone-forming osteoblasts (OBs) [1, 2] and bone resorbing osteoclasts (OCs).[3] OBs and OCs are derived from distinct stem cell pools and serve opposite but coordinated roles during bone remodelling. OBs are derived from mesenchymal stem cells and are responsible for synthesizing matrix proteins that subsequently become mineralized during the process of bone formation. OCs are derived from hematopoietic progenitors of the myeloid lineage, colony-forming unit-granulocyte/macrophage (CFU-GM) and CFU-M, that are responsible for resorbing extracellular matrix, a process called bone resorption. While healthy bone maintains a net balance between the opposing processes of osteoclastic resorption and osteoblastic production, bone loss occurs in diseases such as osteoporosis, arthritis, and tumor metastases, when these two processes are not balanced and bone resorption dominates over bone formation.

Transcription factors and bone cells

Homeostasis of the skeletal system depends on a delicate balance between OBs and OCs, strictly correlated with the action of a number of systemic hor-

mones and transcription factors that regulate the proliferation and differentiation of OBs and OCs.[4, 5] Among these factors, NF-kB, NFATc1, Runx2, and estrogen receptor play a critical role. Many of these molecules are autocrine and paracrine factors produced by OBs.

NF-kB in osteoclastogenesis

M-CSF induces the proliferation of OCs precursor, supports their survival and upregulates the RANK expression, which is a prerequisite for their activity.[6, 7] Several transcription factors have been found crucial for OC differentiation downstream of M-CSF/c-fms and RANKL/RANK signaling. In particular the importance of NF-kB was demonstrated by the absence of multinucleated bone-resorbing cells in NF-kB mutant mice.[8] Furthermore, gene-targeting studies have shown that NF-kB has a crucial role in OC differentiation.[8, 9] NF-kB is a family of dimeric transcription factors that recognize a common DNA sequence called kB site. In mammals, there are five NF-kB proteins: cRel; RelA (p65);RelB; p50;p52.[10] NF-kB proteins reside in the cytoplasm of non-stimulated cells but rapidly enter the nucleus upon cell stimulation with a variety of agonists, including RANKL,[3, 11, 12] where it modulates the expression of a number of target genes critical for osteoclastogenesis.

Several drugs used to fight bone loss in a variety of disorders, such as osteoporosis, act by increasing the frequency of apoptosis of OCs, since it was demonstrated that small changes in OCs apoptosis can result in large changes in bone formation.[13-16] In this respect, targeting of NF-kB transcription factors could be of great interest. Several recent studies indicate indeed that NF-kB activation can block cell-death pathways.[17, 18] For instance, it was shown that NF-kB activation is required to protect cells from the apoptotic cascade induced by Tumour Necrosis Factor and other stimuli.[19, 20] Therefore, experimental strategies aimed at the inhibition of NF-kB activity could greatly facilitate therapeutical approaches to osteopenic disorders.

Among non-viral gene therapy strategies able to inhibit or even block NF-kB activity, the transcription factor decoy (TFD) approach [21, 22] should be taken in great consideration, since NF-kB decoy has been recently used in several preclinical studies and proven to be active in several experimental systems.[23-27]

The technologies of oligodeoxynucleotides (ODN) have received considerable attention because they provide a rational way to design sequence-specific ligands of nucleic acids or DNA-binding regulatory proteins as a tool for the selective regulation of a specific gene expression. In particular, as altered activation of transcription factors has become a better understood component of many pathways of disease pathogenesis, including cancer, viral infection, and chronic inflammatory diseases, the development of molecular strategies targeting transcription activating proteins has emerged as an attractive field of investigation.[28] One of the most successful ODN-based approaches has been the use of a synthetic double-strand ODN (decoy) containing an enhancer element. Nucleic acid molecules with a high affinity for a target transcription factor can be introduced into cells as a "decoy" cis-element to bind to a specific transcription factor. After delivery into the nucleus of the target cell, decoy ODN can bind to free transcription factors and block the interaction of these factors to the promoter region of target genes modulating their expressions. Decoy ODN have been used successfully *in vitro* and, more importantly, *in vivo* in intact animal models.[29-38] Decoy ODN have been used in a variety of forms, ranging from short 10-20 base pairs (bp) ODN to plasmid DNA containing multiple repeats of the consensus sequence.[39] This approach seems to be particularly attractive for several reasons: 1) potential targets are plentiful and readily identifiable; 2) synthesis of decoy ODN is relatively simple and can be targeted to specific issues; 3) knowledge of the exact molecular structure of target transcription factors is not necessary.

Nakamura *et al.* recently demonstrated prevention and regression of atopic dermatitis by ointment containing NF-kB decoy oligodeoxynucleotides in the NC/Nga atopic mouse model;[40] in addition, decoy ODN were demonstrated to be useful to inhibit tumour cell growth and invasion,[41, 42] as anti-inflammatory agents,[43, 44] in myocardial preservation,[45, 48] in cerebral angiopathy.[49] Accordingly, TFD molecules targeting NF-kB transcription factors might bring opportunities for a specific and controlled bone formation with interrupted bone resorption.

Oligonucleotide decoys targeting NF-kB induced apoptosis of human OCs

Since the transcriptional factor NF-kB has been reported to be important for the expression of several OC-specific genes, it was tried to alter OC develop-

ment by inhibiting NF-kB action by the decoy approach. The effects of the double-stranded decoy ODN targeting NF-kB was analysed on apoptosis of mature OCs generated by induction to osteoclastogenesis of mononucleated cells isolated from peripheral blood.[50] Actually primary OCs were treated with the NF-kB HIV-1 (5'-CGC TGG GGA CTT TCC ACG G-3') decoy molecule complexed with lipofectamine (1 μg/mL). Morphological analysis of NF-kB decoy-treated OCs demonstrated cell retraction, in comparison with scramble-ODN control-treated OCs, indicative of apoptosis. To confirm apoptosis, TUNEL staining of fragmented DNA was performed, as reported in Figure 1A. As shown, the treatment with NF-kB decoy significantly increased the percentage of TUNEL-positive cells with respect to scrambled-ODN. In order to provide additional data related to apoptotic pathways involved, the decoy-treated cells were immunostained with antibody directed against Caspase 3. As shown in Figure 1A, multinucleated OCs were strongly positive for Caspase 3 after the decoy treatment. Next, the efficiency of NF-kB decoys was evaluated on the expression of IL-6, a typical target of NF-kB; this evidence was compared with the effect on NF-kB protein expression level. The results obtained demonstrate that treatment with NF-kB decoy does not affect NF-kB content, but causes a strong expression of IL-6 decrease (Figure 1A). These results clarify the mechanism of action of the NF-kB decoy ODNs on human OCs. First, the evidence that no inhibition of accumulation of NF-kB proteins occurs during treatment (Figure 1A) allows to exclude direct effects of NF-kB decoys on NF-kB mRNA accumulation and translation. Second, the strong inhibition of IL-6 expression (Figure 1A) suggests that NF-kB dependent biological functions are impaired, possibly by inhibition of the molecular interactions between NF-kB and target DNA sequences. In conclusion, these results demonstrated that NF-kB decoy administration increased the prevalence of OCs apoptosis in experimental conditions.

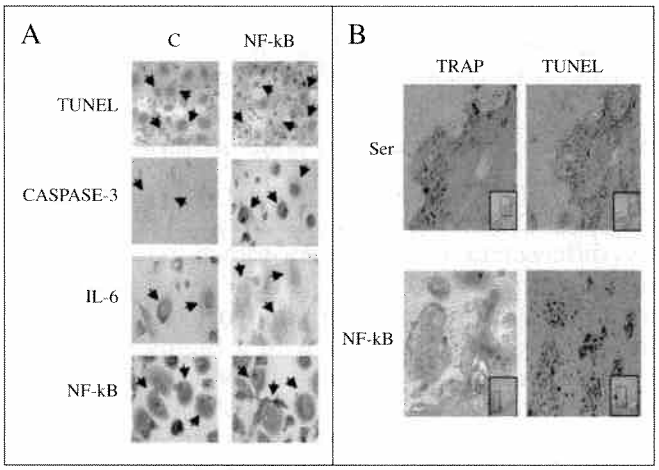

Figure 1.—Effect of NF-kB ODN decoy on osteoclasts. A) hOCs were transiently transfected with DNA (NF-kB) complexed with lipofectamine for 48h. Apoptotic OCs were detected by TUNEL assay and counterstained with haematoxylin. Brown color reaction indicates cells that underwent apoptosis. The detection of the differential expression of Caspase 3, IL-6 and NF-kB in the same experimental conditions was also evaluated by immunocytochemistry. (C) Untreated cells. The presence of human OCs is indicated by arrows (20× magnification); B) paraffin sections of mesial rats tooth surfaces. The same sections were subjected to TRAP and TUNEL assay. Brown staining indicates the presence of apoptotic nuclei. Red staining corresponds to TRAP-positive cytoplasm. After TUNEL assay the sections have been hematoxylin counterstained. NF-kB: NF-kB ODN treated tooth; Scr: scramble ODN treated tooth. Magnification is 20× (large pannel), 10× (bottom inserts).

Induction of OCs apoptosis *in vivo* by NF-kB decoy

Clinical orthodontics offers a good opportunity to study the efficacy of the decoy approach for induction of apoptosis of OCs *in vivo*. In fact, the increased number and activity of OCs is involved in the regulation of alveolar bone resorption during orthodontic tooth movement, and also in the origin of dental problems in diseases of OC activation affecting the maxillo-mandibular bone. *In vivo* experiments aimed at regulating alveolar bone resorption were designed. Ten Wistar male rats were subjected to orthodontic forces, in combination or not with NF-kB decoy treatment, by using a split-mouth design. Examination of paraffin sections of the excised molars showed that orthodontic forces caused a percentage of apoptotic OCs that appear to be highly potentiated by NF-kB but not by scramble ODN (Figure 1B). These results extend to an *in vivo* experimental system the *in vitro* observations on OCs isolated from peripheral blood.[50]

The results confirm that the inhibition of NF-kB activity, through the *in vivo* transfer of NF-kB decoy, results in the suppression of the transcription of key genes controlling OC survival. This clearly supports the effectiveness of a NF-kB decoy strategy by the *in vivo* OCs transfection of decoy ODN containing the NF-kB cis-element. On the basis of the data reported here, it

is likely that the previously set up *in vitro* experimental conditions may be transferred to the study of specific bone disorders and to clinical orthodontics.[51]

NFATc1 in the regulation of bone turnover

It is well known that the nuclear factor for activated T cells (NFAT) signalling regulates OB proliferation, OC differentiation, and the coordination of bone formation and resorption.[52] The NFAT transcription factor family was originally identified in T cells. NFAT has four isoforms, c1-c4, each with a highly conserved DNA binding domain. This family of transcription factors is involved in the regulation of a variety of biological systems such as cardiovascular and muscular systems in addition to the immune system.[53] In particular NFATc1 is a key regulator of bone turnover.[54] The activation of NFATs is mediated by a specific phosphatase, calcineurin, which is activated by calcium/calmodulin signalling. Involvement of NFATc1 directly implicates Ca^{2+} signalling since NFAT activation and subsequent nuclear translocation is directed by the Ca^{2+}/calmodulin dependent serine/threonine phosphatase calcineurin.[53] NFAT then traslocates to the nucleus and binds to specific regions in the promoter of target genes. NFATc1 is essential for OC lineage specification, while active NFATc1 drives OB proliferation. Additionally, calcineurin/NFAT signalling in OBs enhances chemokine expression, which may recruit OC precursors to bone and influence osteoclastogenesis.

The induction of NFATc1 is a hallmark event in the cell fate determination of OCs [54, 55] and a number of OC-specific genes are directly regulated by NFATc1 (trap, calcitonin receptor, cathepsin K).[56-58] NFATc1 is described as a "master regulator" of osteoclastogenesis, due both to its cooperative interaction with AP-1 and NF-kB in osteoclastic gene transcription and its proposed auto-amplification, a strategy known to be utilized for lineage commitment in T cells, cardiac, and skeletal muscle cells.[53-59]

A recent report revealed that NFAT could play a potential role for regulating OB function.[60] Actually NFAT may function at different stages to regulate OB proliferation.[52] Inhibition of the calcineurin/NFAT signalling pathway increases OB differentiation by negative regulation of the expression of Fra-2.[61] The role of calcineurin/NFAT signalling in OBs and OCs is interesting since these two cell types oppose each other's actions. In this respect, NFATc1 should be a good molecular target in order to introduce new therapeutic approach in bone diseases.

OLIGONUCLEOTIDE DECOYS TARGETING NFATc1: EFFECT ON BONE CELLS

The preliminary evidences of the role of NFATc1 both in OCs and in OBs suggest that the inhibition of calcineurin/NFAT pathway may have some positive effect in bone repair. With this in mind, the decoy approach was applied against this transcription factor. For this aim a double-stranded oligonucleotide, whose sequence belongs to the promoter region of the human IL-2 gene, was designed.[62] For these kinds of experiments, NFATcs-ODN decoy molecules were transfected into human primary OCs obtained from precursors present in peripheral blood, and then the percentage of apoptotic cells was verified by TUNEL assay. As shown in Figure 2A, a significant increase of OCs with dark brown nuclei was observed, in respect to the treatment with scramble-ODN. These results indicated that NFATc1-ODN affected specifically OCs viability. These results were also confirmed by the immunocytochemical analysis of Caspase-3 and Fas expression levels (data not shown). The same experiments were applied to another experimental model, human primary OBs obtained from bone specimens collected during oral surgery. The TUNEL assay indicated that no apoptotic cells were present in the culture after NFATc1-ODN treatment, demonstrating the absolute cell-specificity of the decoy effect (Figure 2A).

In a second step, the possible ability of NFATc1-ODN of modulating OB phenotype was also verified. Human OBs were treated with NFATcs-ODN and the effects on Runx2 and ERα expression levels were verified by immunocytochemisty (Figure 2B). Runx2 and ERα were chosen as they are two well-known OB markers. As shown in Figure 2B a significant increase of ERα and Runx2 proteins was observed, with respect to the scramble. In the same experimental condition, a quite good increase of mineralized nodule deposition has also been observed after decoy treatment (data not shown).

In conclusion the effects of transcription factors decoy oligonucleotides on apoptosis of human OCs [63, 64] and reviewed [65] are reported: the first decoy molecules were designed to inhibit NF-kB binding to target sequence, the second to inhibit NFATc1 binding. Both decoy molecules are powerful inducers o

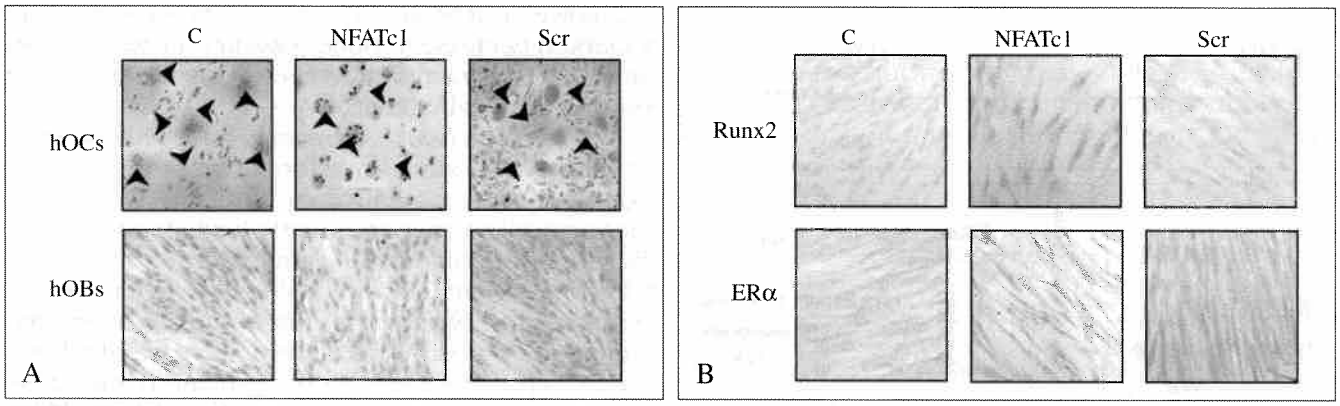

Figure 2.—Effect of NFATc1 ODN decoy on bone cells. A) hOCs and hOBs were transiently transfected with NFATc1-ODN (NFATc1) complexed with lipofectamine for 48 hours. Apoptosis was detected by TUNEL assay and the cells were counterstained with hematoxylin. Brown color reaction indicates cells that underwent apoptosis; B) immunocytochemical analysis of Runx2 and ERα in hOBs after NFATc1-ODN decoy treatment. C): untreated cells; Scr: scramble ODN treated cells. Magnification is 20×.

human OC apoptosis, associated with increase of caspase 3 activity and decrease of IL-6 expression. In addition, evidences indicate that these oligonucleotides did not affect OBs survival. Since OCs are essential for skeletal development and remodelling throughout the life of animals and men, the described approach is of potential clinical interest.

Runx2

The OB lineage derived from mesenchymal stem cells is determined by different transcription factors. Among various transcription factors involved in osteogenic differentiation, Runx2 plays a central role in bone formation because targeted disruption of Runx2 results in the complete lack of bone formation by OBs.[66, 67] Runx2 was identified as a key regulator of OBs specific gene expression. These regulations are thought to occur when the Runx2 binding domain interacts with its related DNA consensus sequence (AACCACA), which is known as OB-specific cis-acting elements (OSE2).[66] The DNA binding domain sites of Runx2 in major bone matrix protein genes including the Col1α1, osteopontin, bone sialoprotein, and osteocalcin genes, have been identified. Runx2 induces the expression of these genes or activates their promoters *in vitro*.[68] Runx2 has all the characteristics of a differentiation regulator in the OB lineage: 1) its expression correlates with osteogenesis during development; 2) Runx2 is necessary for OB differentiation; 3) Runx2 is also sufficient to induce OB differentiation *in vitro* and *in vivo*. *In vivo*, ectopic expression of Runx2 in transgenic animals leads to ectopic endochondral ossification. *In vitro*, overexpression of Runx2 in non OB cells induces OB-specific expression of all bone marker genes, including osteocalcin and bone sialoproteins. Moreover, *in vivo* findings indicate that Runx2 triggers the expression of major bone matrix protein genes at an early stage of OB differentiation, leading to the cells acquiring an osteoblastic phenotype. Runx2 continuously increases during OB differentiation and its activity is subject to many post-translational modifications and coregulatory protein interactions indicating a primary functional role throughout all stages of maturation. Many Runx2 interacting proteins are themselves key regulators of osteogenesis. In addition, Runx2 interacts with several regulatory proteins, resulting in activation or repression of genes which control OB proliferation and differentiation.[69] Several studies have indicated that Runx2 is a context-dependent transcriptional activator and repressor. The transcriptional up- and down-regulation of Runx2 depends on diverse signals and cofactors that affect Runx2 function and location. Cofactor interaction, as well as the possibility that interactions, especially those with other transcription factors, may be either stimulatory or inhibitory depending on the promoter/enhancer context, are important consideration.

Although some transcriptional targets for Runx2 are known, it is believed that the osteogenic action of Runx2 is mediated by additional target genes, and

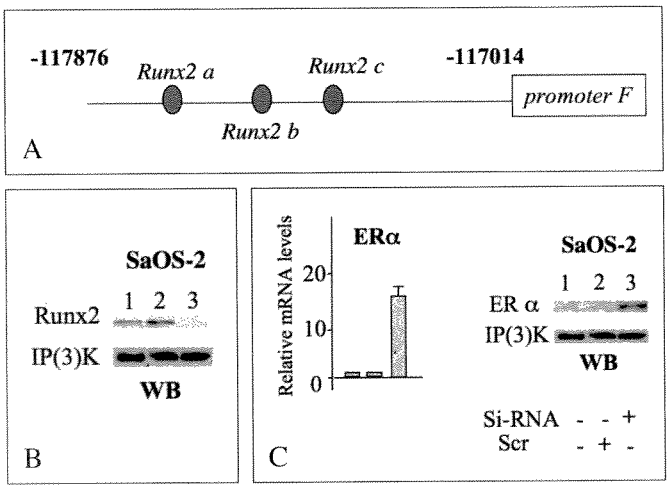

Figure 3.—Effect of siRNA-Runx2 on ERα expression and function. A) The schematic diagram represents the portion of F promoter analyzed, located at -117014.Using the Transfac database and the TF search, several potential transcription factor binding motifs have been identified. In particular the F promoter contains three consensus for Runx2 (a, b, c); B) Western blot with Runx2 antibody shows the specific effect of scramble siRNA (lane 2) and Runx2-siRNA (lane 3) on the endogenous Runx2 protein level (lane 1). Whole cell extracts from SaOS-2 osteosarcoma transfected cells were prepared, equal amounts (25 μg/lane) of cellular proteins were separated on 12% SDS-PAGE gel, transferred to nitrocellulose and probed with anti-Runx2 or anti-IP(3)K antibodies; C) ERα expression analysis in SaOS-2 cells transfected with 150 nM scramble siRNA (lane 2), Runx2-siRNA (lane 3), or remained untreated (lane 1). The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for ERα transcript analysis. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate; the ΔΔCt method was used to compare gene expression data then standard error of the mean (SEM) was calculated. Western blot with antibody against ERα shows the effect of scramble siRNA (lane 2) and Runx2-siRNA (lane 3) on the endogenous ERα protein level (lane 1). IP(3)K was used as a control.

increasing studies are performed in order to identify such Runx2-responsive genes,[70, 71] which may give a new hint to the therapeutic treatment for osteoporosis and other diseases of altered bone mass by stimulating Runx2 expression and then promote their bone formation.

Estrogen receptor

Sex steroids are major hormonal regulators of bone turnover in both sexes. In particular estrogens play a key role in the maintenance of bone homeostasis. The osteoprotective action of estrogen is demonstrable in rodents and is clinically important in human.[5]

It is well established that loss of estrogen leads to a marked decrease in bone mass and increased risks of both bone fracture and cardiovascular disease in postmenopausal women.

Estrogen contributes to maintenance of bone mass either by stimulating bone formation or by reducing bone resorption. Estrogen deficiency in postmenopausal women frequently leads to osteoporosis, the most common skeletal disorder. At the cellular level, there is now considerable evidence that estrogens prolong the lifespan of the OB by inhibiting OB apoptosis.[72] This, in turn, increases the functional capacity of each OB. Estrogens may also enhance bone formation through stimulatory effects on OB transcription factors.[73, 74] At the molecular level the effects of sex steroids are mediated by their receptors (ERα, ERβ). ERs belong to the nuclear receptor gene superfamily and act as ligand-inducible transcription factor.[75] ER dimmers directly or indirectly associate with specific DNA elements, named estrogen response elements, in the target gene promoters,[76, 77] resulting in transcriptional regulation of gene expression.

The transcriptional activation of the ER results in the regulation of target genes that are involved in normal physiological processes such as the maintenance of bone density. Classical receptors (ERα, ERβ) are present both in OBs and OCs,[78, 79] but at lower levels than those found in reproductive tissues.[80] It is now clear that ERα and ERβ are extremely important components of a complex signal transduction pathway that specifically regulates the growth and development of target tissues included bone. In particular, the central role of ERα in the bone is largely described, and a number of estrogen-induced effects on gene expression in OBs have been demonstrated, but little information is available on the transcription factors involved in the control of the production of this unique ERα transcript. Therefore analysis of transcription factors can modulate bone-specific action of ERα, and identification of novel mechanisms of action by which these transcription factors control function of ER in bone cells, would have profound implications for the development of new therapeutic strategies for bone disease.

The human ERα gene is transcribed from at least seven promoters into multiple transcripts that all vary in their 5'-UTRs regulated in a tissue-specific manner. In particular F promoter located at -117014, is one of the multiple promoters of human ERα gene and it is

the only active promoter in bone tissue.[81, 82] Interestingly, the distal F promoter of the human ERα gene, located at -117140 from the +1 initiation transcription start site,[81] contains three potential binding sites for Runx2.

Analysis of F promoter of ERα using siRNA approach

In laboratory, authors investigated the involvement of Runx2 in bone-specific expression of the ERα gene,[83] by conducting a detailed analysis of the Runx2 motifs, Runx2 (a), (b) and (c), present within the F promoter (Figure 3A) and demonstrating that only one of these Runx2 sites, (Runx2 a) is capable of binding Runx2 transcription factor.[83]

To assess the impact of Runx2 levels on controlling ERα gene expression in OBs, short-interfering RNA (siRNA) was used to knock down Runx2 specifically.

RNAi is a nearly ubiquitous pathway that modulates gene-expression by post-transcriptional mechanisms. The basic mechanism of RNAi is considered to be a multi-step process. siRNAs are typically approximately 20 base pairs in length and are complementary to mRNA. Once inside the cell, the siRNA is bound by the proteins of the RNA-induced silencing complex (RISC).[84] The RISC proteins facilitate searching through the genome for RNA sequences that are complementary to one of the two strands of the siRNA duplex. One strand of the siRNA (the sense strand) is lost from the complex, while the other strand (the antisense strand) is matched with its complementary RNA target. Recognition of mRNA by the antisense strand of the siRNA can cause destruction of the mRNA, prevent synthesis of protein, and thereby reduce the level of protein inside cells.

For this study three separate siRNA fragments targeted to the mRNAs for Runx2 were designed. The three fragments were each studied by Western blot to select the most efficacious fragment of the three. The sequences of the most effective fragments are: sense and antisense Runx2 siRNA fragments were: 5-CGAUCUGAGAUUUGUGGGCtt-3; 5-GCCCACAAAUCUCAGAUCGtt-3, and it was demonstrated that Runx2 knockdown (Figure 3B) promotes upregulation of ERα expression, both at mRNA and at protein level, in OBs (Figure 3C). These results confirm the role of Runx2 in the repression of F promoter activity and that Runx2 depletion positively modulates the ERα gene expression.

Conclusions

Finally, this study demonstrates that the osteoblastic master regulator Runx2 transcription factor is also involved in the modulation of human ERα gene expression in OBs.

References

1. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature 2003;423:349-55.
2. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2002;2:389-406.
3. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003;423:337-42.
4. Karsenty G. Convergence between bone and energy homeostases: leptin regulation of bone mass. Cell Metab 2006;4:341-8.
5. Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science 2000;289,1508-14.
6. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K *et al.* Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 999;190:1741-54.
7. Ross FP, Teitelbaum SL. αvβ3 and macrophage colony-stimulating factor: partners in osteoclast biology. Immunol Rev 2005;208:88-105.
8. Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD *et al.* Requirement for NF-kB in osteoclast and B-cell development. Genes Dev 1997;11:3482-96.
9. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R. Osteopetrosis in mice lacking NF-kB1 and NF-kB2. Nat Med 1997;3:1285-9.
10. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol 2004;5:392-401.
11. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER *et al.* A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997;390:175-9.
12. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K *et al.* T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature 2000;408:600-5.
13. Ozaki K, Takeda H, Iwahashi H, Kitano S, Hanazawa S. NF-kB inhibitors stimulate apoptosis of rabbit mature osteoclasts and inhibit bone resorption by these cells. FEBS Lett 1997;410:297-300.
14. Takeuchi T, Tsuboi T, Arai M, Togari A. Adrenergic stimulation of osteoclastogenesis mediated by expression of osteoclast differentiation factor in MC3T3-E1 osteoblast-like cells. Biochem Pharmacol 2000;61:579-86.
15. Rodan GA, Martin TJ. Therapeutic approaches to bone diseases. Science 2000;289:1508-14.
16. Weinstein RS, Manolagas SC. Apoptosis and osteoporosis. Am J Med 2000;108:153-64.
17. Romano MF, Lamberti A, Bisogni R, Tassone P, Pagnini D, Storti G *et al.* Enhancement of cytosine arabinoside-induced apoptosis in human myeloblastic leukemia cells by NF-kappa B/Rel-specific decoy oligodeoxynucleotides. Gene Ther 2000;7:1234-7.
18. Kajino S, Suganuma M, Teranishi F, Takahashi N, Tetsuka T, Ohara H *et al.* Evidence that de novo protein synthesis is dispensable for anti-apoptotic effects of NF-kappaB. Oncogene 2000;19:2233-9.
19. Qin JZ, Bacon P, Chaturvedi V, Nickoloff BJ. Role of NF-kappaB activity in apoptotic response of keratinocytes mediated by interferongamma, tumor necrosis factor-alpha, and tumor-necrosis-factor-related apoptosis-inducing ligand. J Invest Dermatol 2001;117:898-907.

20. Bernard D, Quatannens B, Vandenbunder B, Abbadie C. Rel/NFkappaB transcription factors protect against tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by up-regulating the TRAIL decoy receptor DcR1. J Biol Chem 2001;276:27322-8.
21. Cho-Chung YS, Park YG, Nesterova M, Lee YN, Cho YS. CRE-decoy oligonucleotide-inhibition of gene expression and tumor growth. Mol Cell Biochem 2000;212:29-34.
22. Piva R, Gambari R. Transcription factor decoy (TFD) in breast cancer research and treatment. Technol Cancer Res Treat 2002;1:405-16. 1196 L.
23. Cooper Jr JA, Parks JM, Carcelen R, Kahlon SS, Sheffield M, Culbreth R. Attenuation of interleukin-8 production by inhibiting nuclear factor-kappaB translocation using decoy oligonucleotides. Biochem Pharmacol 2000;59:605-13.
24. Kozlov IA, Kubareva EA, Ivanovskaya MG, Shabarova ZA. Design of new reagents on the base of DNA duplexes for irreversible inhibition of transcription factor NF-kappa B. Antisense Nucleic Acid Drug Dev 1997;7:279-89.
25. Vos IH, Govers R, Grone HJ, Kleij L, Schurink M, De Weger RA *et al.* NFkappaB decoy oligodeoxynucleotides reduce monocyte infiltration in renal allografts. FASEB J 2000;14:815-22.
26. Bonham CA, Peng L, Liang X, Chen Z, Wang L, Ma L *et al.* Marked prolongation of cardiac allograft survival by dendritic cells genetically engineered with NF-kappa B oligodeoxyribonucleotide decoys and adenoviral vectors encoding CTLA4-Ig. J Immunol 2002;169:3382-91.
27. Quan N, Ho E, LaW. Tsai YH, Bray T. Administration of NF-kappaB decoy inhibits pancreatic activation of NF-kappaB and prevents diabetogenesis by alloxan in mice. FASEB J 2001;15:1616-8.
28. Papavassiliou AG. Transcription-factor-modulating agents: precision and selectivity in drug design. Mol Med Today 1998;4:358-66.
29. Morishita R, Gibbons GH, Ellison KE, Nakajima M, Zhang L, Kaneda Y *et al.* A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo. Proc Natl Acad Sci USA 1995;92:5855-9.
30. Bianchi N, Borgatti M, Gambari R. Gene silencing in thalassemia. Minerva Biotec 2008;20:51-5.
31. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A *et al. In vivo* transfection of the *cis* element "decoy" against the nuclear factor-kappa B binding site prevents myocardial infarction. Nat Med 1997;3:894-9.
32. Tomita N, Horiuchi M, Tomita S, Gibbons GH, Kim JY, Baran D *et al.* A novel strategy transcription factor decoy for E2F inhibits proliferation in mesangial cells in vitro. Am J Physiol 1998;27:F278-84.
33. Tomita T, Takeuchi E, Tomita N, Morishita R, Kaneko M, Yamamoto K *et al. In vivo* transfection of NF-kB decoy ODN suppressed the severity of rat collagen-induced arthritis as a gene therapy. Arthritis Rheum 1999;42:2535-42.
34. Tomita N, Morishita R, Lan HY, Yamamoto K, Hashizume M, Notake M *et al. In vivo* administration of a nuclear transcription factor-kappa B decoy suppresses experimental crescentic glomerulonephritis. J Am Soc Nephrol 2000;11:1244-52.
35. Tomita N, Morishita R, Tomita S, Gibbons GH, Zhang L, Horiuchi M *et al.* Transcription factor decoy for NF kappa B inhibits TNFalpha-induced cytokine and adhesion molecule expression *in vivo*. Gene Ther 2000;7:1326-32.
36. Yamasaki K, Asai T, Shimizu M, Aoki M, Hashiya N, Sakonjo H *et al.* Inhibition of NF kappa B activation using a *cis*-element "decoy" of NF kappa B binding site reduces neointimal formation in a porcine balloon-injured coronary artery model. Gene Ther 2003;10:356-64.
37. Tomita N, Kim JY, Gibbons GH, Zhang L, Kaneda Y, Stahl RA *et al.* Gene therapy with E2F transcription factor decoy inhibits cell cycle progression in anti-Thy 1 glomerulonephritis. Int J Mol Med 2004;13:629-36.
38. Nakashima H, Aoki M, Miyake T, Kawasaki T, Iwai M, Jo N *et al.* Inhibition of experimental abdominal aortic aneurysm in the rat by use of decoy oligodeoxy-nucleotides suppressing activity of nuclear factor kappa-B and ets transcription factors. Circulation 2004;109:132-8.
39. Weintraub SJ, Prater C, Dean DC. Retinoblastoma protein switches the E2F site from positive to negative element. Nature 1992;358:259-61.
40. Nakamura H, Aoki M, Tamai K, Oishi M, Ogihara T, Kaneda Y *et al.* Prevention and regression of atopic dermatitis by ointment containing NF-kB decoy oligodeoxynucleotides in NC/Nga atopic mouse model. Gene Ther 2002;9:1221-9.
41. Kawamura I, Morishita R, Tomita N, Lacey E, Aketa M, Tsujimoto S *et al.* Intratumoral injection of oligonucleotides to the NF kappaB binding site inhibits cachexia in a mouse tumor model. Gene Ther 1999;6:91-7.
42. Sharma HW, Perez JR, Higgins-Sochaski K, Hsiao R, Narayanan R. Transcription factor decoy approach to decipher the role of NF-kappa B in oncogenesis. Anticancer Res 1996;16:61-9.
43. Griesenbach U, Cassady RL, Cain RJ, du Bois RM, Geddes DM, Alton EW. Cytoplasmic deposition of NFkappaB decoy oligonucleotides is insufficient to inhibit bleomycin-induced pulmonary inflammation. Gene Ther 2002;9:1109-15.
44. D'Acquisto F, Ialenti A, Ianaro A, Di Vaio R, Carnuccio R. Local administration of transcription factor decoy oligonucleotides to nuclear factor-kappaB prevents carrageenin-induced inflammation in rat hind paw. Gene Ther 2000;7:1731-7.
45. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A *et al. In vivo* transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. Nat Med 1997;3:894-9.
46. Kupatt C, Wichels R, Deiss M, Molnar A, Lebherz C, Raake P *et al.* Retroinfusion of NfkappaB decoy oligonucleotide extends cardioprotection achieved by CD18 inhibition in a preclinical study of myocardial ischemia and retroinfusion in pigs. Gene Ther 2002;9:518-26.
47. Sakaguchi T, Sawa Y, Fukushima N, Nishimura M, Ichikawa H, Kaneda Y *et al.* A novel strategy of decoy transfection against nuclear factor-kappaB in myocardial preservation. Ann Thorac Surg 2001;71:624-9.
48. Suzuki J, Morishita R, Amano J, Kaneda Y, Isobe M. Decoy against nuclear factor-kappa B attenuates myocardial cell infiltration and arterial neointimal formation in murine cardiac allografts. Gene Ther 2000;7:1847-52.
49. Ueno T, Sawa Y, Kitagawa-Sakakida S, Nishimura M, Morishita R, Kaneda Y *et al.* Nuclear factorkappaB decoy attenuates neuronal damage after global brain ischemia: a future strategy for brain protection during circulatory arrest. J Thorac Cardiovasc Surg 2001;122:720-7.
50. Penolazzi L, Lambertini E, Borgatti M, Piva R, Cozzani M, Giovannini I *et al.* Decoy oligodeoxynucleotides targeting NF-kappaB transcription factors: induction of apoptosis in human primary osteoclasts. Biochem Pharmacol 2003;66:1189-98.
51. Penolazzi L, Magri E, Lambertini E, Calò G, Cozzani M, Siciliani G *et al.* Local in vivo administration of a decoy oligonucleotide targeting NF-kappaB induces apoptosis of osteoclasts after application of orthodontic forces to rat teeth. Int J Mol Med 2006;18:807-11.
52. Monte MW, Pan M, Starbuck M, Gallo EM, Deng L III, Karsenty G *et al.* Calcineurin/NFAT signaling in Osteoblasts regulates bone mass developmental Cell 2006;10, 771-82.
53. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 2003;17:2205-32.
54. Asagiri M, Sato K, Usami T, Ochi S, Nishina H, Yoshida H *et al.* Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med 2005;202:1261-9.
55. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H *et al.* Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signalling in terminal differentiation of osteoclasts. Dev Cell 2002;3:889-901.
56. Kim Y, Sato K, Asagiri M, Morita I, Soma K, Takayanagi H,

Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis. J Biol Chem 2005;280:32905-13.
57. Matsumoto M, Kogawa M, Wada S, Takayanagi H, Tsujimoto M, Katayama S *et al.* Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1. J Biol Chem 2004;279: 45969-79.
58. Anusaksathien O, Laplace C, Li X, Ren Y, Peng L, Goldring SR *et al.* Tissue-specific and ubiquitous promoters direct the expression of alternatively spliced transcripts from the calcitonin receptor gene. J Biol Chem 2001;276:22663-74.
59. Takayanagi H. Mechanistic insight into osteoclast differentiation in osteoimmunology. J Mol Med 2005;83:170-9.
60. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K *et al.* NFAT and Osterix cooperatively regulate bone formation. Nat Med 2005;11:880-5.
61. Zhu L, Zaidi S, Moonga BS, Troen BR, Sun L. RANK-L induces the expression of NFATc1, but not of NFkB subunits during osteoclast formation. Bioch Bioph Res Comm 2004;326:131-5.
62. Kaminuma O, Deckert M, Elly C, Liu YC, Altman A. Vav-Rac1-mediated activation of the c-Jun N-terminal kinase/c-Jun/AP-1 pathway plays a major role in stimulation of the distal NFAT site in the interleukin-2 gene promoter. Mol Cell Biol 2001;21:3126-36.
63. Cozzani M, Giovannini I, Naccari R, Penolazzi L, Lambertini E, Borgatti M *et al.* Transcription Factor Decoy (TFD) as a novel approach for the control of osteoclastic resorption. Prog Orthod 2005;6:238-47.
64. Piva R, Penolazzi L, Lambertini E, Giordano S, Gambari R. Induction of apoptosis of human primary osteoclasts treated with a transcription factor decoy mimicking a promoter region of estrogen receptor alpha. Apoptosis 2005;10:1079-94.
65. Piva R, Penolazzi L, Zennaro M, Bianchini E, Magri E, Borgatti M *et al.* Induction of apoptosis of osteoclasts by targeting transcription factors with decoy molecules. Ann N Y Acad Sci 2006;1091:509-16.
66. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997;89:747-54.
67. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K *et al.* Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755-64.
68. Komori T. Regulation of skeletal development by the Runx family of transcription factors. J Cell Biochem 2005;95:445-53.
69. Stein GS, Lian JB, van Wijnen AJ, Stein JL, Montecino M, Javed A *et al.* Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. Oncogene 2004;23:4315-29.
70. Barski A, Frenkel B. ChIP Display: novel method for identification of genomic targets of transcription factors. Nucleic Acids Res 2004;32:e104.
71. Stock M, Schafer H, Fliegauf M, Otto F. Identification of novel genes of the bone-specific transcription factor Runx2. J Bone Miner Res 2004;19:959-72.
72. Kousteni S, Bellido T, Plotkin LI, O_Brien CA, Bodenner DL, Han L *et al.* Nongenotropic, sex-nonspecific signalling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 2001;104:1-20.
73. Zhou S, Zilberman Y, Wassermann K, Bain SD, Sadovsky Y, Gazit D. Estrogen modulates estrogen receptor alpha and beta expression, osteogenic activity, and apoptosis in mesenchymal stem cells (MSCs) of osteoporotic mice. J Cell Biochem Suppl 2001;(Suppl 36):144-55.
74. Gohel A, McCarthy MB, Gronowicz G. Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts in vivo and in vitro. Endocrinology 1999;140:5339-47.
75. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K *et al.* The nuclear receptor superfamily: the second decade. Cell 1995;83:835-9.
76. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889-95.
77. Green S, Chambon P. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet 1988;4:309-14.
78. Komm BS, Terpening CM, Benz DJ, Graeme KA, Gallegos A, Korc M *et al.* Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells. Science 1988;241:81-4.
79. Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC *et al.* Evidence of estrogen receptors in normal human osteoblast-like cells. Science 1988;241:84-6.
80. Zallone A. Direct and indirect estrogen actions on osteoblasts and osteoclasts. Ann N Y Acad Sci 2006;1068:173-9.
81. Kos M, Reid G, Denger S, Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. Mol Endocrinol 2001;15:2057-63.
82. Lambertini E, Penolazzi L, Giordano S, Del Senno L, Piva R. Expression of the human oestrogen receptor-α gene is regulated by promoter F in MG-63 osteoblastic cells. Biochem J 2003;372:831-9.
83. Lambertini E, Penolazzi L, Tavanti E, Schincaglia GP, Zennaro M, Gambari R *et al.* Human estrogen receptor alpha gene is a target of Runx2 transcription factor in osteoblasts. Exp Cell Res 2007;313:1548-60.
84. Tijsterman M, Ketting RF, Plasterk RHA. The genetics of RNA silencing. Annu Rev Genet 2002;36:489-19.
85. Denli AM, Hannon GJ. RNAi: an ever-growing puzzle. Trends Biochem Sci 2003;28:196-201.

Apoptosis of Human Primary Osteoclasts Treated with Molecules Targeting Nuclear Factor-κB

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Osteoclasts (OCs) are involved in several pathologies associated with bone loss, including rheumatoid arthritis, osteoporosis, bone metastasis of myeloma, osteosarcoma, and breast cancer. In this review we determined the effects of natural compounds, including extracts from medicinal plants, on differentiation and survival of human primary OCs obtained from peripheral blood. We found that OCs from umbilical cord blood and peripheral blood behave differently in response to molecules inducing apoptosis in this experimental system. Apoptosis induced by decoy oligonucleotides was reproducibly obtained in OCs from peripheral blood but not in OCs derived from cord blood. With respect to effects of medicinal plants, we found that crude extracts of *Emblica officinalis* are able to induce specifically programmed cell death of mature OCs without altering the process of osteoclastogenesis. *E. officinalis* specifically increased the expression levels of Fas, a critical member of the apoptotic pathway. Gel shift experiments BioPharmaNet demonstrate that *E. officinalis* extracts specifically compete with the binding of a transcription factor involved in osteoclastogenesis NF-κB to its specific target DNA sequences. This might explain the observed effects of *E. officinalis* on the expression levels of IL-6, an NF-κB-specific target gene. We suggest the application of natural products as an alternative tool for therapy applied to bone diseases.

Key words: **osteoclast; rheumatoid arthritis; osteoporosis; apoptosis; medicinal plants; *Emblica officinalis***

Introduction

Osteoclasts (OCs) are multinucleated cells of hematopoietic origin and are the primary bone-resorbing cells.[1–4] OCs are involved in several pathologies associated with bone loss, including rheumatoid arthritis, osteoporosis, bone metastasis of myeloma, osteosarcoma, and breast cancer. Accordingly, molecules inducing apoptosis of OCs but not in on osteoblasts are expected to be of great interest for therapeutic intervention in pathologies caused by bone loss.

In this respect, we recently reported[5,6] and reviewed[7] the effects of transcription factor decoy oligonucleotides on apoptosis of human OCs. The first decoy molecules were designed to inhibit nuclear factor (NF)-κB binding[5] to a target sequence, the second to increase estrogen receptor-α (ER-α) expression.[6] We found that both decoy molecules are potent inducers of apoptosis of human OCs and are able to increase caspase 3 activity and decrease IL-6

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Natural Compounds and Their Role in Apoptotic Cell Signaling Pathways: Ann. N.Y. Acad. Sci. 1171: 448–456 (2009). doi: 10.1111/j.1749-6632.2009.04906.x

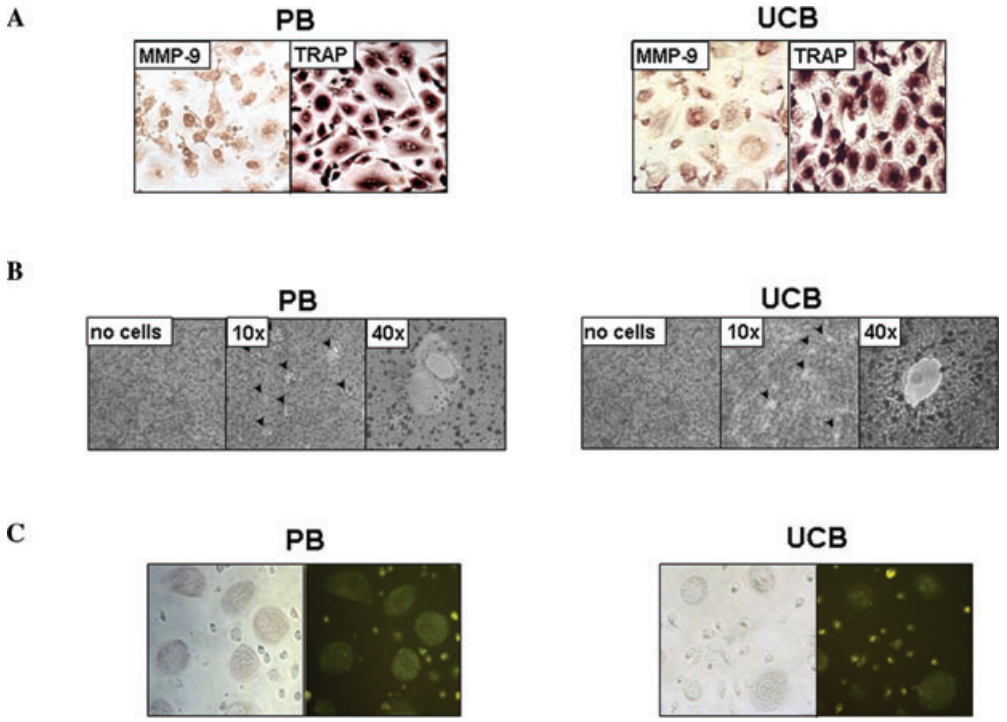

Figure 1. Phenotypical and functional characterization of osteoclasts (OCs)/peripheral blood (PB) and OCs/umbilical cord blood (UCB). (**A**) Human primary OCs obtained after 21 days of culture from PB or UCB were immunostained for matrix metallopeptidase 9 and stained for tartrate-resistant acid phosphatase (TRAP) to enumerate the attached TRAP-positive multinucleated cells. Cells were photographed at a magnification of 20×. (**B**) The pit formation ability of osteoclasts obtained from PB or UCB was assayed using culture dishes that were bottom coated with calcium-phosphate film [Osteoclast Activity Assay Substrate (OAAS), Oscotect Inc., Seoul, Korea]. Cells were cultured in contact with the OAAS plate and removed with a solution of 5% sodium hypochlorite on the day 21. Bone resorption activity was measured by direct observation under phase-contrast microscopy. (**C**) Oligonucleotide uptake ability of OCs/PB and OCs/UCB. The cells were incubated for 3 h with 1 μg of fluorescein-labeled 20-mer double-stranded oligonucleotide complexed with 4 μg of LipofectAMINE (Invitrogen, Carlsbad, CA), washed, and examined under phase-contrast or fluorescent light, using a fluorescent filter.

expression. In addition, we provided evidence indicating that these oligonucleotides are active *in vivo* in inducing OC apoptosis.[8,9] Because OCs are essential for skeletal development and remodeling throughout the life of animals and humans, the described approach is of potential clinical importance.

From these data two further issues were lacking. The first one was the possible differential effects of the employed decoy oligonucleotide OC derived from different histological compartments. The second one was to extend the observation of the induction of apoptosis to other molecules exhibiting inhibitory effects on NF-κB activity.

Osteoclasts Derived from Umbilical Cord Blood Are Less Prone to Apoptosis than Osteoclasts from Peripheral Blood

It is well documented that for *in vitro* analysis human OCs could be obtained from the precursors present in peripheral blood (PB) or umbilical cord blood (UCB), but there has been no

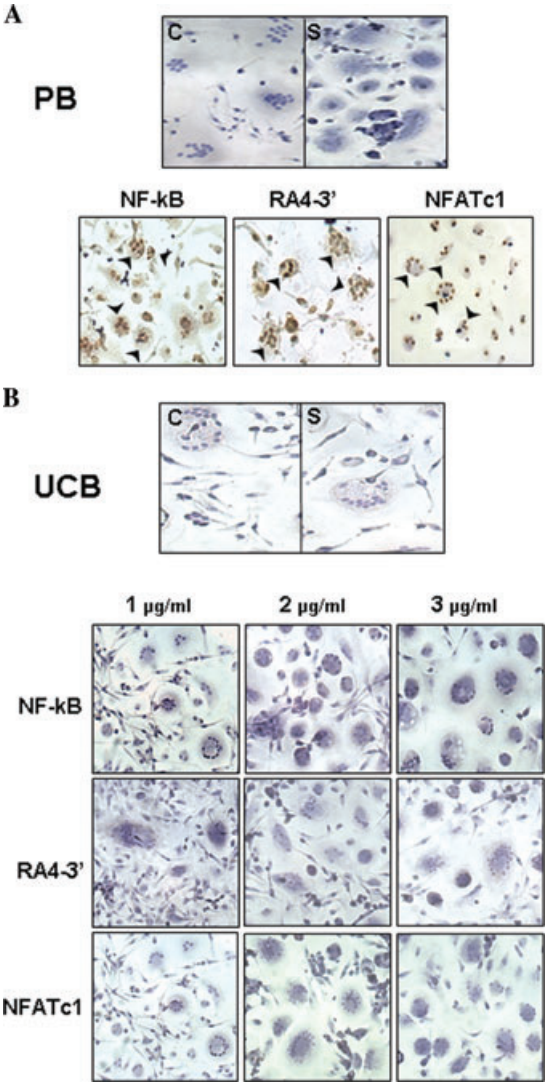

Figure 2. Treatment of OCs/PB and OCs/UCB with specific transcription factor decoys. In presence of different decoy molecules able to rectuit NF-κB, NFATc1 or a transcription factor that negatively affects ERα expression (RA4-3′). The on apoptosis of OCs obtained from PB (**A**) or UCB (**B**) was evaluated after 14 days of culture. Detection of apoptosis was performed by TdT-mediated dUTP nick end labeling (TUNEL) assay, after 48 h 1 µg/mL decoy treatment. OCs/UCB were also treated with 2 and 3 µg/mL administered every 48 h for 6 days. Brown color reaction (arrows in the panels) indicates cells that underwent apoptosis. Cells were photographed at a magnification of 20×. C, untreated control OCs; S, OCs treated with unrelated scramble oligonucleotide.

detailed analysis of how the kind of source may affect the behavior of these cells. In the present study we analyzed the behavior of OCs after transfection with specific transcription factor decoy molecules AGAINST NF-κB or NFATc1 (nuclear factor of activated T cell cytoplasmic.

Phenotypical and Functional Characterization of OCs/PB and OCs/UCB

PB and UCB-derived mononuclear cells were set in culture as previously described.[10] Briefly, PB was collected from healthy normal volunteers after informed consent. Full-term UCB samples ($n = 10$) were obtained from the umbilical vein immediately after vaginal delivery, with the informed consent of the mother approved by the Ethical Committee of Ferrara University and S. Anna Hospital. PB and UCB mononuclear cells (PBMCs and CBMCs) were plated in 24-well plates or in chamber slides and then cultured in Dulbecco's MEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 10 U/mL streptomycin for 14 days. On day 14 the PB-derived OCs generated in the presence of 10% fetal bovine serum (FBS), parathyroid hormone (PTH) 10^{-7} mol/L, macrofage colony-stimulating factor (M-CSF) (25 ng/mL), and receptor activator of NF-κB ligand (RANKL; 30 ng/mL) were multinucleated tartrate-resistant acid phosphatase (TRAP)-positive cells and expressed matrix metallopeptidase 9 (MMP-9) OC-associated antigen, confirming the cell phenotype at the molecular level (panel A, Fig. 1).[5,6] The same characteristics were shown by spontaneously generated UCB-derived OCs. In order to compare the different activity of OCs from different origins, we tested their function in terms of bone resorption ability. As shown in panel B of Figure 1, resorption lacunae were detected at a comparable level in OCs from the two groups. These results indicate that the source of OCs did not affect pit formation capability, confirming the functionality

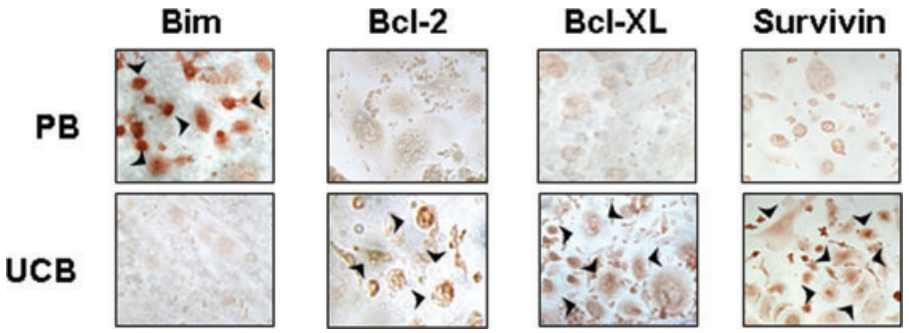

Figure 3. Expression of proapoptotic and survival proteins in OCs/PB and OCs/UCB. Control cells were immunocytochemically analyzed for basal expression of Bim, Bcl2, Bcl-XL, and survivin. Cells were photographed at a magnification of 20×. Arrowheads indicate immunopositivity.

of OCs/UCB and OCs/PB. Moreover, the two types of OCs retain comparable DNA uptake ability, as shown in a representative experiment indicated in panel C, Figure 1. 20-Mer fluorescein-labeled double-stranded oligonucleotides are widely distributed in all nuclei of OCs/PB and OCs/UCB.

Effect of Transcription Factor Decoy on OCs/PB and OCs/UCB

Following these preliminary results, we treated OCs/PB and OCs/UCB with specific decoy oligonucleotides (ODN) able to remove NF-κB or NFATc1 transcription factors or increase ER-α expression, and we then analyzed the level of apoptosis. Apoptotic cells were detected by the DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI), according to the manufacturer's instructions. Measurement of apoptosis was calculated as a percentage of apoptotic nuclei (dark-brown nuclei) versus total nuclei of multinucleated TRAP-positive cells evaluated in three independent experiments. Even if they showed a comparable transfection efficiency, the effect of the decoy treatment in the OCs/UCB was quite different from that observed in OCs/PB (Fig. 2A, B). As shown in Figure 2 (panel A), OCs/PB were mostly apoptotic because they were TdT-mediated dUTP nick end labeling (TUNEL) positive; in contrast OCs/UCB did not exhibit features characteristic of programmed cell death after the same decoy treatment (panel B, Fig. 2).

The Expression of Survival Proteins in OCs/UCB

To further study the different sensitivity to apoptotic stimuli, the basal expression of Bim, Bcl-2, Bcl-XL, and survivin was tested. Bim is a pro-apoptotic member of the Bcl-2 family, and it has been recently showed that regulation of its levels is critical for controlling OC apoptosis.[11] For this kind of study an immunocytochemistry analysis was performed employing the streptavidin–biotin method using an Ultraystain polyvalent-HRP (horseradish peroxidase) immunostaining kit (Ylem, Italy).[5] As shown in Figure 3, OCs/PB, but not OCs/UCB, are strongly immunopositive for Bim. The resistance to apoptosis may be ascribed to the presence of anti-apoptotic factors, including Bcl-2, Bcl-XL, and survivin,[12–14] for which we observed a strong staining in OCs/UCB but not in OCs/PB (Fig. 3). Therefore, our data support the possibility that the loss of prosurvival Bcl-2 and the presence of pro-apoptotic Bim might be critical in driving OC/PB apoptosis and that, conversely, the presence of Bcl-2, Bcl-XL, and survivin in OCs/UCB plays a critical role in blocking OC apoptosis and promoting OC survival.

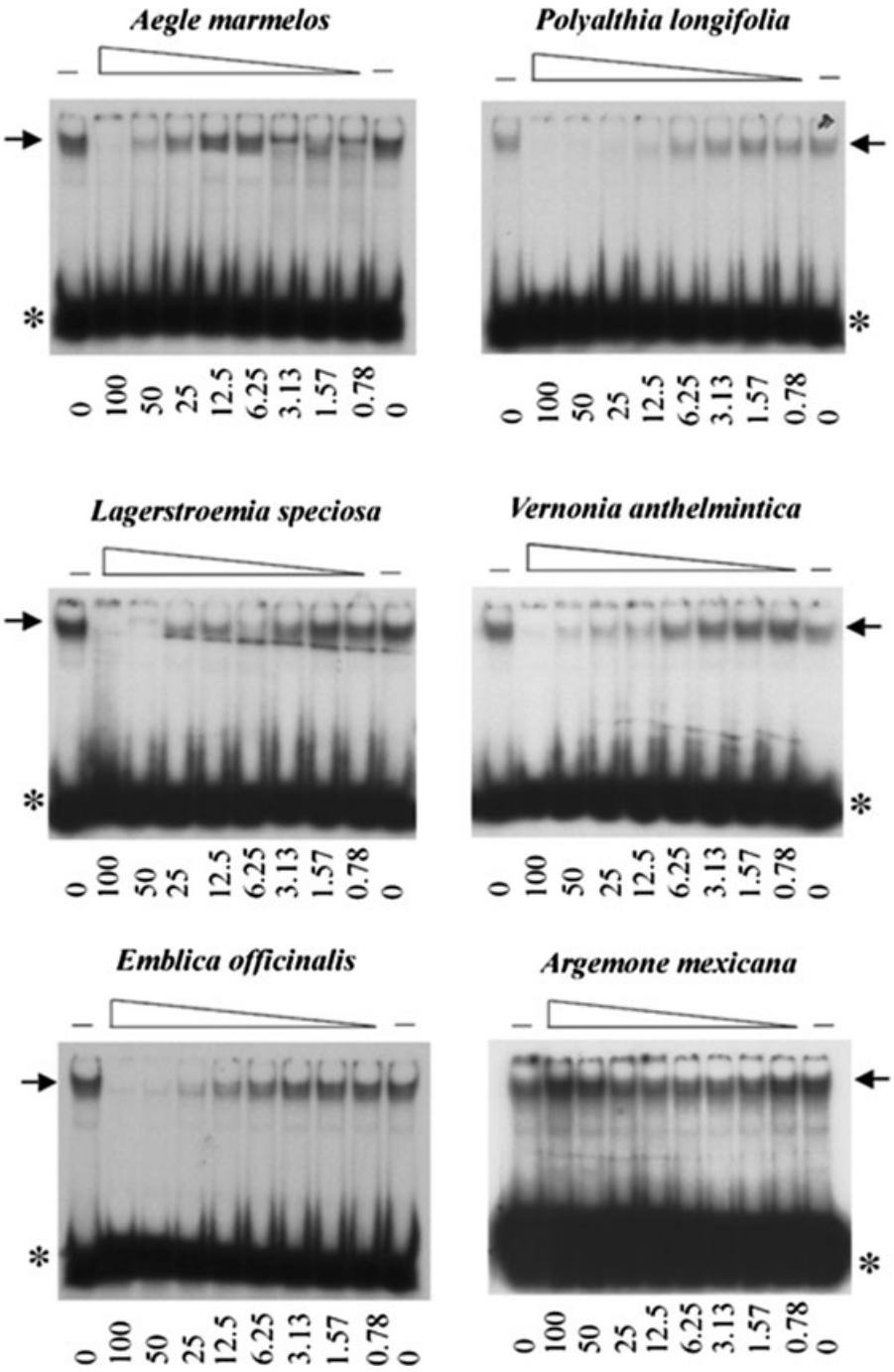

Figure 4. Extracts from medicinal plants differentially inhibit molecular interactions between NF-κB and target DNA sequences. The effects on NF-κB–DNA interactions of the indicated amounts of extracts (μg/binding reaction) from *Aegle marmelos, Polyalthia longifolia, Lagerstroemia speciosa, Vernonia anthelmintica, Emblica officinalis,* and *Argemone mexicana* were studied by electrophoretic mobility shift assay. *, free probe; arrows, NF-κB–DNA complexes.

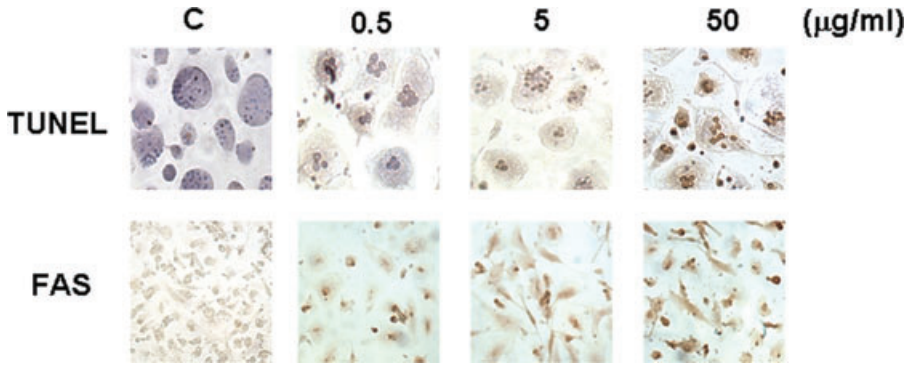

Figure 5. Detection of apoptosis by TUNEL assay in human primary osteoclasts. The presence of apoptotic OCs after treatment with 0.5, 5, and 50 μg/mL of *E. officinalis* extract for 60 h is shown. Brown color reaction indicates cells that underwent apoptosis. In the lower panel, the immunocytochemical analysis of FAS receptor expression levels in human primary OCs subjected to the same experimental conditions is reported. C, control cells; cells were photographed at a magnification of 20×.

Inhibitors of NF-κB Activity from the Natural World: *Emblica officinalis* Extracts

Recent results support the concept that some medicinal plants and natural products derived from them are of great interest in developing therapeutic strategies against bone disorders, including rheumatoid arthritis and osteoporosis.[15–20] For instance, Yin *et al.* demonstrated that water extracts of *Dioscorea spongiosa* stimulate osteoblast proliferation, exhibiting at the same time a potent inhibitory activity on osteoclastogenesis.[21] Moreover, fruit extracts of *Psoralea corylifolia*[22] and *Cnidium monnieri*[23] exhibit osteoblastic proliferation stimulating activity in osteoblast-like UMR106 cells *in vitro*. Several plant extracts inhibiting OC differentiation also display strong anti-inflammatory properties.[21–23]

Among medicinal plants, *Emblica officinalis* is certainly of interest.[21–32] This medicinal plant has played an important medicinal role for centuries in the Indian system of medicine. Fruits of *E. officinalis* are used for the treatment of a number of diseases, such as dyslipidemia[24] and atherosclerosis,[25] as hepatoprotective,[26] antibacterial,[27] and anti-inflammatory agents.[28] In many cases *E. officinalis* has been shown to be a potent free-radical scavenging agent, thereby preventing carcinogenesis and mutagenesis; for this purpose hypotheses to determine the mechanism of its action have been suggested.[29]

In Vitro Effects of *E. officinalis* Extracts on NF-κB Transcription Factor Activity

The transcription factor NF-κB is involved in inflammation and it plays a critical role in OC differentiation, regulating the expression of a large number of OC-specific genes. The ability of *E. officinalis* extracts to interfere with NF-κB binding properties was investigated. *E. officinalis* extracts were incubated in the presence of 5 μg of K562 cell nuclear extracts with an oligonucleotide containing a *cis* element of the long terminal repeat of HIV-1, representing the DNA binding site for NF-κB. DNA–protein interactions were then evaluated by electrophoretic mobility shift assay.

Representative results are shown in Figure 4; full results are reported by Lampronti *et al.*[33] High inhibitory activity of *E. officinalis* was found in comparison was performed with extracts from *Oroxylum indicum*, *Santalum yasi*,

Cuscuta reflexa, Argemone mexicana, Cassia sophera, Paederia foetida, Hygrophila auriculata, and *Ocimum sanctum*.[33] Extracts showing high activity on NF-κB interactions were also those derived from *Aegle marmelos, Moringa oleifera*, *Vernonia anthelmintica*, *Rumex maritimus*, *Lagerstroemia speciosa, Hemidesmus indicus*, *Polyalthia longifolia*, *Terminalia arjuna*, *Saraca asoka*, and *Aphanamixis polystachya* (Fig. 4).[28]

E. officinalis Extracts Induce Osteoclast Apoptosis

A TUNEL test was performed on OCs from peripheral blood after exposure for up to 60 h to quantities of 0.5, 5, and 50 μg/mL of *E. officinalis* extract. As shown in Figure 5, a low but significant level of apoptosis (20%) was induced by 0.5 μg/mL of extract, while at 5 and 50 μg/mL a dramatic increase (50% and 98%, respectively) in TUNEL-positive nuclei was observed. Times of exposure shorter than 60 h were also tested (24 and 48 h) without obtaining significant differences from untreated cells (data not shown). These results were confirmed by immunocytochemical analysis of FAS receptor expression. FAS receptor is a well-known apoptosis-related protein and, as shown in Figure 5, its expression increased in OCs treated with *E. officinalis* extracts at all three concentrations used.

Conclusions

Induction of apoptosis of OCs could be an important strategy for not only interfering with rheumatoid arthritis complications of the bone skeleton that result in joint destruction but also preventing and reducing osteoporosis.

In the present review we report data suggesting the use of primary cultures of human OCs (hOCs) from PB as a good tool to test the potential use of *E. officinalis* fruit extracts in the therapy of human pathologies associated with bone loss. In fact *E. officinalis* extracts significantly induce apoptosis in hOCs as a result of specific inhibition of NF-κB activity. In this context, the possible use of natural products, including plant extracts and nutriaceuticals, is under debate.

In addition, our experimental data indicate that different susceptibility to apoptosis between OCs differentiated from hematopoietic precursor cells present in PB and UCB exists. Because OCs are extensively employed to investigate cell response to therapies for the treatment of bone loss associated with several diseases, we conclude that the source of potential OC precursors used for *in vitro* analysis may influence the cell response to biological modifiers. This may be important in future therapeutic designs for limiting the number of bone resorbing cells and for testing the effects of antiresorptive agents.

Acknowledgments

R.G. is funded by the Consorzio Interuniversitario di Biotecnologie, Italy, Associazione Italiana Ricerca sul Cancro, Associazione Veneta per la Lotta alla Talassemia, Italy, SPINNER e PRIITT (EU, Obiettivo 2), Fondazione Cassa di Risparmio di Padova e Rovigo, the STAMINA Project of Ferrara University, and the UE ITHANET Project. This work was supported by grants from the Italian Cystic Fibrosis Research Foundation (grant # 15/2004).

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Szekanecz, Z. & A.E. Koch. 2007. Macrophages and their products in rheumatoid arthritis. *Curr. Opin. Rheumatol.* **19:** 289–295.
2. Hadjidakis, D.J. & I.I. Androulakis. 2006. Bone remodeling. *Ann. N. Y. Acad. Sci.* **1092:** 385–396.
3. Giuliani, N., F. Morandi, S. Tagliaferri & V. Rizzoli. 2006. Targeting pathways mediating bone disease. *Curr. Pharm. Biotechnol.* **7:** 423–429.

4. Blair, H.C. & M. Zaidi. 2006. Osteoclastic differentiation and function regulated by old and new pathways. *Rev. Endocr. Metab. Disord.* **7:** 23–32.
5. Penolazzi, L., E. Lambertini, M. Borgatti, *et al*. 2003. Decoy oligodeoxynucleotides targeting NF-kappaB transcription factors: induction of apoptosis in human primary osteoclasts. *Biochem. Pharmacol.* **66:** 1189–1198.
6. Piva, R., L. Penolazzi, E. Lambertini, *et al*. 2005. Induction of apoptosis of human primary osteoclasts treated with a transcription factor decoy mimicking a promoter region of estrogen receptor alpha. *Apoptosis* **10:** 1079–1094.
7. Piva, R., L. Penolazzi, M. Zennaro, *et al*. 2006. Induction of apoptosis of osteoclasts by targeting transcription factors with decoy molecules. *Ann. N. Y. Acad. Sci.* **1091:** 509–516.
8. Penolazzi, L., E. Magri, E. Lambertini, *et al*. 2006. Local in vivo administration of a decoy oligonucleotide targeting NF-kappaB induces apoptosis of osteoclasts after application of orthodontic forces to rat teeth. *Int. J. Mol. Med.* **18:** 807–811.
9. Piva, R., L. Penolazzi, E. Lambertini, *et al*. 2005. Induction of apoptosis of human primary osteoclasts treated with a transcription factor decoy mimicking a promoter region of estrogen receptor alpha. *Apoptosis* **10:** 1079–1094.
10. Matsuzaki, K., K. Katayama, Y. Takahashi, *et al*. 1999. Human osteoclast-like cells are formed from peripheral blood mononuclear cells in a coculture with SaOS-2 cells transfected with the parathyroid hormone (PTH)/PTH-related protein receptor gene. *Endocrinology* **140:** 925–932.
11. Akiyama, T., P. Bouillet, T. Miyazaki, *et al*. 2003. Regulation of osteoclast apoptosis by ubiquitylation of proapoptotic BH3-only Bcl-2 family member Bim. *EMBO J.* **22:** 6653–6664.
12. Strasser, A., L. O'Connor & V.M. Dixit. 2000. Apoptosis signaling. *Annu. Rev. Biochem.* **69:** 217–245.
13. Sevilla, L., A. Zaldumbide, F. Carlotti, *et al*. 2001. Bcl-XL expression correlates with primary macrophage differentiation, activation of functional competence, and survival and results from synergistic transcriptional activation by Ets2 and PU.1. *J. Biol. Chem.* **276:** 17800–17807.
14. Tamm, I., Y. Wang, E. Sausville, *et al*. 1998. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res.* **58:** 5315–5320.
15. Putnam, S.E., A.M. Scutt, K. Bicknell, *et al*. 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. *Phytother. Res.* **21:** 99–112.
16. New, S.A. 2003. Intake of fruit and vegetables: implications for bone health. *Proc. Nutr. Soc.* **62:** 889–899.
17. Hidaka, S., Y. Okamoto, Y. Yamada, *et al*. 1999. A: Japanese herbal medicine, Chujo-to, has a beneficial effect on osteoporosis in rats. *Phytother. Res.* **13:** 14–19.
18. Funk, J.L., J.N. Oyarzo, J.B. Frye, *et al*. 2006. Turmeric extracts containing curcuminoids prevent experimental rheumatoid arthritis. *J. Nat. Prod.* **69:** 351–355.
19. Ramprasath, V.R., P. Shanthi & P. Sachdanandam. 2006. Curative effect of Semecarpus anacardium Linn. nut milk extract against adjuvant arthritis – with special reference to bone metabolism. *Chem. Biol. Interact.* **160:** 183–192.
20. Sohail, M.T., M.I. Chaudhry, M.K. Usman, *et al*. 2005. Efficacy and tolerance of atrisin in degenerative and inflammatory joint disorders. *Phytother. Res.* **19:** 365–368.
21. Yin, J., Y. Tezuka, K. Kouda, *et al*. 2004. Antiosteporotic activity of the water extract of *Dioscorea spongiosa*. *Biol. Pharm. Bull.* **27:** 583–586.
22. Wang, D., F. Li & Z. Jiang. 2001. Osteoblastic proliferation stimulating activity of *Psoralea corylifollia* extracts and two of its flavonoids. *Planta Med.* **67:** 748–749.
23. Meng, F., Z. Xiong, Y. Sun & F. Li. 2004. Coumarins from *Cidium monnieri* (L.) and their proliferation stimulating activity on osteoblast-like UMR106 cells. *Pharmazie* **59:** 643–645.
24. Anila, L. & N.R. Vijayalakshmi. 2002. Flavonoids from *Emblica officinalis* and Mangifera indica, effectiveness for dyslipidemia. *J. Ethnopharmacol.* **79:** 81–87.
25. Thakur, C.P. & K. Mandal. 1984. Effect of Emblica officinalis on cholesterol-induced atherosclerosis in rabbits Indian. *J. Med. Res.* **79:** 142–146.
26. Bhattacharya, A., M. Kumar, S. Ghosal & S.K. Bhattacharya. 2000. Effect of bioactive tannoid principles of Emblica officinalis on iron-indued hepatic toxicity in rats. *Phytomedicine* **7:** 173–175.
27. Ahmad, I., Z. Mehmood & F. Mohammad. 1998. Screening of some Indian medicinal plants for their antimicrobial properties. *J. Ethnopharmacol.* **62:** 183–193.
28. Asmawi, M.Z., H. Kankaanranta, E. Moilanen & H. Vapaatalo. 1993. Anti-inflammatory activities of *Emblica officinalis* Gaertn leaf extracts. *J. Pharm. Pharmacol.* **45:** 581–584.
29. Jeena, K.J., G. Kuttan & R. Kuttan. 2001. Antitumor activity of *Emblica officinalis*. *J. Ethnoph.* **75:** 65–69.
30. Sancheti, G., A. Jindal, R. Kumari & P.K. Goyal. 2005. Chemopreventive action of Emblica officinalis on skin carcinogenesis in mice. *Asian Pac. J. Cancer Prev.* **6:** 197–201.
31. Sultana, S., S. Ahmad, N. Khan & T. Jahangir. 2005. Effect of Emblica officinalis (Gaertn) on CCl4 induced hepatic toxicity and DNA synthesis in Wistar rats. *Indian J. Exp. Biol.* **43:** 430–436.

32. Scartezzini, P., F. Antognoni, M.A. Raggi, *et al*. 2006. Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of Emblica officinalis Gaertn. *J. Ethnopharmacol.* **104:** 113–118.

33. Lampronti, I., M.T.H. Khan, M. Borgatti, *et al*. Inhibitory effects of Bangladeshi medicinal plant extracts on interactions between transcription factors and target DNA sequences. *Evid. Based Complement Alternat. Med*. **5:** 303–312.

Cell. Mol. Life Sci. (2009) 66:3641–3653
DOI 10.1007/s00018-009-0149-5

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Slug gene expression supports human osteoblast maturation

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Received: 15 May 2009 / Revised: 17 August 2009 / Accepted: 25 August 2009 / Published online: 11 September 2009

Abstract This study aims to define the function of Slug transcription factor in human normal osteoblasts (hOBs). To date, Slug is considered exclusively a marker of malignancy in bone tissue. Here, we identified, for the first time, a role for Slug in hOBs using a knockdown approach. We demonstrated that Slug is positively correlated with osteoblast markers, including Runx2, osteopontin, osteocalcin, Collagen type 1, Wnt/β-catenin signaling mediators, and mineral deposition. At the same time, Slug silencing potentiates the expression of Sox-9, a factor indispensable for chondrogenic development. These data, with the finding that Slug is in vivo recruited by the promoters of Runx2 and Sox-9 genes, suggest that, in hOBs, Slug may act both as positive and negative transcriptional regulator of Runx2 and Sox-9 genes, respectively. In summary, our results support the hypothesis that Slug functions as a novel regulator of osteoblast activity and may be considered a new factor required for osteoblast maturation.

Electronic supplementary material The online version of this article (doi:10.1007/s00018-009-0149-5) contains supplementary material, which is available to authorized users.

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Keywords Human osteoblasts · Slug · Wnt signaling · Runx2 · Sox-9

Introduction

Osteoblasts, bone producing cells, originate from multipotent mesenchymal stem cells and are responsible for the secretion of the organic extracellular matrix of bone, both during development and later during the remodeling of mature bone [1]. Osteoblast differentiation and activity are regulated by multiple signaling pathways and specific factors that, by influencing gene expression, cell proliferation, and migration, control bone mass homeostasis. Among these factors, Wingless-type proteins (Wnts) and bone morphogenetic proteins (BMP) drive early events, while the helix-loop-helix proteins Twist and Id maintain proliferation [2–5]. Subsequently, transcriptional regulation of osteoblast differentiation, maturation, and activity is mainly due to key regulators including Runx2, Msx1 and 2, Osterix, NFAT2, c-fos, c-jun, ATF4, fra-2, jun D, Dlx3 and 5 [5–12]. In recent years, the knowledge of the molecular mechanisms that drive the function of these factors in the regulation of bone cell biology has greatly improved; nevertheless, many aspects have not yet been investigated. In particular, the intricate cross-talk among the different pathways is only partially known. In addition, the expression and function of the previously mentioned factors in osteoblasts have been extensively analyzed in experimental animal models, tumor cells, or cellular lines, whereas data about these molecules in human primary osteoblasts cultures are very scarce.

Accumulated evidence has shown that lymphocyte enhancer binding factor 1/T cell factor (LEF1/TCF) transcription factors, the nuclear effectors of the Wnt/β-catenin

signaling pathway, influence osteoblast proliferation, function, and regeneration, enhancing expression of Runx2, the master transcription regulator of osteoblasts [13]. Nevertheless, most downstream bone-specific target genes of this pathway are only partially known. Among these, Slug has been recently implicated in osteosarcoma progression as a Wnt-responsive molecule strongly correlated with a loss of tumor suppressors such as E-cadherin [14, 15]. We recently demonstrated that Slug gene is also expressed in normal human osteoblasts, and Lef/Tcf cis elements present on its promoter act in regulating its transcription through a direct binding of Lef-1, TCF-1, and TCF-4 in vivo (submitted manuscript).

Human Slug, also named Snail 2, belongs to the Snail family of genes encoding zinc-finger transcription factors [14]. It is expressed at different stages of development in different tissues, and mediates epithelial–mesenchymal transition [14–16]. Moreover, Slug is involved in a broad spectrum of biological functions, such as cell differentiation, cell motility, cell-cycle regulation, and apoptosis [17]. Slug is also expressed in most normal adult human tissues, but little is known about its potential functions.

In order to identify new potential osteoblast-specific proteins, in the current study we investigated the role of Slug in relation to the expression of Wnt/β-catenin signaling mediators and bone-related genes in human mature osteoblasts (hOBs). For this purpose, we evaluated the effects of Slug gene knockdown on osteoblast maturation by using siRNA strategy. In addition, in order to correlate the expression level of Slug and its activity in regulating the transcription of genes that are crucial for osteoblast differentiation, we investigated Slug in vivo occupancy of the E boxes regulatory sites present in the Runx2 and Sox-9 gene promoters.

Our findings describe an unknown regulatory function of Slug and provide clear evidence for a pivotal role of Slug in regulating osteoblast maturation in human.

Materials and methods

Isolation and culture of osteoblasts and chondrocytes

Bone tissues were harvested from different patients undergoing total knee replacement for osteoarthritis (mean ± SD age 65 ± 10 years). The study was approved by the Istituto Ortopedico Rizzoli (Bologna, Italy) ethical committee and informed consent was obtained from each patient. Trabecular bone was obtained from the inner portion of the tibial plateau. Bone chips were removed from the tibial plateau, collected in a V-glass tube containing 1.5 ml of 1:1 mixture of DMEM/Ham's F12 K no calcium (Gibco, Invitrogen, Paisley, Scotland, UK) and supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), 25 µg/ml ascorbic acid, 4 mM glutamine (Sigma–Aldrich, St. Louis, MO, USA) and 2 mM calcium chloride (referred to as enzyme medium) as previously reported [18] and according to the methods described by Robey and Termine [19].

Primary chondrocytes were obtained from patients with osteoarthritis undergoing knee arthroplasty. The chondrocytes were isolated from minced tissue by sequential enzymatic digestion as previously described [20].

Immunocytochemistry

A sample of 10^4 human osteoblasts (at passage 2) were seeded in 8-well chamber slide and allowed to adhere for 96 h. Human osteoblasts were fixed in 4% PFA for 20 min at room temperature and then hydrated with TBS 1% BSA for 5 min at room temperature. The slides were incubated with monoclonal antibodies anti-human -CD45 (Dako Cytomation, Glostrup, Denmark), -CD146 (Nocastra, Newcastle, UK), -CD105 (produced from the hybridoma cell line, clone SH2; ATCC, Rockville, MD), -Runx-2, -osteocalcin, (all purchased from R&D Systems, Minneapolis, MN), -alkaline phosphatase, -collagen type 1 (both obtained from DSHB, Department of Biological Sciences, Iowa city, IA), -bone sialoprotein, (Fisher Scientific,Pittsburg, PA, USA), -estrogen receptor alpha (Upstate Biotechnology, Lake Placid, NY), and -collagen type 2 (Millipore, 900 Middlesex Tpk Billerica, USA) for 1 h at room temperature. The slides were washed three times with TBS 1% BSA and then sequentially incubated with multilinker biotinilated secondary antibody and alkaline phosphatase-conjugated streptavidin (Kit BioGenex, San Ramon, CA, USA) at room temperature for 20 min. The slides were developed using fast red as substrate, counterstained with haematoxylin, mounted with glycerol jelly, and evaluated in a brightfield microscope. Negative and isotype matched controls were performed. Positive cells were manually counted by two evaluators blinded to marker evaluated. For each well, we randomly selected 20 fields at high magnification (×40). Results were expressed as the percentage of positive cells on the total number of cells counted.

Analysis of osteoblast activity

For alkaline phosphatase staining, the Alkaline phosphatase (ALP) Leukocyte kit (Sigma) was used. To perform the test, prefixed mono-layered cells were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (AMPD). The presence of sites of ALP activity appeared as blue cytoplasmatic staining.

ALP activity was evaluated in hOBs by the hydrolysis of *p*-nitrophenylphosphate (PNPP). The enzyme activity, expressed as μmol/min/μg of protein, was evaluated 6 days after siRNA/Slug2 treatment. One unit was defined as the amount of enzyme which hydrolyzed 1 nmol/PNPP per minute.

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma) in the cells cultured for up to 21 days in osteogenic medium consisting in DMEM, high-glucose, supplemented with 10% FBS, 10 mM *β*-glycerophosphate, 0.1 mM dexamethasone, and 50 mM ascorbate. In the committed cells, the osteogenic medium was changed every 3 days as well as RNA interference treatment where indicated. The cells were then fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature, washed five times with deionized water, and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope. Matrix mineralization was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer ("Quantity one" software, Biorad). The surface and the number of all mineralized nodules were quantified in 2 wells per condition at day 14 and 21 of culture.

Small interfering RNA (siRNA) transfection

Three sets of Stealth RNAi duplexes and corresponding Stealth control were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). Stealth RNAi compounds are 25 mer dsRNA containing proprietary chemical modifications that enhance nuclease stability and reduce off-target effects.

The following Stealth RNAi sequences were used: siRNA/Slug1 sense: 5′-CCGUAUCUCUAUGAGAGUUACUCCA-3′, antisense: 5′-UGGAGUAACUCUCAUAGAGAUACGG-3′; siRNA/Slug2 sense: 5′-CCCUGGUUGCUUCAAGGACACAUUA-3′, antisense: 5′-UAAUGUGUCCUUGAAGCAACCAGGG-3′; siRNA/Slug3 sense: 5′-GGCUCAUCUGCAGACCCAUUCUGAU-3′, antisense: 5′-AUCAGAAUGGGUCUGCAGAUGAGCC-3′.

The most effective fragments used for targeting human Slug were siRNA/Slug2.

Twenty-four hours before siRNA transfection, hOBs were seeded in triplicate at density of $16 \times 10^3/cm^2$ in DMEM with 10% FBS. Cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturer's instructions. Transfected cells were incubated for 6 days at 37°C before gene silencing analysis. As a negative control for the siRNA treatment, Medium GC Stealth RNAi Negative Control Duplex (Invitrogen) was used. Knockdown of Slug expression was verified by Real-Time RT-PCR.

Real-time quantitative RT-PCR

Cells from three wells were harvested and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and as previously described [21]. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). TaqMan technology, the Assays-On-Demand kit for human Slug, Lef-1, *β*-catenin, Runx2, Sox-9, OPN, OC, Col1a1, RANKL, and c-myc, were used. The mRNA levels of target genes were corrected for GAPDH mRNA levels (endogenous control). All PCR reactions were performed in triplicate for each sample and were repeated three times. All experimental data were expressed as the mean ± SEM.

Western blotting

For western blot analysis, the cells were washed twice with ice-cold PBS and cell lysates were prepared as previously reported [22]. Then, 10 μg of each sample was electrophoresed on a 12% SDS-polyacrylamide gel. The proteins were then transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, USA). After blocking with PBS-0.05% Tween 20 and 5% dried milk, the membrane was probed with the following antibodies: Slug (L40C6) from Cells Signaling Technology (Danvers, CA, USA), Runx2 (sc-10758) and Sox-9 (sc-20095), from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Lef-1 (L7901) from Sigma Aldrich, IP3 K (06-195), and Active-*β*-Catenin (05-665) from Upstate Biotechnology (Lake Placid, NY).

After washing with PBS-Tween, the membranes were incubated with peroxidase-conjugated anti-rabbit antibody (1:50000) or anti-mouse (1:2000) (Dako, Glostrup, Denmark) in 5% non-fat milk. Immunocomplexes were detected using Supersignal West Femto Substrate (Pierce, Rockford, IL, USA). Anti-IP(3)K was used to confirm equal protein loading.

Osteocalcin assay

Osteocalcin secretion was measured in cell culture supernatants collected from osteoblasts plated in 24-well dishes and cultured with D-MEM, 10% FCS in presence or in absence of 30 nM siRNA/Slug2 for 6 days. Prior to measurements, the media were collected, centrifuged at 1,300*g* for 5 min, and tested by using a human osteocalcin enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer's instructions (DRG Diagnostics, Germany).

Osteocalcin levels were corrected with total protein content and expressed as nanograms per micrograms of cell protein and each treatment was performed in duplicate.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described [21] using the standard protocol supplied by Upstate Biotechnology with their ChIP assay reagents.

The cells were cross-linked with 1% formaldehyde for 10 min at 37°C, washed in ice-cold PBS, and suspended in SDS lysis buffer for 10 min on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors, and precleared with 80 µl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 5 µg of anti- Slug, (sc-10436) (Santa Cruz Biotechnology), overnight at 4°C. Immunocomplexes were mixed with 80 µl of DNA-coated protein A-agarose followed by incubation for 1 h at 4°C. Beads were collected and sequentially washed 5 times with 1 ml each of the following buffers: low salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH-8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-pH 8.1), and TE buffer. The immunocomplexes were eluted two times by adding a 250-µl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$ and the cross-linking reactions were reversed by incubation at 65°C for 4 h. Further, the samples were digested with proteinase K (10 mg/ml) at 42°C for 1 h, DNA was recovered by phenol/chloroform extractions, ethanol precipitated using 1 µl of 20 mg/ml glycogen as the carrier, and suspended in sterile water. For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (input). PCR was performed to analyze the presence of DNA precipitated by Slug-specific antibody, and by using specific primers (Table 1) to amplify fragments of the Runx2 and Sox-9 gene promoters.

Each PCR reaction was performed with 5 µl of the bound DNA fraction or 2 µl of the input. The PCR was performed as follows: preincubation at 95°C for 5 min, 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 62°C, and 1 min at 72°C, with one final incubation at 72°C for 5 min. No-antibody control was included in each experiment.

Statistical analysis

Data are presented as the mean ± SEM from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance and the Student's *t* test. A P value <0.05 was considered statistically significant.

Table 1 Immunocytochemical analysis of human primary osteoblasts (hOBs)

Markers	Percentage of positive cells				
	hOB1	hOB2	hOB3	hOB4	hOB5
CD45	Negative	Negative	Negative	Negative	Negative
ALP	5	15	15	12	15
CD146	2	2	4	2	4
CD105	100	100	100	100	100
Col1a1	50	60	60	60	60
Runx2	80	80	80	80	80
BSP	100	100	100	100	100
OC	60	80	90	100	80
ER-α	100	100	100	100	100

Results

Phenotypical characterization and osteogenic potential of human osteoblasts

Human primary osteoblast cultures (hOBs) were generated from bone chips removed from the tibial plateau as previously described [18] and as reported in "Materials and methods". We first analyzed a panel of nine phenotypic markers in cells at the second passage in culture. All osteoblasts were highly positive for the typical osteogenic markers, including Runx2, collagen type 1 (Col1a1), bone sialoprotein (BSP), and osteocalcin (OC), and weakly positive to ALP. In addition, the cells were positive for estrogen receptor alpha (ERα), a protein that is known to be associated to osteoblast differentiation. The samples were negative for a typical hematopoietic marker (CD45), only partially positive for a mesenchymal marker such as CD146, and positive for CD105. After this analysis, the cells that we used may be considered mature osteoblasts because they express low levels of CD146 and ALP, and high levels of CD105, as previously reported [18]. The percentage of positive cells for the markers analyzed by immunocytochemistry in five hOB samples is shown in Table 1, and the immunocytochemical staining of a representative sample is reported in Fig. 1a. These immunocytochemical data have also been confirmed by FACS analysis (data not shown). Next, the cells were characterized for their osteogenic capacity. All hOBs exhibited an evident extracellular matrix mineralization after 21 days of culture under osteogenic conditions (a representative sample is shown in Fig. 1b).

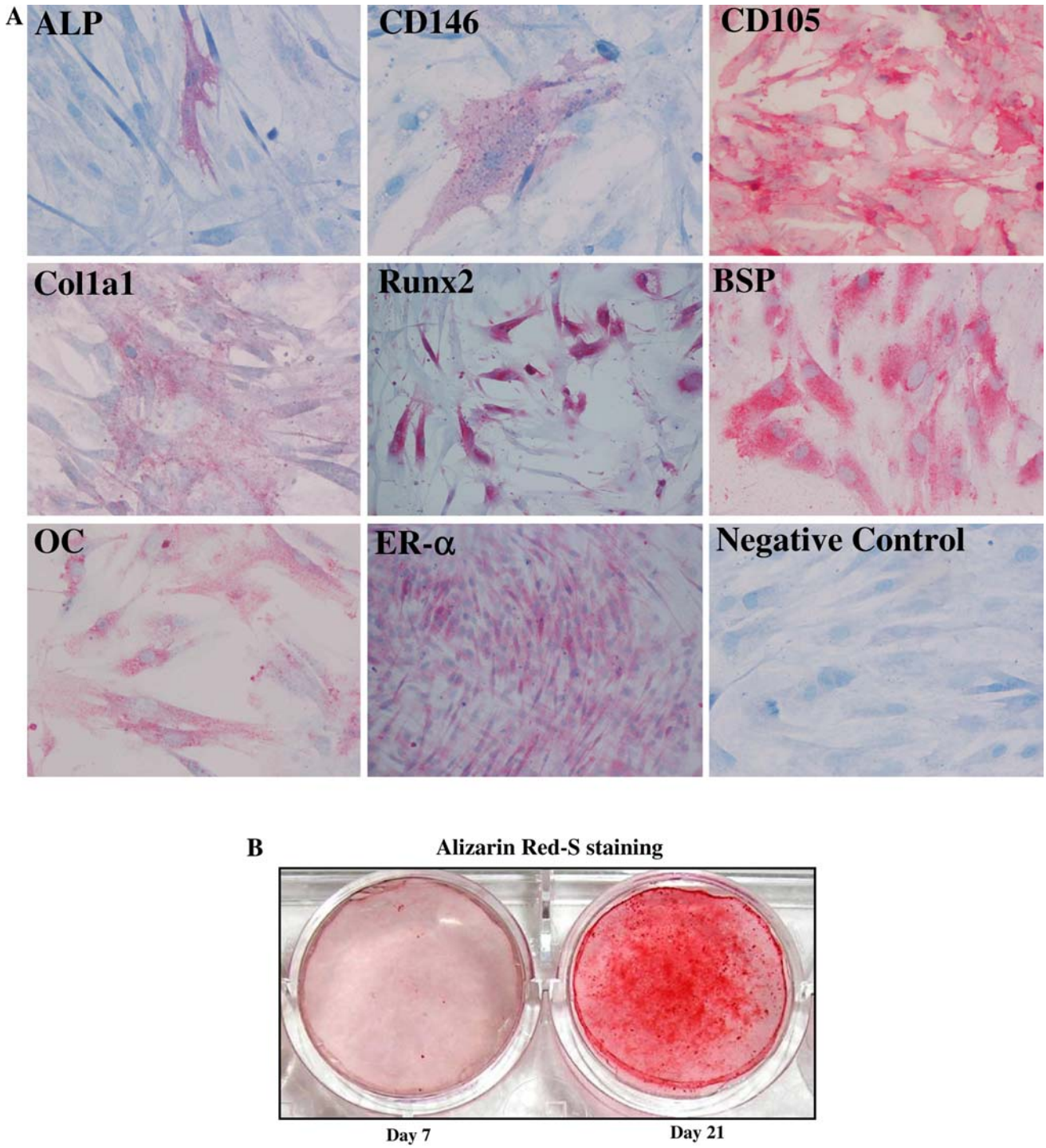

Fig. 1 Phenotypical characterization of hOBs. Five hOB samples were subjected to immunocytochemical analysis for ALP, CD146, BSP, OC, CD105, Col1a1, Runx2, and ER-α phenotypical markers. **a** The staining showed the local expression of the markers analyzed in a representative sample (×20 magnification). **b** The formation of extracellular matrix by cells treated with β-glycerophosphate, ascorbic acid, and dexamethasone. Mineral formation was examined by Alizarin Red S staining. The deposition of calcium salts was observed in osteogenic cultures at day 21

Slug knockdown and Wnt signaling

We firstly confirmed by western blot analysis on whole cell extracts (Fig. 2a) that all hOB samples express Slug protein at comparable levels, approximately the same that were found in Slug-positive MDA-MB-231 breast cancer cells [23]. Then, to test whether Slug expression may be correlated with osteoblast phenotype, we used a Slug

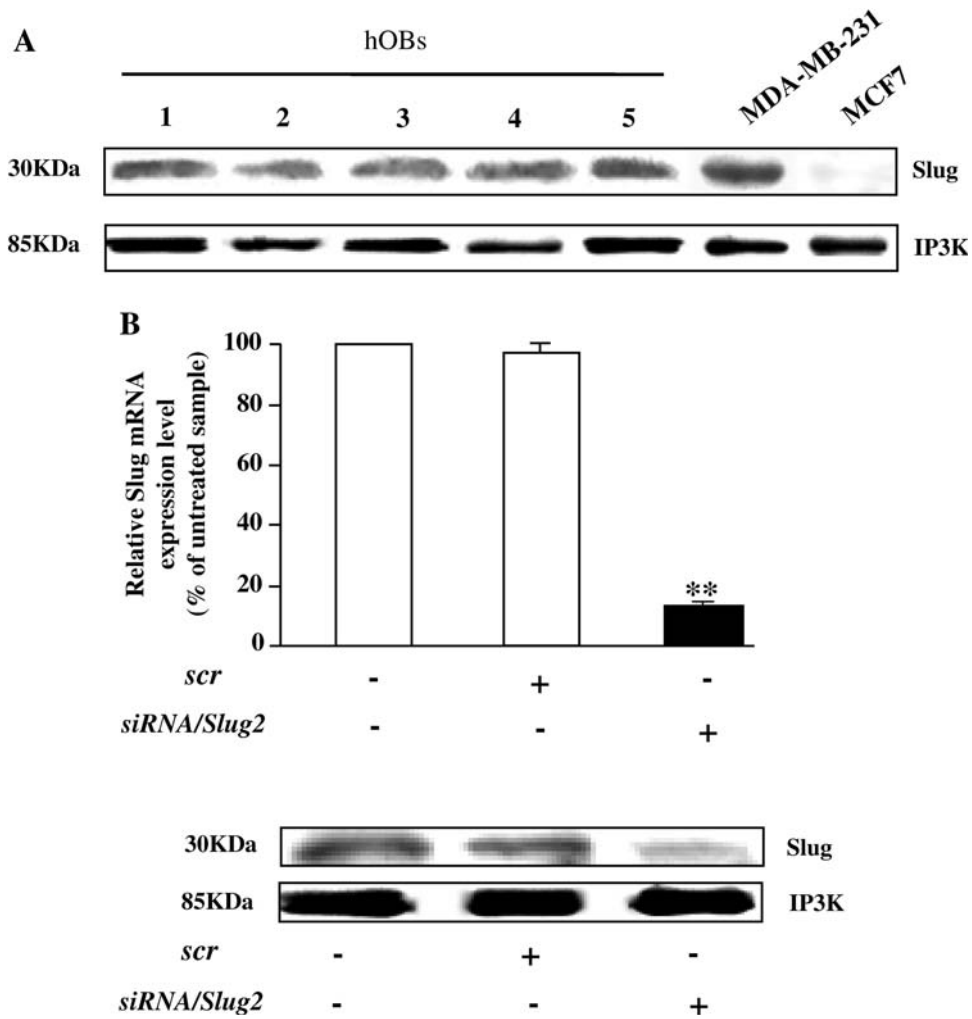

Fig. 2 Silencing of Slug gene expression by siRNA/Slug2 in hOBs. **a** Western blot analysis of endogenous Slug expression in hOBs. 10 μg of whole cell lysates from five hOBs samples were assayed on a 12% SDS-polyacrylamide gel. The proteins were visualized using Supersignal Femto Substrate (Pierce). Size markers are reported (*KDa*). IP3 K was used as a loading control. Positive and negative controls for Slug band (Slug-positive MDA-MB-231 breast cancer cells, and Slug-negative MCF7 breast cancer cells, respectively) are shown. **b** hOBs were transfected with siRNA/Slug2 or a non-relevant siRNA (*scr*). Slug expression was determined both at mRNA and protein level, and revealed by quantitative RT-PCR and western blot analysis, respectively. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug2 over control ratio. Results represent means ± SEM of five hOBs samples (**$P < 0.01$). At the *bottom*, representative western blot of siRNA/Slug2 treated cells shows a specific decrease of endogenous Slug protein level

knockdown approach using siRNA. For this purpose, three siRNAs against Slug were designed, siRNA/Slug1, siRNA/Slug2, and siRNA/Slug3, in order to down-regulate Slug expression. Transfection of hOBs and osteosarcoma SaOS2 cell line with these siRNAs resulted in the down-regulation of Slug transcript by 30% (siRNA/Slug1 and siRNA/Slug3) or 80% (siRNA/Slug2) (electronic supplementary material, Fig. 1). Therefore, we blocked Slug endogenous production treating the cells for 6 days with siRNA/Slug2 30 nM and obtaining a strong inhibition of Slug mRNA and protein, as revealed by quantitative RT-PCR and western blot, respectively (Fig. 2b).

In order to establish a role of Slug in osteoblasts, we analyzed the effects of Slug silencing on the expression of mediators of a central pathway in bone metabolism, such as Wnt signaling.

As shown in Fig. 3a, Slug knockdown significantly reduced the protein levels of two important Wnt mediators, Lef-1 and β-catenin, in all the analyzed samples. Attempts to detect a corresponding reduction of mRNA expression level of Lef-1 and β-catenin met with variable success, and we found approximately the same Lef-1 and β-catenin mRNA levels both in siRNA-transfected cells and in control cells (data not shown). This suggests that, for what concerns these two genes, the regulation mediated by Slug silencing is mainly at the protein level that is independent of mRNA. Therefore, we hypothesized that the levels of Slug may interfere with Wnt signaling modulating the levels of Lef-1 and β-catenin, and that Slug may consequently act by controlling the expression of specific genes in osteoblasts.

In order to strengthen this hypothesis, the expression of downstream target genes of canonical Wnt signaling such as Runx2, Sox-9, osteocalcin, RANKL, and c-myc were examined in Slug silenced cells. As shown in Fig. 3b, the expression of Runx2, the master transcription regulator of osteoblasts previously identified as a Lef-1/β-catenin target gene [13], was markedly decreased in Slug silenced cells compared to untreated osteoblasts. On the contrary, Slug knockdown induced expression of Sox-9, a factor indispensable for chondrogenic development [24], in four out five samples. In this case, the effects of Slug silencing were

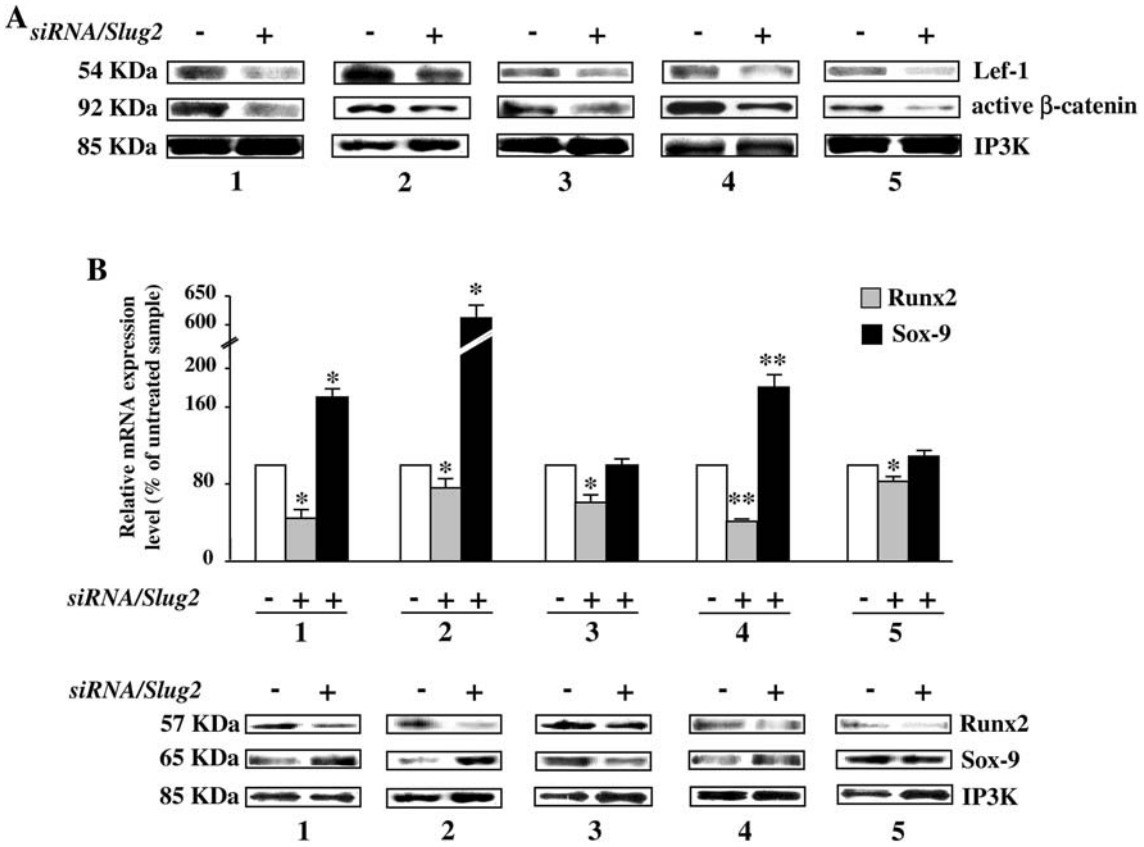

Fig. 3 Effect of Slug interference on Wnt signaling target genes in hOBs. After siRNA/Slug2 treatment the expression of bone-related Wnt target genes was analyzed in five hOB samples. **a** Western blot analysis of Lef-1 and active β-catenin protein levels. Size markers are reported (*KDa*). IP3 K was used as a loading control. **b** Runx2 and Sox-9 expression analysis in siRNA/Slug2 treated cells. mRNA and protein levels were determined by quantitative RT-PCR and western blot analysis, respectively. **c** Osteocalcin, c-myc, and RANKL expression analysis in siRNA/Slug2 treated cells. Osteocalcin protein was measured in cell culture supernatants by ELISA. The level of c-myc and RANKL expression was determined by quantitative RT-PCR. In all quantitative RT-PCR experiments the results, after correction to GAPDH content, are expressed as siRNA/Slug2 over control ratio. Results represent means ± SEM of triplicate determinations ($*P < 0.05$, $**P < 0.01$)

both at mRNA and protein level. Accordingly, in four out five samples, the amount of secreted osteocalcin, which is a marker of late osteoblast differentiation positively modulated by Wnt signaling [25], was significantly reduced by Slug knockdown (Fig. 3c), as well as the expression of c-myc which is induced in response of activation of Wnt signaling [26] (Fig. 3d). On the contrary, mRNA levels of the receptor activator of NFkB ligand (RANKL), the expression of which is repressed by Wnt signaling [27], was significantly increased in the siRNA/Slug2 hOBs-treated samples (Fig. 3e). As a whole, these results suggest that Slug knockdown affects Wnt signaling and consequently its downstream target genes in human osteoblasts.

Slug interacts in vivo with the promoters of Runx2 and Sox-9

Investigations were then performed to address whether Slug may be directly involved in the control of specific gene transcription in hOBs. For this purpose, the promoters of Runx2 and Sox-9 were chosen for chromatin immunoprecipitation (ChIP) analysis. At first, we searched putative Slug binding sites, named E boxes [28], in the promoter regions of human Runx2 and Sox-9 genes by using a program for predicting transcription factor binding sites (TFSEARCH, www.cbrc.jp/research/db/TFSEARCH.html). As shown in Fig. 4, many E boxes were found in the promoter of both genes; their functionality was then investigated, analyzing the in vivo association between Slug and the promoter sequences by ChIP binding assays (Fig. 4). To this aim, human primary osteoblasts were exposed to formaldehyde to cross-link proteins and DNA, and sonicated to fragment the chromatin. Specific antibody against Slug was used to immunoprecipitate the protein–DNA complexes. The presence of the promoter-specific DNA region before immunoprecipitation was confirmed by PCR (input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate

C

Osteocalcin amount (ng/μg total protein ± S.D.)

	1	2	3	4	5
Control	102.52 ± 4.5	36.97 ± 5.49	32.61 ± 4.86	29.71 ± 6.7	38.55 ± 8.1
siRNA/Slug2	78.41 ± 2.86	20.18 ± 1.54	18.87 ± 3.65	15.8 ± 2.19	81.92 ± 2.38

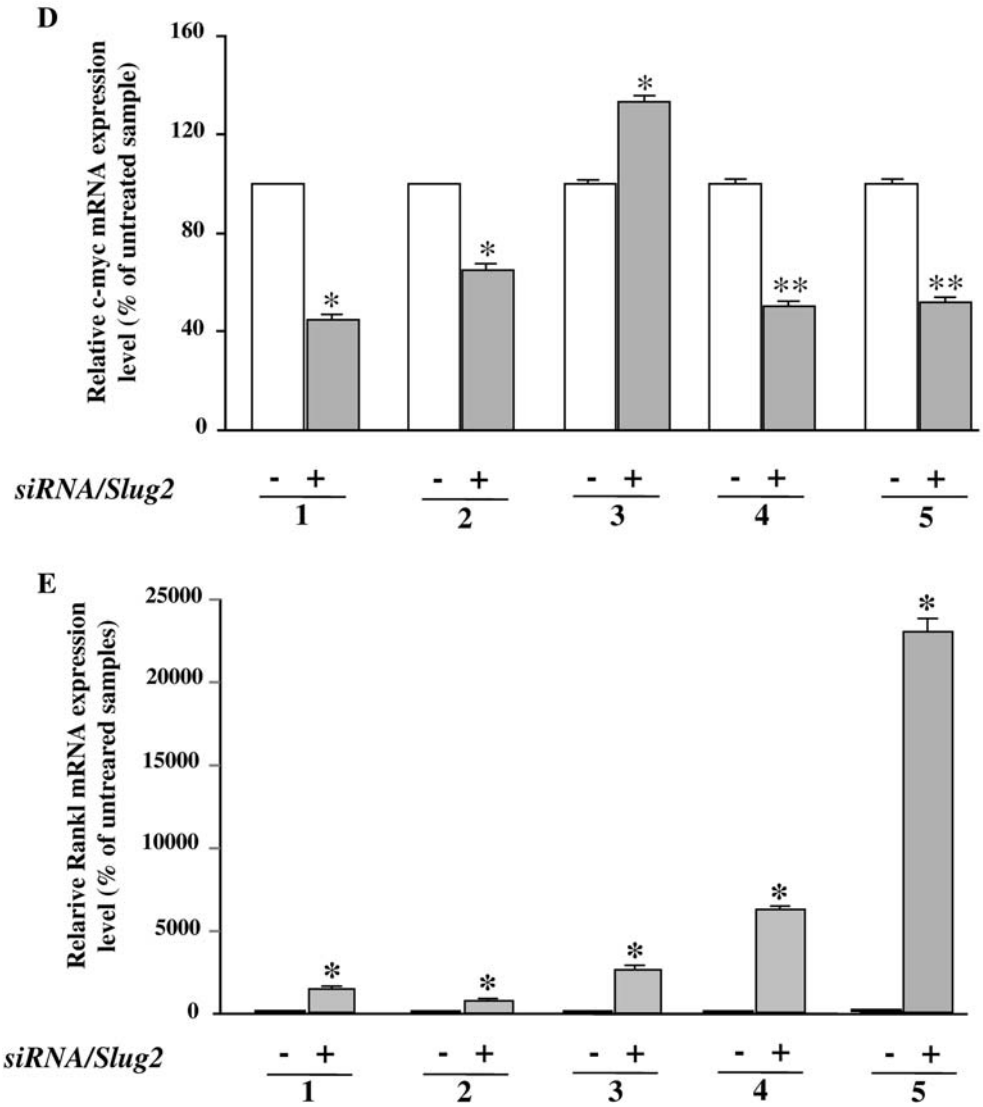

Fig. 3 continued

specific PCR products spanning the putative Slug binding sites from −3,000 bp to +1 bp in the promoter of both genes. Slug recruitment was assessed at the different promoter regions, as indicated in Fig. 4, by using specific sets of primers (Table 2). Slug occupancy was detected at the region 2 and 3 of Runx2 gene (Fig. 4a) and at the region 2 of Sox-9 gene (Fig. 4b).

These ChIP experiments were repeated four times with identical results and demonstrate that both Runx2 and Sox-9 are Slug target genes in human osteoblasts.

Slug knockdown inhibits maturation of osteoblasts and supports differentiation of chondrocytes

Afterwards, the effect of Slug silencing on osteoblast maturation was examined analyzing ALP activity and the formation of mineralized matrix. As shown in Fig. 5a, there was a significative decrease of ALP activity after 6 days of siRNA/Slug2 treatment. Accordingly, Slug silenced cells showed a reduced mineralization ability; in fact, 14 days after the initiation of osteoblast induction in osteogenic

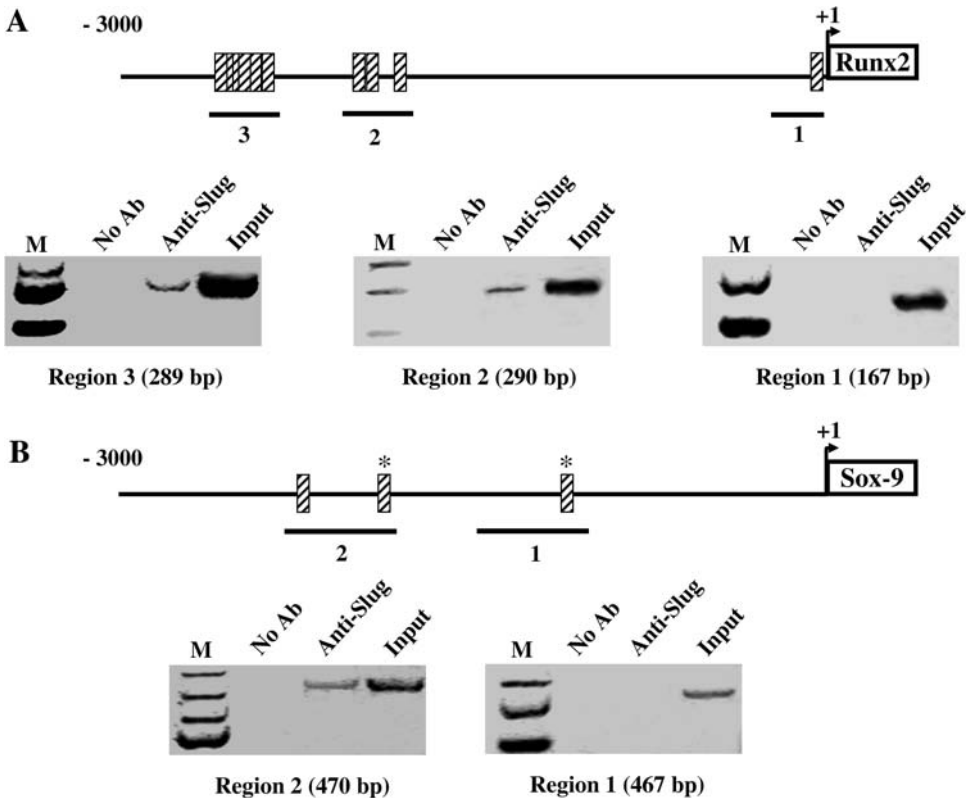

Fig. 4 In vivo recruitment of Slug on Runx2 and Sox-9 gene promoters. Protein–DNA complexes were formaldehyde-cross-linked in hOBs in vivo. Chromatin fragments from these cells were subjected to immunoprecipitation with antibody against Slug. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the primers reported in Table 2, and specific for the indicated promoter regions. PCR fragments were resolved in 1.5% agarose gels. Aliquots of chromatin taken before immunoprecipitation were used as *Input* positive controls whereas chromatin eluted from immunoprecipitations lacking antibody were used as no antibody (*NoAb*) controls. **a** The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the reported regions of Runx2 promoter. **b** The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the reported regions of Sox-9 promoter. The specific molecular weights of PCR fragments are shown in parentheses. The relative positions of Slug putative binding sites (*striped boxes*) are indicated. *Sites showing 100% homology with consensus-binding site (CAGGTG)

Table 2 PCR primers used for chromatin immunoprecipitation assay (ChIP)

Gene	Primer sequences	Product size (bp)
Runx2	Forward F1:5′-ATATCCTTCTGGATGCCAGG-3′	167
	Reverse R1:5′-AAGCACTATTACTGGAGAGGC-3′	
	Forward F2:5′-GTTTCAGTGAATGCTAATGTAG-3′	290
	Reverse R2:5′-AAGCGTTCATTTAACATGCAG-3′	
	Forward F3:5′-CAAGAGCTTTATTTGCATTGAC-3′	282
	Reverse R3:5′-TTGTCCTCTGTGAGGCCTAT-3′	
Sox9	Forward F1:5′-GATAGTGTCCTCACTTCGCA-3′	467
	Reverse R1:5′-TCCACTCTGGCGGAGTCATG-3′	
	Forward F2:5′-CAGCCACCACCATCCAAGTT-3′	470
	Reverse R2:5′-GAAGGGCATTGTGTGTACAG-3′	

medium, a reduction up to 86% of mineral deposition was observed in Slug silenced cells in comparison with non-silenced cells (Fig. 5b). This decrease in osteoblast maturation was accompanied by a significative decrease of other correlated markers including Col1a1 and classical Runx2 targets such as osteopontin and osteocalcin (Fig. 5c).

Since we have here demonstrated that Slug knockdown-induced expression of Sox-9 in hOBs, investigations were

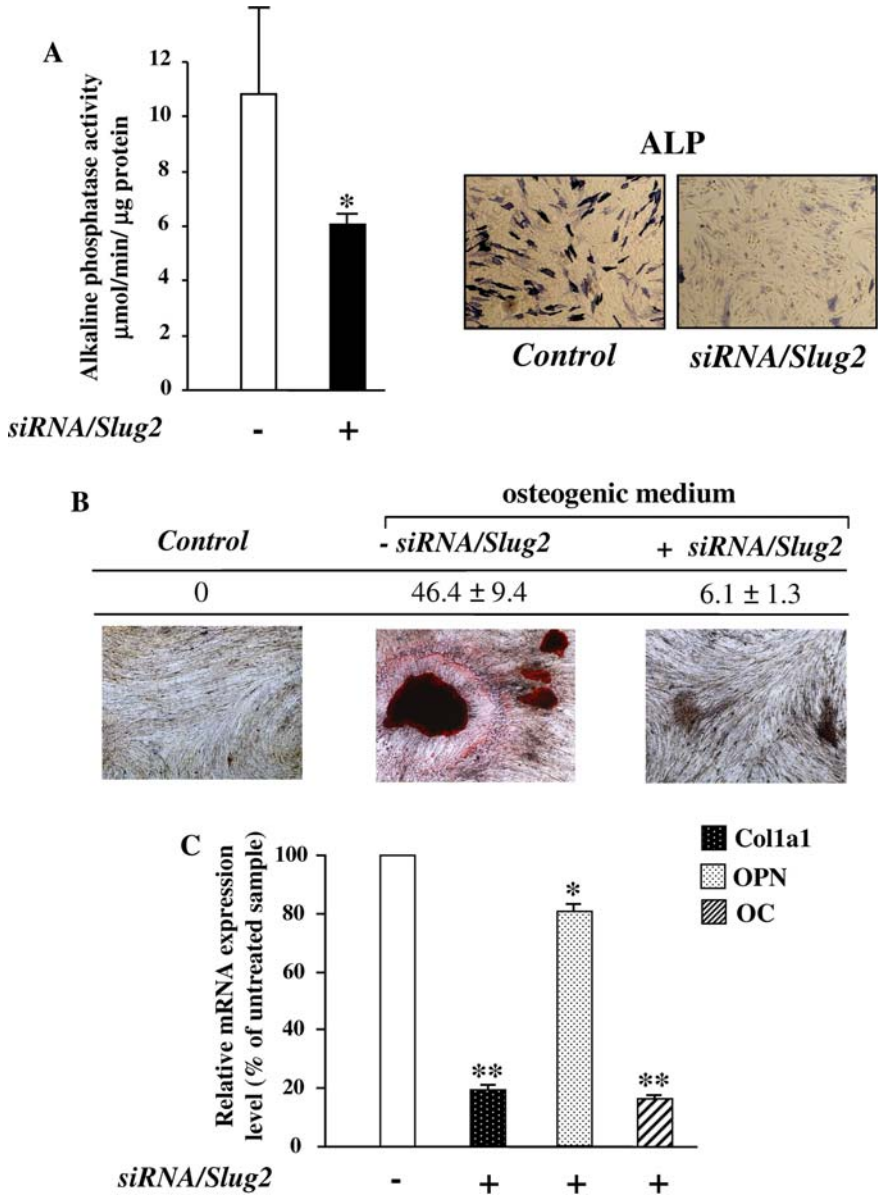

Fig. 5 Effect of Slug interference on osteoblast maturation. After siRNA/Slug2 treatment the cells were analyzed for the presence of alkaline phosphatase activity (*ALP*), formation of mineralized matrix and expression of osteoblast maturation markers. **a** Alkaline phosphatase activity was evaluated by PNPP hydrolysis and ALP Leukocyte kit. The presence of sites of ALP activity appeared as blue cytoplasmic staining as shown in the reported representative sample (×20 magnification). **b** Mineral formation was examined by Alizarin Red S staining in the cells cultured with β-glycerophosphate, ascorbic acid, and dexamethasone (osteogenic medium). The deposition of calcium salts was observed in osteogenic cultures at day 14, but not in control cells (not cultured in osteogenic medium), and was quantified by measuring the number and surface of mineralized nodules using a digital image analyzer ("Quantity one" software, Biorad). The ratio of the surface to the number of nodules in a representative hOB sample is reported. **c** The expression of *Col1a1*, osteopontin (*OPN*) and osteocalcin (*OC*) was determined by quantitative RT-PCR in the cells cultured in osteogenic medium. The results, after correction to GAPDH content, are expressed as siRNA/Slug2 over control ratio. Results represent means ± SEM of triplicate determinations (*$P < 0.05$, **$P < 0.01$)

then performed to evaluate the potential pro-chondrogenic effect of Slug knockdown in human undifferentiated chondrocytes at passage 6–8. In these conditions, the cells express very low level of Collagen type 2 [29]. Slug mRNA expression was blocked in these cells with the same approach and effectiveness as in hOBs (Fig. 6a). Runx2 and Sox-9 expression was then tested. As shown in Fig. 6b, a significative upregulation of both these genes, required for chondrocyte maturation [30], was found in all analyzed samples after Slug silencing. This effect was accompanied by a significant increase of Collagen type 2 evaluated by immunocytochemistry (Fig. 6c).

These preliminary findings demonstrate that when endogenous Slug levels are suppressed chondrocytes are inclined to differentiate, supporting the idea that Slug may play a critical role as pro-chondrogenic factor.

Discussion

To our knowledge, this is the first study to examine the role of Slug on normal human primary osteoblasts. Much is known about the role of Slug during development and its action in malignant progression [14–17, 31]; nevertheless, its expression and function in normal adult tissues has not been elucidated. To date, for what concerns bone tissue, Slug is considered exclusively a marker of malignancy and, consequently, an attractive potential target for therapeutic

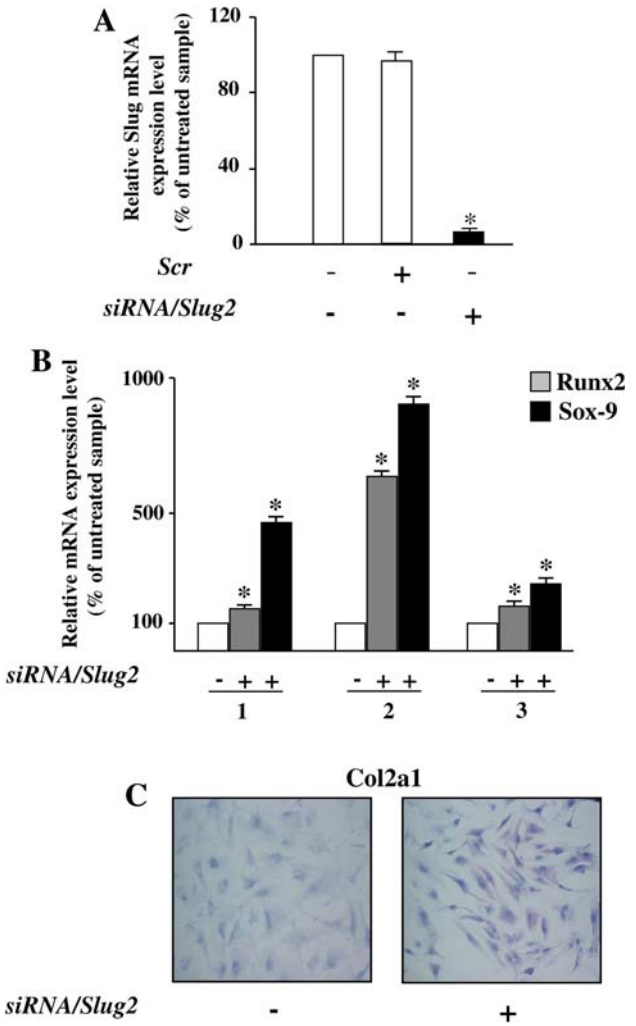

Fig. 6 Silencing of Slug gene expression by siRNA/Slug2 in undifferentiated chondrocytes. **a** Undifferentiated chondrocytes were transfected with siRNA/Slug2 or a non-relevant siRNA (*scr*). Slug expression was determined at mRNA, and revealed by quantitative RT-PCR. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug2 over control ratio. Results represent means ± SEM of three chondrocytes samples ($*P < 0.05$). **b** Runx2 and Sox-9 expression analysis in siRNA/Slug2 treated cells. mRNA levels were determined by quantitative RT-PCR analysis. **c** Collagen type 2 expression analysis in siRNA/Slug2 treated cells. The presence of protein was determined by immunocytochemistry and appeared as violet cytoplasmic staining, as shown in the reported representative sample (×20 magnification)

modulation of bone metastasis and osteosarcoma invasiveness [31], blocking its expression and potentially repressing any detrimental downstream effects.

However, there are no data about Slug expression and regulation in human normal osteoblasts. Here, we identified a role for Slug in normal human osteoblast maturation. Gene expression analysis showed that Slug is positively correlated with osteoblast markers, such as Runx2, and Wnt/β-catenin signaling. A requirement for Slug in osteoblast maturation is supported by Slug knockdown data. In fact, the suppression of Slug mediated by siRNA weakens Wnt/β-catenin signaling, decreases ALP activity, as well as osteoblast mineralization, osteopontin, osteocalcin, and Col1a1, demonstrating that Slug may be considered a novel osteogenic factor. At the same time, Slug silencing does not reflect a global suppression of gene expression. In fact, the expression of RANKL which is suppressed by Wnt signaling in osteoblasts [27] was enhanced in response to Slug knockdown. Furthermore, we observed that Slug silencing potentiates the expression of Sox-9 both in hOBs and in undifferentiated chondrocytes. This is particularly interesting in the context of maturation and differentiation of these cells because Sox-9 is an indispensable factor for chondrogenic development [24, 30] activating cartilage-specific genes, but also acts as a transcriptional repressor for osteoblast differentiation [32] interacting with Runx2 and repressing its function. Therefore, our findings suggest that Slug is required for supporting osteogenic maturation, and its suppression also has a potential pro-chondrogenic effect. Thus, Slug could affect phenotypical changes in response to alteration of its expression levels favoring the dominance of Runx2 function over Sox-9 or vice versa. This is in agreement with several observations demonstrating that, in order to achieve differentiation towards a desired lineage, it is important to direct the stem cell differentiation with correct levels of transcription factors [2, 33]. Additional investigations will be required to understand if Slug plays a role in cell fate determination between chondrocytes and osteoblasts that are derived from common mesenchymal progenitors [34].

Our observation that the Slug gene silencing may decrease Runx2 and increase Sox-9 expression in hOBs was further validated by the in vivo occupancy of the E boxes regulatory sites present in the Runx2 and Sox-9 gene promoters. This suggests that Slug may act, at the same time, both as positive and negative transcriptional regulator of Runx2 and Sox-9 genes, respectively, in human osteoblasts. This supports a role for Slug in maintaining high levels of Runx2 and low levels of Sox-9 to promote osteoblast maturation. Nevertheless, further studies will be needed to characterize the action of Slug on the promoter sequences of these genes, and to establish whether Slug interacts with other transcription factors both in osteoblasts and their precursors.

Several observations indicate that Sox-9 and Runx2 are targets of one of the most crucial signaling for normal skeletogenesis, Wnt/β-catenin pathway [13, 24, 35–38]. Interestingly, we demonstrated that the levels of β-catenin and Lef-1 proteins, two important mediators of Wnt/β-catenin signaling, correlate with Slug expression levels and significantly decrease in Slug silenced cells. Consistent with these findings, we have recently shown that Slug gene expression is positively regulated by Lef-1 which directly binds to Lef/Tcf cis elements present on its promoter (submitted manuscript).

Previous studies have established that Wnt/β-catenin signaling events are mediated by the existence of a large signalosome in which inputs from Wnt signaling, steroid receptors, BMPs, and kinases converge to induce differentiation of osteoblast precursors [39, 40]. However, there are still many unresolved issues about osteoblast regulation through these different pathways. In this scenario, Slug might represent a new regulation factor required for human osteoblast differentiation and maturation. Recent data indicate that, in addition to Slug, another member of the zinc-finger Snail family, Snail1, is involved in bone cell differentiation, but with opposite effect [41]. In fact, in this study, the authors demonstrated that Snail1 controls bone mass by repressing the transcription of Runx2 and vitamin D receptor genes in murine osteoblasts. This suggests that Snail proteins may be involved in the complex dinamics of osteoblast differentiation and maturation processes through different mechanisms. Alternatively, it is plausible that the effects of a transcription factor on the regulation of bone specific genes are different in murine and human cells, and depend on different moments of osteoblastic maturation. This concept may explain the different results from another group demonstrating that Snail enhances expression of osteoblast markers in the MC3T3-E1 cell line [42]. In this study, in addition to Snail, the authors investigated the contribution of many helix-loop-helix (HLH) transcription factors such as Twist proteins that compete with all the Snail factors for the same E-box motifs on the target genes. In agreement with several lines of evidence, the authors indicate that Twist proteins inhibit osteoblast differentiation by interfering with Runx2 function. The evidence that we here obtained on Slug in human osteoblasts supports the conclusions of this study, revealing that the integrated activities of negative and positive E-box-related regulatory factors may control osteoblast differentiation. Therefore, even if the gene regulatory networks are highly intricate, it is possible that further characterization of the role of Snail together with HLH transcriptional regulators in human bone differentiation may provide new insights into the discovery of new molecular targets to use in bone repair and engineering [43].

Acknowledgments This research was supported by grants from MIUR COFIN-2007, Regione Emilia Romagna, Programma di Ricerca Regione Universita' 2007–2009, the Fondazione Cassa di Risparmio di Padova e Rovigo. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

References

1. Karsenty G, Wagner EF (2002) Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2:389–406
2. Soltanoff CS, Yang S, Chen W, Li YP (2009) Signaling networks that control the lineage commitment and differentiation of bone cells. Crit Rev Eukaryot Gene Expr 19:1–46
3. Hartmann C (2006) A Wnt canon orchestrating osteoblastogenesis. Trends Cell Biol 16:151–158
4. Wu X, Shi W, Cao X (2007) Multiplicity of BMP signaling in skeletal development. Ann N Y Acad Sci 1116:29–49
5. Hayashi M, Nimura K, Kashiwagi K, Harada T, Takaoka K, Kato H, Tamai K, Kaneda Y (2007) Comparative roles of Twist-1 and Id1 in transcriptional regulation by BMP signaling. J Cell Sci 120:1350–1357
6. Marie PJ (2008) Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys 473:98–105
7. Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, van Wijnen AJ, Stein JL, Stein GS (2004) Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. Crit Rev Eukaryot Gene Expr 14:1–41
8. Xiao G, Jiang D, Ge C, Zhao Z, Lai Y, Boules H, Phimphilai M, Yang X, Karsenty G, Franceschi RT (2005) Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. J Biol Chem 280:30689–30696
9. Hoffmann HM, Catron KM, van Wijnen AJ, McCabe LR, Lian JB, Stein GS, Stein JL (1994) Transcriptional control of the tissue-specific, developmentally regulated osteocalcin gene requires a binding motif for the Msx family of homeodomain proteins. Proc Natl Acad Sci USA 91:12887–12891
10. Hassan MQ, Javed A, Morasso MI, Karlin J, Montecino M, van Wijnen AJ, Stein GS, Stein JL, Lian JB (2004) Dlx3 transcriptional regulation of osteoblast differentiation: temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. Mol Cell Biol 24:9248–9261
11. Deng ZL, Sharff KA, Tang N, Song WX, Luo J, Luo X, Chen J, Bennett E, Reid R, Manning D, Xue A, Montag AG, Luu HH, Haydon RC, He TC (2008) Regulation of osteogenic differentiation during skeletal development. Front Biosci 13:2001–2021
12. Machwate M, Jullienne A, Moukhtar M, Lomri A, Marie PJ (1995) c- fos protooncogene is involved in the mitogenic effect of transforming growth factor-beta in osteoblastic cells. Mol Endocrinol 9:187–198
13. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 280:33132–33140
14. Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 3:155–166
15. Conacci-Sorrell M, Simcha I, Ben-Yedidia T, Blechman J, Savagner P, Ben-Ze'ev A (2003) Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. J Cell Biol 163:847–857
16. Hajra KM, Chen DY, Fearon ER (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res 62:1613–1618
17. Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: Implications in development and cancer. Development 132:3151–3161
18. Tonnarelli B, Manferdini C, Piacentini A, Codeluppi K, Zini N, Ghisu S, Facchini A, Lisignoli G (2008) Surface-dependent modulation of proliferation, bone matrix molecules, and inflammatory factors in human osteoblasts. J Biomed Mater Res 89:687–696
19. Robey PG, Termine JD (1985) Human bone cells in vitro. Calcif Tissue Int 37:453–460
20. Grigolo B, Lisignoli G, Piacentini A, Fiorini M, Gobbi P, Mazzotti G, Duca M, Pavesio A, Facchini A (2002) Evidence for redifferentiation of human chondrocytes grown on a hyaluronan-based biomaterial (HYAff 11): molecular, immunohistochemical and ultrastructural analysis. Biomaterials 23:1187–1195

21. Lambertini E, Tavanti E, Torreggiani E, Penolazzi L, Gambari R, Piva R (2008) ER alpha and AP-1 interact in vivo with a specific sequence of the F promoter of the human ER alpha gene in osteoblasts. J Cell Physiol 216:101–110
22. Lambertini E, Penolazzi L, Tavanti E, Schincaglia GP, Zennaro M, Gambari R, Piva R (2007) Human estrogen receptor alpha gene is a target of Runx2 transcription factor in osteoblasts. Exp Cell Res 313:1548–1560
23. Tripathi MK, Misra S, Khedkar SV, Hamilton N, Irvin-Wilson C, Sharan C, Sealy L, Chaudhuri G (2005) Regulation of BRCA2 gene expression by the SLUG repressor protein in human breast cells. J Biol Chem 280:17163–17171
24. Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD, de Crombrugghe B (2004) Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev 18:1072–1087
25. Zhu ED, Demay MB, Gori F (2008) Wdr5 is essential for osteoblast differentiation. J Biol Chem 283:7361–7367
26. Katoh M (2008) WNT signaling in stem cell biology and regenerative medicine. Curr Drug Targets 9:565–570
27. Spencer GJ, Utting JC, Etheridge SL, Arnett TR, Genever PG (2006) Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis in vitro. J Cell Sci 119:1283–1296
28. Mittal MK, Myers JN, Misra S, Bailey CK, Chaudhuri G (2008) In vivo binding to and functional repression of the VDR gene promoter by SLUG in human breast cells. Biochem Biophys Res Commun 372:30–34
29. Okazaki K, Li J, Yu H, Fukui N, Sandell LJ (2002) CCAAT/enhancer-binding proteins beta and delta mediate the repression of gene transcription of cartilage-derived retinoic acid-sensitive protein induced by interleukin-1 beta. J Biol Chem 277:31526–31533
30. Komori T (2009) Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res. doi 10.1007/s00441-009-0832-8
31. Guo Y, Zi X, Koontz Z, Kim A, Xie J, Gorlick R, Holcombe RF, Hoang BH (2007) Blocking Wnt/LRP5 signaling by a soluble receptor modulates the epithelial to mesenchymal transition and suppresses met and metalloproteinases in osteosarcoma Saos-2 cells. J Orthop Res 25:964–971
32. Zhou G, Zheng Q, Engin F, Munivez E, Chen Y, Sebald E, Krakow D, Lee B (2006) Dominance of Sox9 function over Runx2 during skeletogenesis. Proc Natl Acad Sci USA 103:19004–19009
33. Davis LA, Zur Nieden NI (2008) Mesodermal fate decisions of a stem cell: the Wnt switch. Cell Mol Life Sci 65:2658–2674
34. Zou L, Zou X, Li H, Mygind T, Zeng Y, Lü N, Bünger C (2006) Molecular mechanism of osteochondroprogenitor fate determination during bone formation. Adv Exp Med Biol 585:431–441
35. Topol L, Chen W, Song H, Day TF, Yang Y (2009) Sox9 inhibits Wnt signaling by promoting beta-catenin phosphorylation in the nucleus. J Biol Chem 284:3323–3333
36. Hamidouche Z, Haÿ E, Vaudin P, Charbord P, Schüle R, Marie PJ, Fromigué O (2008) FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signaling-dependent Runx2 expression. FASEB J 22:3813–3822
37. Dong YF, Soung do Y, Schwarz EM, O'Keefe RJ, Drissi H (2006) Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. J Cell Physiol 208:77–86
38. Day TF, Guo X, Garrett-Beal L, Yang Y (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 8:739–750
39. Kousteni S, Almeida M, Han L, Bellido T, Jilka RL, Manolagas SC (2007) Induction of osteoblast differentiation by selective activation of kinase-mediated actions of the estrogen receptor. Mol Cell Biol 27:1516–1530
40. Bodine PV, Komm BS (2006) Wnt signaling and osteoblastogenesis. Rev Endocr Metab Disord 7:33–39
41. de Frutos CA, Dacquin R, Vega S, Jurdic P, Machuca-Gayet I, Nieto MA (2009) Snail1 controls bone mass by regulating Runx2 and VDR expression during osteoblast differentiation. EMBO J 28:686–696
42. Zhang Y, Hassan MQ, Li ZY, Stein JL, Lian JB, van Wijnen AJ, Stein GS (2008) Intricate gene regulatory networks of helix-loop-helix (HLH) proteins support regulation of bone-tissue related genes during osteoblast differentiation. J Cell Biochem 105:487–496
43. Leucht P, Minear S, Ten Berge D, Nusse R, Helms JA (2008) Translating insights from development into regenerative medicine: the function of Wnts in bone biology. Semin Cell Dev Biol 19:434–443

Reproductive Biology and Endocrinology

Debate

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Influence of obstetric factors on osteogenic potential of umbilical cord-derived mesenchymal stem cells

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Published: 5 October 2009

Reproductive Biology and Endocrinology 2009, **7**:106 doi:10.1186/1477-7827-7-106

Received: 12 August 2009
Accepted: 5 October 2009

This article is available from: http://www.rbej.com/content/7/1/106

Abstract

Wharton's jelly from the umbilical cord is a noncontroversial source of mesenchymal stem cells (WJMSCs) with high plasticity, proliferation rate and ability to differentiate towards multiple lineages. WJMSCs from different donors have been characterized for their osteogenic potential. Although there is large evidence of WJMSCs plasticity, recently scientific debate has focused on MSCs selection, establishing predictable elements to discriminate the cells with most promising osteoprogenitor cell potential.

In the present study a comparative study between the presence of osteoblastic markers and different parameters that pertain to both the newborn and the mother was performed. Umbilical cords were collected after all patients signed the informed consent and local ethical commettee approved the study. Obstetric parameters, including baby's gender and birth weight, mother's age at delivery, gestational stage at parturition and mode of delivery were examined. After characterization and expansion, WJMSCs were analyzed for two osteoblastic markers, alkaline phosphatase (ALP) activity, and the expression level of RUNX-2 transcription factor, and for their ability to deposit mineralized matrix after osteogenic induction.

We found that osteoblastic potential was not influenced by baby's gender and mode of delivery. On the contrary, the highest degree of osteoblastic potential has been shown by WJMSCs with RUNX-2 high basal levels, selected from umbilical cords of the heaviest term babies.

Even if further evaluation is required, our hypothesis is that our findings may help in selecting the optimal umbilical cord donors and in collecting high potential Wharton's jelly-derived osteoprogenitors efficiently.

Background

The umbilical cord is a noncontroversial source of mesenchymal stem cells (MSCs) [1,2]. Recently MSCs isolated from Wharton's Jelly (WJMSCs), a mucoid connective tissue of the umbilical cord, were shown to have the ability to differentiate towards multiple lineages, including adipose, bone, and neuronal lineages [3,4].

Work from several laboratories suggests that these cells, which are very abundant, have potential in therapeutic and tissue engineering field, and indicates that they may be successfully collected and stored for both preclinical work, and banking services [5,6].

It is important to point out that patients who receive umbilical cord stem cells are at a lower risk of developing graft versus host disease, than those who receive bone marrow transplants [7].

Even if there is a considerable debate about MSC plasticity, there are numerous recent reviews and papers on MSCs describing molecular signals that have been identified in driving MSC differentiation down osteoblast lineage, and molecules that are known playing an important role in achieving the desired cellular response such as bone morphogenetic proteins (BMP), dexamethasone, ascorbic acid, and β-glycerophosphate [8,9]. Bone defect repair has been one of the first applications of MSCs, and clinical potential of the use of these cells for bone tissue repair is now extensively explored [10-13].

Nevertheless, bone tissue engineering applications require that MSCs must possess certain reproducible characteristics such as maintenance of the differentiated phenotype. In this scenario, an area of intense research activity, is devoted to improve human MSC characterization, isolation, and expansion [14-16].

Starting from these considerations, we sought to establish further elements for selection of the most desirable cell source for obtaining, inside a WJMSCs collection, the cells with most promising ability to differentiate into osteoblasts.

In the present study five different obstetric parameters, including baby's gender and birth weight, mother's age at delivery, gestational stage at parturition and mode of delivery, were correlated with osteoblastic markers, such as ALP activity, RUNX-2 expression and with the ability of WJMSCs to differentiate along osteogenic lineage.

The hypothesis that the correlation among these parameters may help the selection of optimal umbilical cord donors to collect WJMSCs with most promising osteoprogenitor cell potential is discussed.

Discussion

Human umbilical cord collection and WJMSCs analysis

Human umbilical cord and umbilical cord blood taken after delivery of the newborn, from samples that would be inevitably discarded, have been regarded as an alternative source for transplantation and therapy because of their haematopoietic and mesenchymal cell components [14]. The increasing interest in mesenchymal progenitors for tissue repair widely promoted the characterization of early predictive parameters for plasticity, inducibility and practical utility of these cells [10].

As previously reported [17] Wharton's jelly is an ideal and uncontroversial source for mesenchymal stem cells (WJMSCs) due to the simple collection procedure and the high homogeneity of cell population which is obtained.

In this study, we prepared primary cultures of WJMSCs from 60 donating subjects whose characteristics are shown in Table 1. Five obstetric factors, including baby's gender and birth weight, mother's age at delivery, gestational stage at parturition and mode of delivery were examined. By flow cytometric analysis (Figure 1A and 1B) and double staining with propidium iodide (PI) and Calcein-AM (Figure 1C) before and after cryopreservation, we demonstrated that all WJMSCs samples showed a comparable mesenchymal property and the same level of viability. These characteristics indicate that the quality of the cells is not influenced by the examined obstetric factors. In order to assess the effect of these maternal and neonatal factors on osteoblastic potential of WJMSCs, we focused on the WJMSCs showing the highest levels of mesenchymal and adhesion markers (CD90/Thy-1, CD29/β-1 integrin, CD44/hyaluronan receptor, and CD105/SH2, endoglin), but not expressing hematopoietic/endothelial markers (CD34 and CD45). 20 samples were induced to osteoblast differentiation (see legend of Figure 2), and the propensity to differentiate into osteoblasts was demonstrated through the different ability of the cells to deposit mineralized matrix. A very high heterogeneity in response to treatment with osteogenic medium was observed, and all attempts to detect a correlation between this ability and one of the examined clinical parameters met not success. It is important to underline that heterogeneity in the behaviour of our WJMSC samples is in agreement with the data obtained by other researchers [18,19] and suggests that a great variability is often present after the recover of these staminal cells.

WJMSCs and osteoblastogenesis

On the basis of these observations, we conducted further analyses adjusting for two specific markers of osteoblast differentiation: the activity of Alkaline Phosphatase (ALP) [20] and the expression levels of Runt-related transcription factor 2 (RUNX-2) which increases transcription of

Table 1: Characteristics of subjects

Sample	Mother's age (ys)	Weeks of pregnancy	Mode of delivery	Gender	Birth weight (Kg)
1	26	29	CS	F	1.25
2	31	32	CS	M	1.74
3	30	40	CS	F	3
4	30	32	CS	M	1.9
5	30	32	CS	M	1.91
6	37	40	SP	F	3.59
7	21	41	SP	F	3.75
8	35	40	SP	F	3.17
9	33	42	SP	M	3.05
10	31	40	SP	M	3.69
11	38	38	CS	F	3.32
12	38	38	CS	F	2.6
13	38	39	SP	M	3.45
14	36	40	SP	F	3.6
15	29	40	CS	F	3.22
16	33	38	SP	M	3.7
17	34	38	CS	F	3.45
18	35	35	CS	M	3.31
19	34	40	SP	M	3.21
20	35	40	SP	M	3.18
21	32	38	CS	F	3.6
22	33	35	CS	M	2.41
23	33	35	CS	M	2.53
24	40	40	SP	F	3.75
25	38	39	SP	M	3.36
26	29	37	CS	F	3.62
27	29	40	SP	M	3.4
28	39	39	CS	M	3.62
29	33	40	SP	F	3.12
30	32	35	SP	F	3.17
31	29	37	CS	M	2.14
32	37	39	CS	M	3.37
33	20	40	SP	F	3.02
34	41	39	CS	F	2.73
35	35	37	CS	F	2.77
36	34	38	CS	F	2.9
37	38	38	SP	F	3.05
38	34	40	SP	M	3.7
39	32	36	SP	M	2.93
40	27	38	SP	M	3.1
41	30	34	SP	M	2.6
42	31	41	SP	F	3.25
43	27	33	CS	M	1.915
44	27	33	CS	F	2.005
45	26	39	SP	M	3.35
46	34	42	CS	M	3.51
47	23	37	CS	F	2.95
48	30	39	CS	M	4.24
49	33	39	CS	F	3.3
50	37	39	CS	M	2.84
51	33	39	CS	M	3.27
52	32	40	CS	M	3.9
53	31	37	CS	F	1.98
54	35	40	SP	M	3.95
55	23	37	SP	M	3.11
56	40	38	CS	M	2.85
57	37	39	CS	F	3.3
58	25	35	CS	M	3.57
59	25	35	CS	F	2.38
60	27	29	CS	M	2.1

The recorded clinical parameters are: mother's age, weeks of pregnancy, mode of delivery, newborn gender and birth weight.
F (female) M (male) CS (caesarian delivery) SP (spontaneous delivery)
Umbilical cords were collected, after mothers' consent and approval of "Ethical commettee of University of Ferrara" and "Ethical commettee of Sant'Anna Hospital". The study population consisted of 60 healthy pregnant women voluntary enrolled between February 1, 2008 and March 1, 2009.

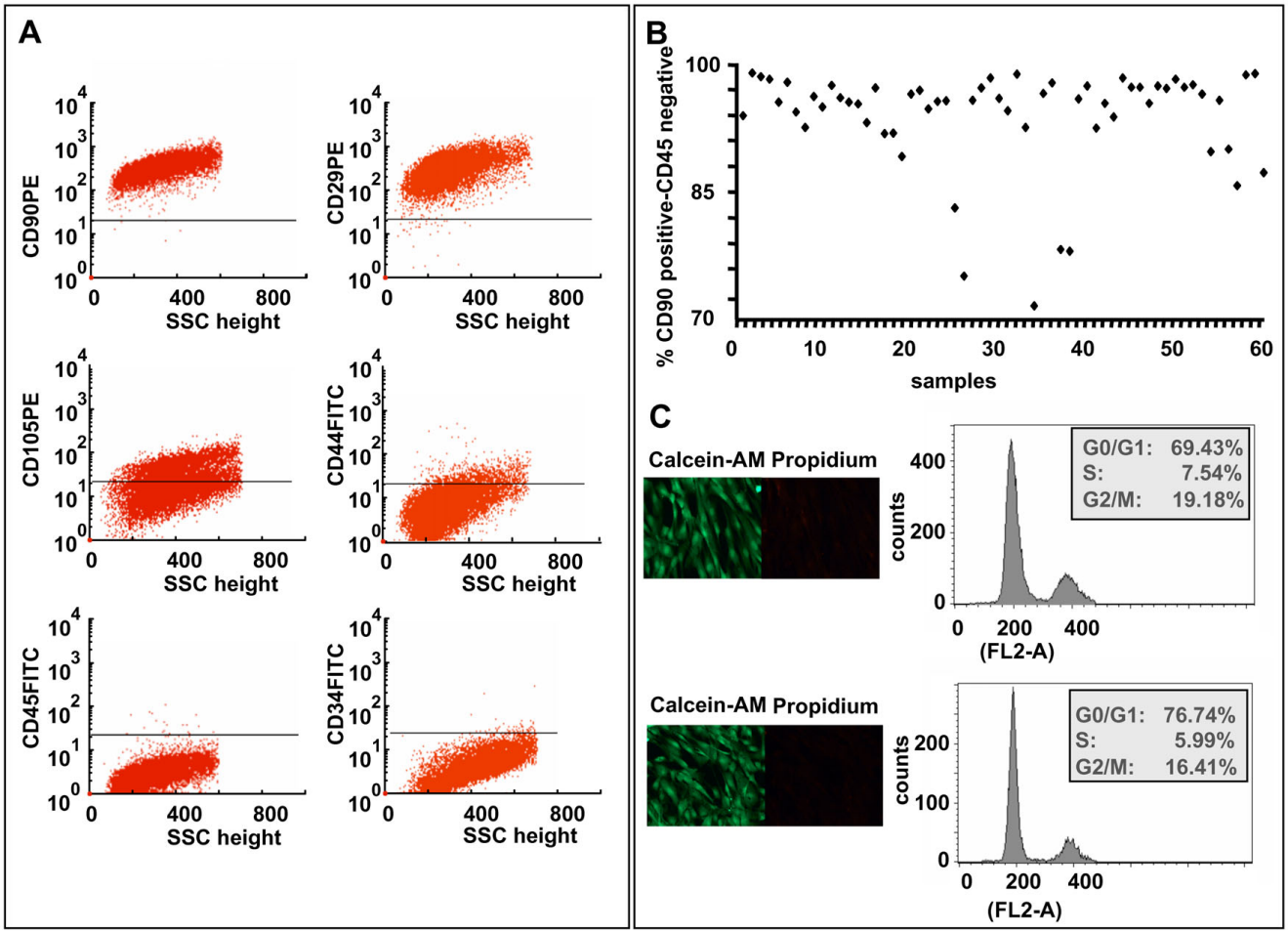


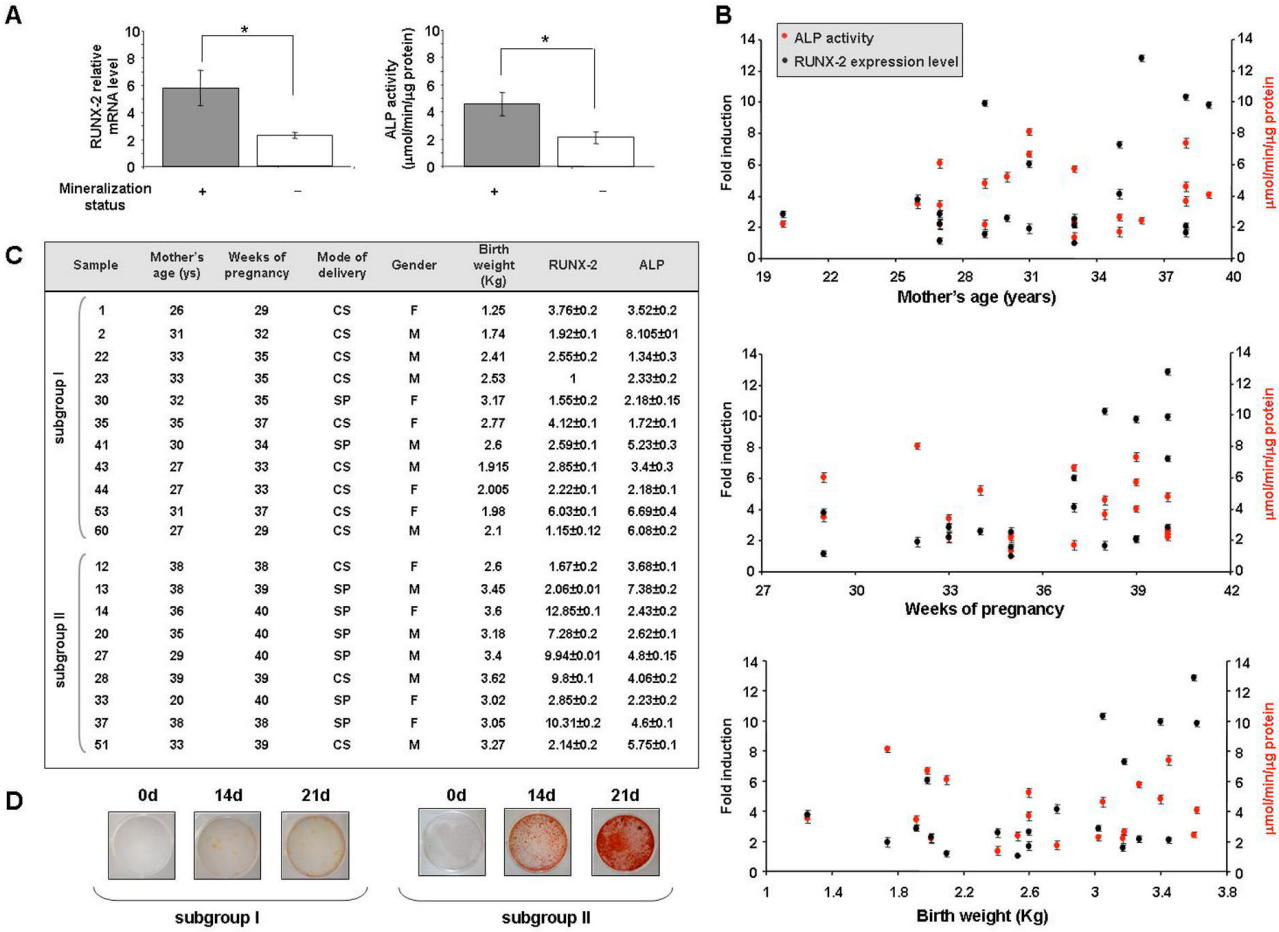
Figure 1

Small pieces (2-3 mm^2) of cords were processed within 4 hours and cultured in DMEM-LG. At ~70-80% confluence, cells were scraped off by 0.05% trypsin/EDTA (Gibco, Grandisland, USA), and analyzed for expression of mesenchymal stem cell surface markers [17], by flow cytometric analysis, as reported (representative experiment) in panel **A**. The gated cells were negative for the hematopoietic line markers CD45 and CD34, partially positive for CD105 and CD44, and positive for the mesenchymal stem cells markers CD90 and CD29. **B**) Schematical distribution of the cell surface parameters of the 60 samples analyzed. **C**) Comparison of the cell culture viability before and after thawing of a cryopreserved sample (high and low panel, respectively). The viability of WJMSCs analyzed by double staining with propidium iodide (PI) and Calcein-AM (Cellstain double staining kit, Sigma Aldrich, St Luis, MO, USA) is indicated. Cells were propidium iodide stained and then analyzed, before and after cryopreservation, for their DNA content, by using BD Immunocytometry Systems DNA QC Particles (BD, New Jersey, USA). The cytofluorimetric profile was analyzed and the percentage of the cell population distribution in the different phases has been reported.

osteoblast specific genes [17]. As shown in Figure 2A, focusing on basal levels of ALP and RUNX-2, it has been possible to demonstrate that these parameters can be predictive of osteoblastic potential of WJMSCs. In fact, the samples with high basal levels of RUNX-2 and ALP are more prone to deposit mineral matrix if compared to WJMSC with low levels of these two proteins. These observations suggest that it may be possible to discriminate among different WJMSC samples those will have a positive outcome towards osteoblastic differentiation.

Osteoblastogenesis and clinical parameters

In a next step, we analyzed whether the basal levels of RUNX-2 and ALP correlate with the examined obstetrics factors (Figure 2B). We found that the infant gender and mode of delivery didn't significantly correlate ($P > 0.05$) with basal RUNX-2 expression and ALP activity. On the other hand, the age of the mother at delivery, has a significant impact on the basal ALP activity but doesn't affect RUNX-2 expression level. Samples collected from mothers which were <32 years old give origin to WJMSCs with high



	Sample	Mother's age (ys)	Weeks of pregnancy	Mode of delivery	Gender	Birth weight (Kg)	RUNX-2	ALP
subgroup I	1	26	29	CS	F	1.25	3.76±0.2	3.52±0.2
	2	31	32	CS	M	1.74	1.92±0.1	8.105±01
	22	33	35	CS	M	2.41	2.55±0.2	1.34±0.3
	23	33	35	CS	M	2.53	1	2.33±0.2
	30	32	35	SP	F	3.17	1.55±0.2	2.18±0.15
	35	35	37	CS	F	2.77	4.12±0.1	1.72±0.1
	41	30	34	SP	M	2.6	2.59±0.1	5.23±0.3
	43	27	33	CS	M	1.915	2.85±0.1	3.4±0.3
	44	27	33	CS	F	2.005	2.22±0.1	2.18±0.1
	53	31	37	CS	F	1.98	6.03±0.1	6.69±0.4
	60	27	29	CS	M	2.1	1.15±0.12	6.08±0.2
subgroup II	12	38	38	CS	F	2.6	1.67±0.2	3.68±0.1
	13	38	39	SP	M	3.45	2.06±0.01	7.38±0.2
	14	36	40	SP	F	3.6	12.85±0.1	2.43±0.2
	20	35	40	SP	M	3.18	7.28±0.2	2.62±0.1
	27	29	40	SP	M	3.4	9.94±0.01	4.8±0.15
	28	39	39	CS	M	3.62	9.8±0.1	4.06±0.2
	33	20	40	SP	F	3.02	2.85±0.2	2.23±0.2
	37	38	38	SP	F	3.05	10.31±0.2	4.6±0.1
	51	33	39	CS	M	3.27	2.14±0.2	5.75±0.1

Figure 2

After characterization in terms of mesenchymal stem cell surface markers expression and cell viability, the osteogenic differentiation of WJMSCs was assessed in the first- and second-passage cultures. Cells were cultured for 21 days in Osteogenic Differentiation Medium (Osteogenic BulletKit, PT-3924 & PT-4120, Lonza, Basel, Switzerland) or in DMEM-LG as a control. The ability of the cells to become mature osteoblasts was evaluated in terms of mineral matrix deposition assessed by Alizarin Red staining (AR-S, Sigma Aldrich, St Luis, MO, USA). The cells fixed in 70% ethanol for 1 h at room temperature, were washed with PBS, stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature, washed five times with deionized water and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was then observed at different magnification using a Leitz microscope. In panel **A** the mineralization status (+, positive or -, negative) was correlated with the basal level (at day "0") of RUNX-2 expression and ALP activity. RUNX-2 expression was examined by quantitative TaqMan (ABI PRISM 7700, Applied Biosystems Inc, Foster City, CA, USA) RT-PCR (Assay-on-demand, Hs00231692_m1). The data were normalized on the basis of GAPDH expression and reported as relative mRNA expression levels. ΔΔCt method was used to compare gene expression data. ALP activity was measured by the hydrolysis of p-nitrophenylphosphate (PNPP, Sigma Aldrich, St Luis, MO, USA) [22] and one unit was defined as the amount of enzyme which hydrolyzed 1 μmol/PNPP per minute. Cell protein content was determined according to the Lowry method [23]. (* = $P < 0.05$). **B**) Relationship between molecular and obstetric parameters. Basal levels of RUNX-2 expression and ALP activity were related to mother's age, weeks of pregnancy and birth weight in 20 WJMSC samples. **C**) The 20 samples of panel **B** were subdived in the two reported subgroups: premature birth (subgroup I) and full term birth (subgroup II). All examined molecular and obstetric parameters are reported. F (female), M (male), CS (Caesarian delivery), SP (spontaneous delivery). **D**) The ability to deposit mineralized matrix was valuated at the indicated times (0, 14, 21 days) in two representative samples of the two subgroups, by Alizarin red staining.

ALP activity. Interestingly, birth weight of the infant was shown to significatively impact on RUNX-2 basal expression level which, as reported in Figure 2B, decreases with the decreasing of the baby's weight. The same relationship was found for the duration of pregnancy. In fact, it was found that WJMSCs from babies born before the 37 weeks of gestation express lower basal level of RUNX-2 than the full term borns. It is very likely that WJMSCs recovered from premature birth contain a high number of undifferentiated cells with high plasticity, a condition which is not actually required for osteoblast differentiation.

As a whole, these findings led us to focus on two parameters, weeks of pregnancy and consequently birth weight of the baby, and RUNX-2 basal levels, subdividing the collected samples in the two subgroups reported in Figure 2C: subgroup I, premature birth with low levels of RUNX-2, and subgroup II, full term birth with high levels of RUNX-2. The ability of the samples belonging to these two subgroups to complete the event of cellular maturation, that is the deposition of mineralized matrix, was then compared. Two representative samples of the two subgroups (Figure 2D) demonstrate that, samples from subgroup I showed a null mineralization status also after 21 day of cell culture in osteogenic medium, whereas samples from subgroup II showed a high level of mineralization beginning from day 14. These findings suggest that maximal WJMSCs osteoblastic potential can be obtained by primary cultures with RUNX-2 high basal levels, selected from the heaviest term babies.

Another clinical observation that is important to do is that the cases below 37 weeks of gestation were all treated with 24 mg of bethametasone two hours before delivery. Such a therapy is routinely given to all the pregnant women delivering prematurely in order to prevent respiratory distress syndrome in the newborns. Therefore, a possible influence of this hormone on stem cells behaviour can be hypothesized. At this regard it has been recently reported that glucocorticoids play an essential role in favouring stem cells differentiation towards adipocyte lineage, thus inhibiting bone and muscle lineages [21]. Such an influence appears to be exerted by inducing myostatin, a potent molecule that regulates muscle development. Therefore, based on the above evidences a possible absence of osteoblastogenesis could have been expected in the samples derived from women delivering prematurely. Since we observed a low or null mineralization status in WJMSCs from such patients, it could be possible to point out a correlation between the two events. However, our analysis showed that this evidence is not always occurring, indicating that the short term high dose of bethametasone administered wasn't ever effective in inhibiting bone lineage.

Conclusion

To conclude, we should indicate that the analysis of the basal level of RUNX-2 and ALP activity may allow a quick testing of a high number of mesenchymal precursors cultured in vitro and select the more suitable to potentially use for bone tissue engineering application. In addition, for the same aim, our results suggest that it is preferred to recruit the samples from full term borns without paying attention to mother's age.

Even if further evaluation is required, our hypothesis is that our findings may help in selecting the optimal umbilical cord donors and in collecting high potential Wharton's jelly-derived osteoprogenitors efficiently.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LP partecipated together with RP and TF in the design of the study. LP, RV, ET and AC carried out the experiments. Data analysys was performed by EL, LP, RV and RP. SB and FV collected the samples and performed clinical analysis. The manuscript was written by RP and LP. GC, RV and FV critically read the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by grants from Regione Emilia Romagna, Programma di Ricerca Regione Università 2007-2009. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

References

1. Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE: **Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors.** *Stem Cells* 2005, **23:**220-229.
2. Can A, Karahuseyinoglu S: **Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells.** *Stem Cells* 2007, **25:**2886-2895.
3. Fu YS, Cheng YC, Lin MY, Cheng H, Chu PM, Chou SC, Shih YH, Ko MH, Sung MS: **Conversion of Human Umbilical Cord Mesenchymal Stem Cess in Wharton's Jelly to dopaminergic Neurons in vitro: Potential therapeutic application for Parkinsonism.** *Stem Cells* 2006, **24:**115-124.
4. Lu LL, Liu YJ, Yang SG, Zhao QJ, Wang X, Gong W, Han ZB, Xu ZS, Lu YX, Liu D, Chen ZZ, Han ZC: **Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials.** *Hematologica* 2006, **91(Suppl 8):**1017-1026.
5. Kestendjieva S, Kyurkchiev D, Tsvetkova G, Mehandjiev T, Dimitrov A, Nikolov A, Kyurkchiev S: **Characterization of mesenchymal stem cells isolated from the human umbilical cord.** *Cell Bio Int* 2008, **32:**724-732.
6. Sesharedy K, Troyer D, Weiss ML: **Method to Isolate Mesenchymal-Like Cells from Wharton's Jelly of Umbilical Cord.** *Methods Cell Biol* 2008, **86:**101-119.
7. Oh W, Kim DS, Yang YS, Lee JK: **Immunological properties of umbilical cord blood-derived mesenchymal stromal cells.** *Cell Immunol* 2008, **251:**116-123.
8. Ciavarella S, Dammacco F, De Matteo M, Loverro G, Silvestris F: **Umbilical Cord Mesenchymal Stem Cells: Role of Regulatory Genes in Their Differentiation to Osteoblasts.** *Stem CellsDev* in press.

9. Hildebrandt C, Buth H, Thielecke H: **Influence of cell culture media conditions on the osteogenic differentiation of cord blood-derived mesenchymal stem cells.** *Annals of Anatomy* 2009, **191:**23-32.
10. Bajada S, Mazakova I, Richardson JB, Ashammakhi N: **Updates on stem cells and their applications in regenerative medicine.** *J Tissue Eng Regen Med* 2008, **2:**169-183.
11. Diao Y, Ma Q, Cui F, Zhong Y: **Human umbilical cord mesenchymal stem cells: Osteogenesis *in vivo* as seed cells for bone tissue engineering.** *J Biomed Mater Res A* 2009, **91:**123-131.
12. Wang L, Milind S, Bonewald LF, Detamore MS: **Signalling strategies for osteogenic differentiation of human umbilical cord mesenchymal stromal cells for 3D bone tissue engineering.** *J Tissue Eng Regen Med* 2009, **3:**398-404.
13. Wang L, Sesharedy K, Weiss ML, Detamore MS: **Effect of Initial Seeding Density on Human Umbilical Cord Mesenchymal Stromal Cells for Fibrocartilage.** *Tissue Eng Part A* 2009, **15:**1009-1017.
14. Secco M, Zucconi E, Vieira NM, Fogaça LL, Cerqueira A, Carvalho MD, Jazedje T, Okamoto OK, Muotri AR, Zatz M: **Mesenchymal stem cells from umbilical cord: do not discard the cord!** *Neuromuscul Disord* 2008, **18:**17-18.
15. Troyer DL, Weiss ML: **Wharton's jelly-derived cells are a primitive stromal cell population.** *Stem Cells* 2008, **26:**591-599.
16. Sarugaser R, Ennis J, Stanford WL, Davies JE: **Isolation, propagation, and chaarcterization of human umbilical cord perivascular cells (HUCPVCs).** *Methods Mol Biol* 2009, **482:**269-279.
17. Penolazzi L, Tavanti E, Vecchiatini R, Lambertini E, Vesce F, Gambari R, Mazzitelli S, Mancuso F, Luca G, Nastruzzi C, Piva R: **Encapsulation of Mesenchymal Stem Cells from Wharton's Jelly in Alginate Microbeads.** *Tissue Eng Part C Methods* 2009 in press.
18. Markov V, Kusumi K, Tadesse MG, William DA, Hall DM, Lounev V, Carlton A, Leonard J, Cohen RI, Rappaport EF, Saitta B: **Identification of cord blood-derived mesenchymal stem/stromal cell populations with distinct growth kinetics, differentiation potentials, and gene expression profiles.** *Stem Cells Dev* 2007, **16:**53-73.
19. Zhang ZY, Teoh SH, Chong MS, Schantz JT, Fisk NM, Choolani MA, Chan J: **Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells.** *Stem Cells* 2009, **27:**126-37.
20. Lambertini E, Tavanti E, Torreggiani E, Penolazzi L, Gambari R, Piva R: **ER alpha and AP-1 interact in vivo with a specific sequence of the F promoter of the human ER alpha gene in osteoblasts.** *J Cell Physiol* 2008, **216:**101-110.
21. Feldman BJ: **Glucocorticoids influence on Mesenchymal Stem Cells and Implications for metabolic Disease.** *Pediatric Res* 2009, **65:**249-251.
22. Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Smith DD, Winkler ME, Derynck R, Mundy GR: **Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro.** *Proc Natl Acad Sci USA* 1986, **83:**2228-2232.
23. Waterborg JH, Matthews HR: **The Lowry method for protein quantitation.** *Methods Mol Biol* 1994, **32:**1-4.

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RESEARCH ARTICLE Open Access

SLUG: a new target of lymphoid enhancer factor-1 in human osteoblasts

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Abstract

Background: Lymphoid Enhancer Factor-1 (Lef-1) is a member of a transcription factor family that acts as downstream mediator of the Wnt/β-catenin signalling pathway which plays a critical role in osteoblast proliferation and differentiation. In a search for Lef-1 responsive genes in human osteoblasts, we focused on the transcriptional regulation of the SLUG, a zinc finger transcription factor belonging to the Snail family of developmental proteins. Although the role of SLUG in epithelial-mesenchymal transition and cell motility during embryogenesis is well documented, the functions of this factor in most normal adult human tissues are largely unknown. In this study we investigated SLUG expression in normal human osteoblasts and their mesenchymal precursors, and its possible correlation with Lef-1 and Wnt/β-catenin signalling.

Results: The experiments were performed on normal human primary osteoblasts obtained from bone fragments, cultured in osteogenic conditions in presence of Lef-1 expression vector or GSK-3β inhibitor, SB216763. We demonstrated that the transcription factor SLUG is present in osteoblasts as well as in their mesenchymal precursors obtained from Wharton's Jelly of human umbilical cord and induced to osteoblastic differentiation. We found that SLUG is positively correlated with RUNX2 expression and deposition of mineralized matrix, and is regulated by Lef-1 and β-catenin. Consistently, Chromatin Immunoprecipitation (ChIP) assay, used to detect the direct Lef/Tcf factors that are responsible for the promoter activity of SLUG gene, demonstrated that Lef-1, TCF-1 and TCF4 are recruited to the SLUG gene promoter "*in vivo*".

Conclusion: These studies provide, for the first time, the evidence that SLUG expression is correlated with osteogenic commitment, and is positively regulated by Lef-1 signal in normal human osteoblasts. These findings will help to further understand the regulation of the human SLUG gene and reveal the biological functions of SLUG in the context of bone tissue.

Background

Lymphoid Enhancer binding Factor-1 (Lef-1) is a nuclear high mobility group (HMG) protein that mediates gene transcription in response to canonical Wnt/β-catenin signaling pathway [1-3]. Wnt signaling controls normal and abnormal development in a variety of tissues including skeleton, and accumulated evidence has shown that Lef-1 influences osteoblast proliferation, maturation, function, and regeneration both *in vitro* and *in vivo* [4-7]. Nevertheless, the exact mechanism by which Lef-1 affects osteoblast differentiation is unknown. In a search for Lef-1 responsive genes in human osteoblasts, we focused on the transcriptional regulation of the SLUG gene for the reasons reported below.

SLUG, also named SNAIL2, is a member of a superfamily of zinc-finger transcription factors that play a central role in the patterning of vertebrate embryos [8-10]. It is implicated in the induction of epithelial mesenchymal transitions (EMT) at specific stages of normal development and tumor progression, acting as a transcriptional repressor of genes encoding components of cell-cell adhesive complexes in the epithelia [11-17]. Several signalling pathways inducing EMT cellular event and including FGF, WNT, TGF-β, BMP, EGF, HIF, Notch, PTH, integrins and SCF/c-Kit have been shown

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to converge in SNAIL genes induction, as well reviewed by Barrallo-Gimeno et al. [18], and as previously reported [9,10,19].

SLUG and its family members also have important roles in other processes, including protection of cells from programmed cell death, regulation of cytoskeletal elements [18], adipocyte differentiation [20] and migration of neural crest cells [21,22]. Although the expression of SLUG has been found in most normal adult human tissues [23-25], little is known about its potential functions.

It is important to underline that the vertebrate neural crest, formed at the border between the neural plate and the non-neural ectoderm during neurulation, is able, under SLUG control, to give rise to different cell types including neurons, glia, facial chondrocytes, osteoblasts, and melanocytes [8,26,27]. In addition, craniofacial abnormalities have been observed in association with cerebral malformations and cutaneous lesions in some neurocutaneous syndromes, emphasizing an important inductive role of the neural tube in the development of non-neural tissues mediated through neural crest and differentiating genes such as SLUG and Sox10 [28,29]. Overall, these observations encourage investigation on SLUG expression and functions in adult cells, including osteoblasts.

We recently demonstrated, by a knockdown approach, that SLUG is involved in the differentiation and maturation process of normal human osteoblasts [30]. Nevertheless, so far, no data have been presented on SLUG regulation in these cells and their precursors. Only one previous investigation has demonstrated that Wnt signaling regulates SLUG expression, in a tumor model, such as an osteosarcoma cell line, mediating cancer invasion [31].

The presence of putative *cis* elements for Lef-1, in human SLUG gene promoter has raised the possibility that Lef-1 may be implicated in the modulation of SLUG expression as previously demonstrated in other species such as chick and Xenopus [32,33]. In this study we demonstrated that SLUG is expressed in both normal human osteoblasts and their mesenchymal precursors, and that Lef-1 is recruited "*in vivo*" to its promoter acting as a positive transcriptional regulator.

Results

SLUG expression in human osteoblasts and their mesenchymal precursors

Lef-1 has been shown to play a role in osteoblast differentiation and function. Owing to the relationship between Lef-1, β-catenin and SLUG recently found in some epithelial-mesenchymal transition cellular models [34,35], we hypothesized that Lef-1 and SLUG may also be correlated in osteoblast lineage cells. To test this idea SLUG expression was examined during osteoblast differentiation and compared with Lef-1 expression levels. SLUG mRNA levels were measured in human mesenchymal stem cells (hMSCs) obtained from umbilical cord Wharton's Jelly and induced towards osteogenesis, as previously described [36]. RNA was collected after 0, 7, 14, 21, and 28 days in culture and evaluated by quantitative RT-PCR. As shown in Figure 1A, these cells differentiate along the osteoblast lineage in osteogenic medium as confirmed by the positive staining for extracellular calcium deposition. Abundant SLUG mRNA was detected in the cells at all times tested, and was induced as the cultures progressed. Lef-1 was less abundant, but significantly increased during the osteogenesis. RUNX2, a determinant transcription factor for osteoblastogenesis [37], was also expressed at all stages, and was induced as the cultures progressed, confirming that each time point represented increasingly mature osteoprogenitors.

In order to confirm that the expression profile that we found was associated with osteoblast phenotype, SLUG, Lef-1 and RUNX2 expression levels were measured in human primary osteoblasts obtained from five bone specimens (hOBs). All these samples were positive for alkaline phosphatase (ALP) activity, a well-known osteoblast differentiation marker, and were able to form mineralized nodular structures after 14 days in osteogenic condition (see a representative experiment in the panel of Figure 1B). As shown in Figure 1B, SLUG, Lef-1 and RUNX2 were detected in all hOB samples analyzed. The level of SLUG mRNA in hOBs was also compared with that found in different osteoblast-like cell lines [Additional file 1].

To further characterize the potential involvement of SNAIL family members in osteogenesis, the expression of SNAIL1 and SNAIL3 was examined in the same set of experiments. SNAIL1 has been recently reported to act on the osteoblast population regulating bone cells differentiation and contributing to bone remodeling in mice [38]. In agreement with this previous study, we found that SNAIL1 was expressed at early stages of osteoblast differentiation and then downregulated for differentiation to proceed (Figure 1A). In hOB samples SNAIL1 was expressed at substantial levels (Figure 1B). The expression of SNAIL3 [39] was detectable at very low levels in the hMSCs induced towards osteogenesis (Figure 1A), and at low levels in hOBs (Figure 1B).

SLUG expression is positively modulated by Lef-1

hOBs were then transfected with expression vector containing hLef-1 cDNA (K14-myc-hLEF1) as described in the Methods section. As shown in Figure 2, SLUG expression significantly increased in Lef-1 overexpressing cells, both at mRNA and protein level, as

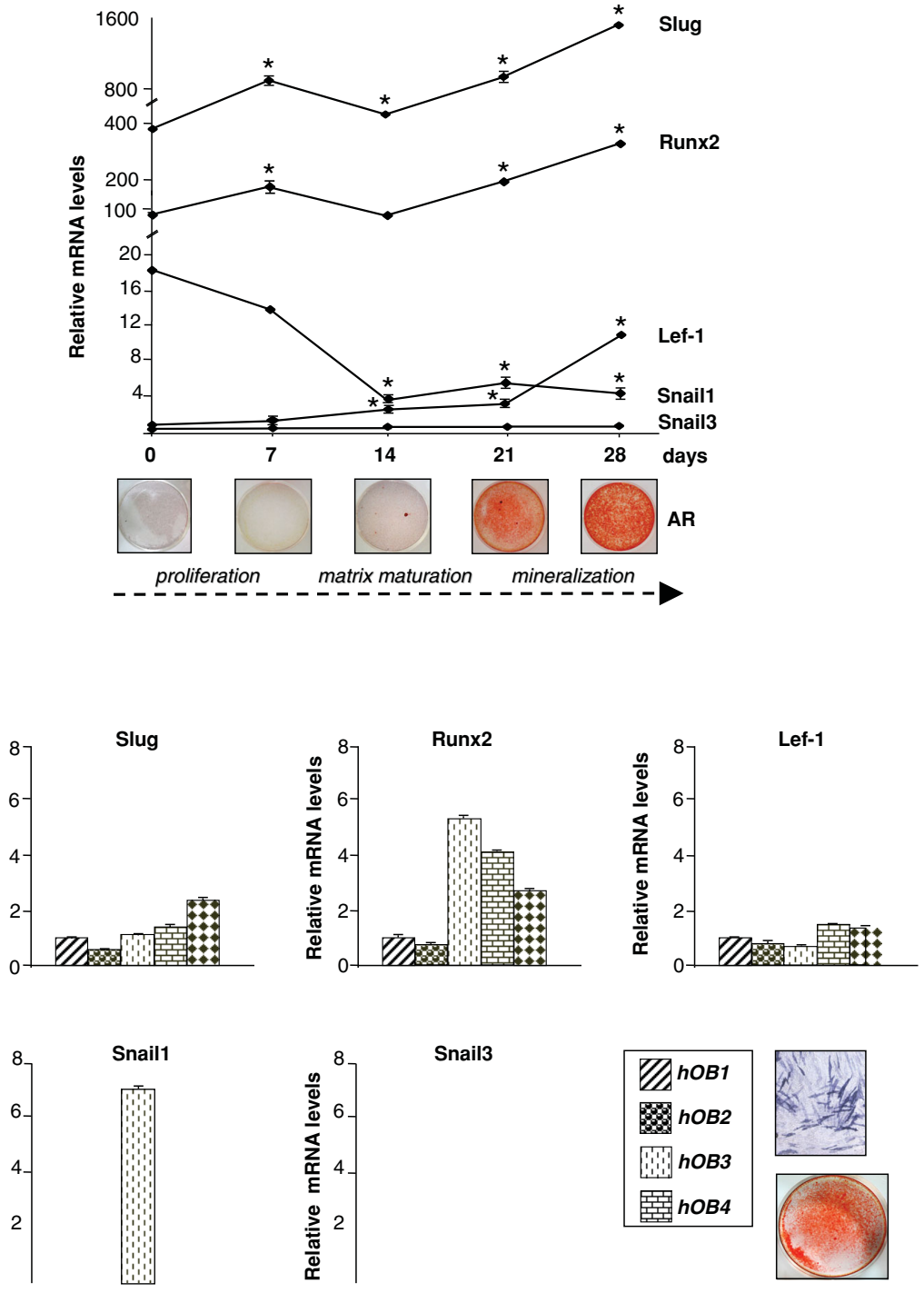

Figure 1 Detection of SLUG expression by quantitative RT-PCR. The level of SLUG, RUNX2 Lef-1, SNAIL1 and SNAIL3 expression was examined by quantitative RT-PCR in three hMSC samples cultured up to 28 days in osteogenic medium (A) and in five hOB samples (B). The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for SLUG, RUNX2, Lef-1, SNAIL1 and SNAIL3 transcript analysis. The experiments were carried out in triplicate, the expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. ΔΔCt method was used to value the gene expression; standard error of the mean (SEM) was calculated. The commitment to osteoblastic lineage of hMSCs was evaluated by Alizarin Red staining for extracellular calcium deposition. The authentic osteoblast phenotype was confirmed in hOBs by staining for alkaline phosphatase (ALP) activity and mineralized matrix deposition (AR, Alizarin Red staining). * = $p < 0.05$ (respect to day 0).

demonstrated by RT-PCR (Figure 2A) and Western blot analysis (Figure 2B). The significant increase of Lef-1 in the cells transfected with hLef-1 expression vector was confirmed by the same Western blot analysis (Figure 2B). As expected, forced expression of Lef-1 increased Slug expression to higher levels in SaOS-2 osteoblast-like cells than in hOBs, because of a higher intrinsic transfection facility of this cell line.

The ability of Lef-1 to activate transcription of SLUG gene was then tested on the human SLUG promoter (Figure 3). We chose to focus on an approximately 1 Kb fragment upstream of the transcription start site in the SLUG gene since it contains sequences involved in the regulation of promoter activity mediated by β-catenin [34]. In addition to the previously identified TCF binding site at -859/-855 position [34,35], we identified, in this region, another five potential consensus binding sites for the Lef/Tcf family by using the programs Transcription Element Search Software TESS for transcription factor search and MatInspector 7.4 program (Figure 3A). The sequence was cloned upstream of the Luc reporter gene in the pGL3basic vector, and the construct, (named 982 bp luc-construct), was assayed after osteoblast transfections performed with or without Lef-1 expression plasmid. As shown in Figure 3B, transient transfection with the luciferase reporter 982 bp luc-construct resulted in an increase in luciferase activity relative to the empty, promoterless pGL3-basic vector, demonstrating that this DNA fragment contains significant promoter activity in hOBs (5-10 fold increase). Co-transfection with plasmid encoding Lef-1 produced a significant increase in Luc activity as compared with cells containing the 982 bp luc-construct reporter plasmid. This increase was dramatic in Lef-1 overexpressing SaOS-2 cells. On the contrary, the same experiments performed in the non-osseous SLUG-negative MCF7 breast cancer cell line revealed no promoter activity.

As a whole, these data indicate that Lef-1 upregulates SLUG gene expression in normal human osteoblasts.

Lef-1 is recruited to the SLUG promoter "in vivo"

Next, we investigated whether Lef-1 could, "*in vivo*", physically bind with the human SLUG promoter. Considering that in addition to Lef-1, among TCF family members, both TCF-1 and TCF-4 are expressed in osteoblasts [2], the analysis was addressed to all three proteins. The binding of transcription factor to the SLUG promoter was verified by performing *in vivo* chromatin immunoprecipitation (ChIP) assays (Figure 3C). To this aim, hOBs were exposed to formaldehyde to cross-link proteins and DNA, and were sonicated to fragment the chromatin. Specific antibody against Lef-1, TCF-1 and TCF-4 were used to immunoprecipitate the protein-DNA complexes. After immunoprecipitation, DNA was extracted from the beads and used as a template to generate specific PCR products. The presence of the promoter specific DNA region before immunoprecipitation was confirmed by PCR (input). In the SLUG promoter fragment used for the reporter assay, three different regions were identified, as depicted in Figure 3A, and analyzed by a set of primers spanning the six consensus binding sites for the Lef/Tcf family. The amplified product sizes (bp) were 178 for region 1, 164 for region 2, and 165 for region 3. The results showed that the promoter region 3, containing the previously identified TCF binding site at -859/-855 position [34,35], was significantly immunoprecipitated by Lef-1 and TCF4 antibodies, but that Lef-1 was mostly associated with the promoter region 1 and not at all with the promoter region 2 (Figure 3C). On the contrary, we found that region 2 was rather occupied by TCF-1 and TCF-4. Therefore, the observation that the endogenous SLUG gene expression may be increased by Lef-1 was further validated by the *in vivo* occupancy of the Lef/TCF regulatory sites in the SLUG gene promoter.

Activation of Wnt signaling by GSK-3β inhibitor increases SLUG promoter activity

It has been demonstrated that β-catenin promotes Lef/Tcf interaction with target DNA sequence in many cellular contexts. In order to support the role of Lef/Tcf transcription factors in SLUG expression regulation, we next investigated whether β-catenin activation was involved in SLUG expression regulation. We used a treatment with SB216763 as a model for β-catenin activation (Figure 4A). This compound binds and specifically inhibits glycogen synthase kinase GSK-3β. GSK-3β is a serine/threonine kinase, originally identified as a kinase that is involved in glucose metabolism, but recent research has determined that it acts on a wide variety of substrates, including transcription factors, and is a key regulator in many signalling pathways [40]. This enzyme is known to be a key negative regulator of canonical Wnt/β-catenin and PI3K/Akt signalings [41]; hence, its inhibition activates Wnt signalling selectively via the β-catenin/TCF pathway and results in relocation of stabilized β-catenin to the nucleus. As expected, the SB216763-treated cells transfected with the β-catenin/Tcf transcription reporter construct -TOPflash reporter system- showed an increase in TOPflash activity up to 4-fold (Figure 4B). The β-catenin/Tcf transcription reporter assay was recognised as an important assessment method for evaluation of the Wnt pathway activity. As TOPflash has three TCF-binding sites, it could be applied to represent the activation of the Wnt pathway. In fact, our data showed that SB216763 treatment positively affected β-catenin expression, as revealed by Western blot reported in Figure 4C. The dose- and

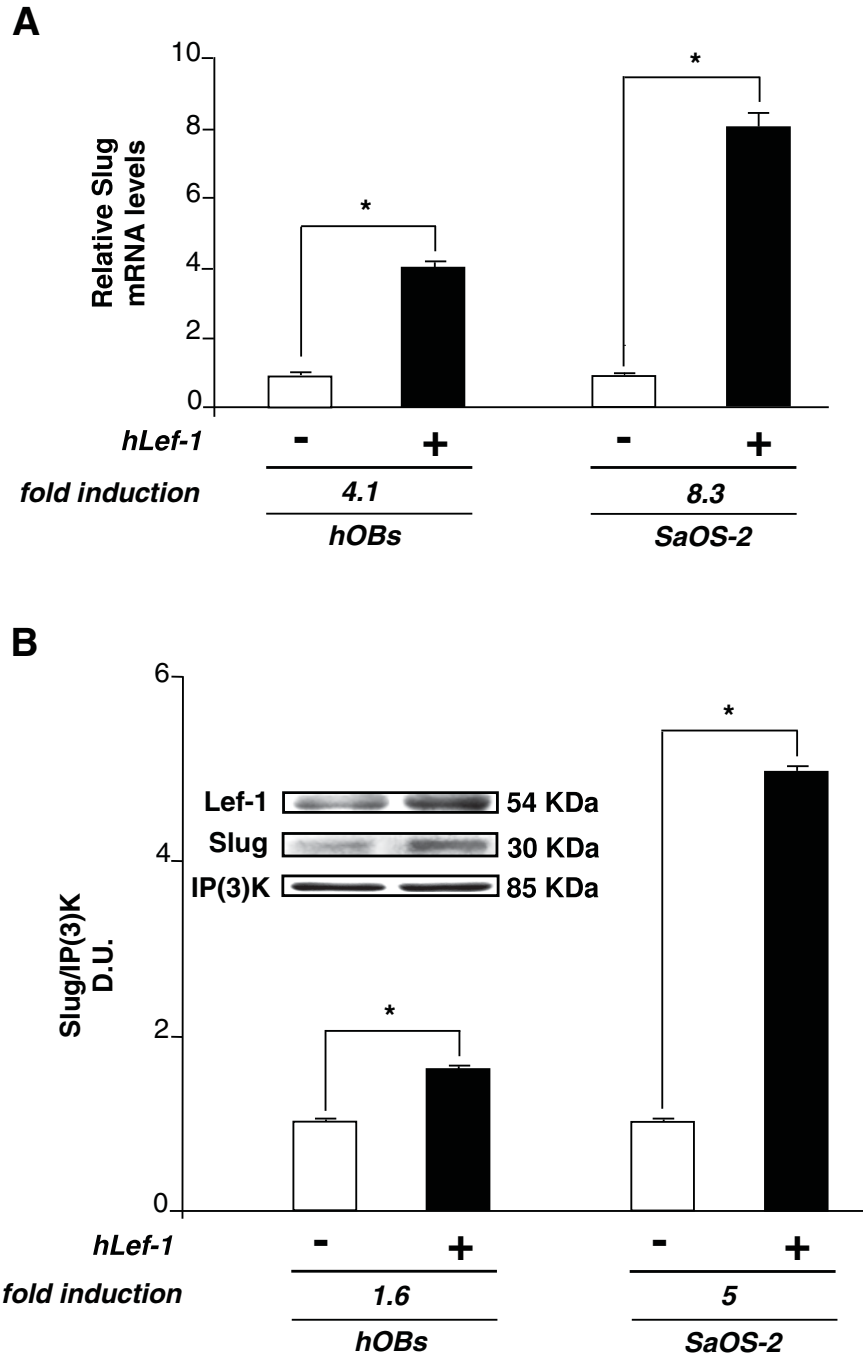

Figure 2 Effect of Lef-1 overexpression on SLUG expression in hOBs. The effect of Lef-1 overexpression was examined at mRNA (A) and protein (B) level. (A) SLUG mRNA was evaluated by quantitative RT-PCR in hOBs and SaOS-2 osteoblast-like cells transfected with 2.5 μg of hLef-1 (K14-myc-hLEF1) expression plasmid. The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for SLUG transcript analysis. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. Results are representative of three independent experiments carried out in triplicate. ΔΔCt method was used to compare gene expression data; standard error of the mean (SEM) was calculated. * = $p < 0.05$. (B) SLUG protein levels were examined by Western blot analysis in hOBs and SaOS-2 osteoblast-like cells transfected with 2.5 μg of hLef-1 expression plasmid. Whole cell lysates were prepared and 25 μg of protein run on a 12% SDS-polyacrylamide gel. The proteins were visualized using Supersignal West Femto Substrate (Pierce). The quantitative presentation of the protein levels were performed by densitometric analysis using Anti-IP(3)K as control. D.U. = densitometric units. This experiment was repeated three times with similar results. A representative SLUG and Lef-1 Western blot analysis with size markers (KDa) is reported. * = $p < 0.05$.

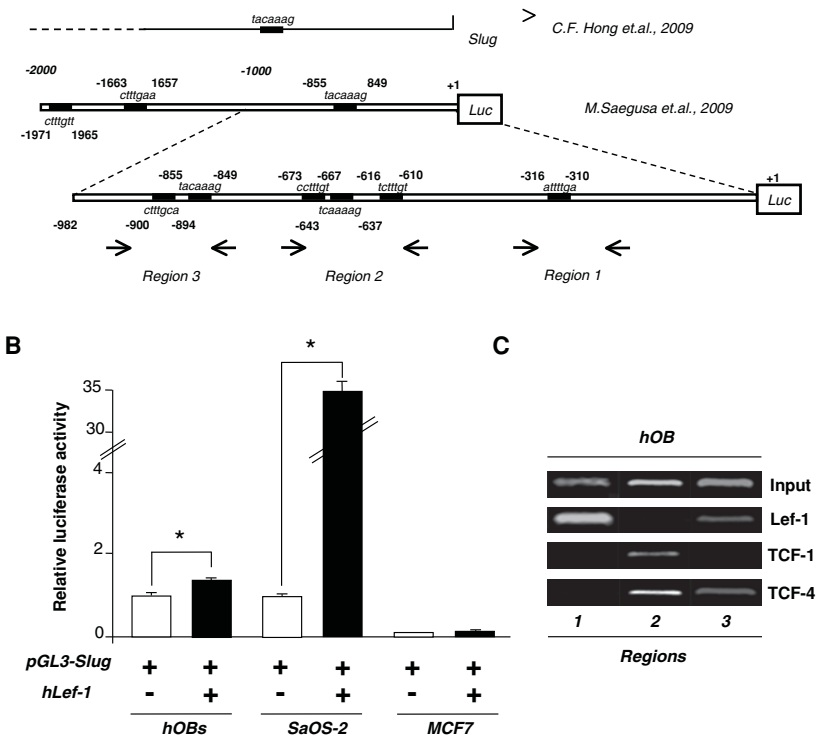

Figure 3 Lef-1 affects the activity of human SLUG promoter "*in vitro*" and binds it "*in vivo*". (A) The SLUG promoter region under investigation is reported. The positions of putative Lef/Tcf binding sites are enclosed by rectangles and are compared with those recently investigated by others. Positions of PCR primers used in ChIP experiments are also reported. (B) The DNA construct containing the human SLUG promoter region was cloned into upstream of the firefly luciferase (LUC) reporter gene. hOBs and SaOS-2 osteoblast-like cells were transfected with the pGL3-SLUG Luc reporter vector containing the sequence from +1 to -982 of the human SLUG promoter (pGL3-SLUG 982 bp), in the absence (-) or presence (+) of 2.5 μg of hLef-1 expression plasmid. The results of reporter gene assays were normalized with protein concentration and β-gal activity for transfection efficiency and the data are represented as ratios of luciferase units to β-galactosidase units. MCF7 breast cancer cell line was used as negative control. All experiments were performed in triplicate and the average of the ratio of the reporter activity + SEM is shown. * = $p < 0.05$. (C) Recruitment of Lef1/TCF transcription factors to the human SLUG promoter is demonstrated by "*in vivo*" chromatin immunoprecipitation (ChIP) binding assays. Soluble chromatin was prepared from hOBs and immunoprecipitated with the indicated specific antibodies against Lef-1, TCF-1, and TCF-4. The associations of the transcription factors to bound precipitated DNA were monitored on the human SLUG promoter regions 1, 2 and 3 by PCR with the primers indicated in the scheme. Input represents a positive control using the starting material (0.2%) prior to immunoprecipitation. Representative agarose gels are shown.

time-response to SB216763 cell treatment was analyzed in terms of SLUG mRNA levels in osteoblast-like cell lines [Additional file 2]. The same analysis demonstrated that the increase in β-catenin mediated by SB216763 was correlated with a significative increase in SLUG and RUNX2 expression both at protein (Figure 4C) and mRNA level (data not shown). Therefore, on the whole, this suggests that the canonical Wnt signaling positively affects SLUG expression in normal human osteoblasts via the β-catenin/TCF pathway because, by potentiating β-catenin, SLUG expression increases.

Discussion

In this paper we have demonstrated that the transcription factor SLUG is present in normal human osteoblasts and their mesenchymal precursors. Osteoblasts are the primary cell type responsible for the bone remodeling process, and alterations in this pathway can lead to osteopenic disorders such as osteoporosis. Therefore, any new marker or mechanism associated with differentiation of these cells represent very relevant information for the study of bone biology and bone-related diseases in general.

We have shown that SLUG expression increases during osteogenesis, and is positively regulated by Lef-1, an osteoblastic transcription factor which we found *in vivo* recruited by specific *cis* elements present in the SLUG promoter. In the SLUG promoter region of approximately 1 Kb upstream of the transcription start site, we found at least six potential consensus binding sites for the Lef/Tcf family, and not just one only at -859/-855 position, as recently reported [34,35]. We found that the

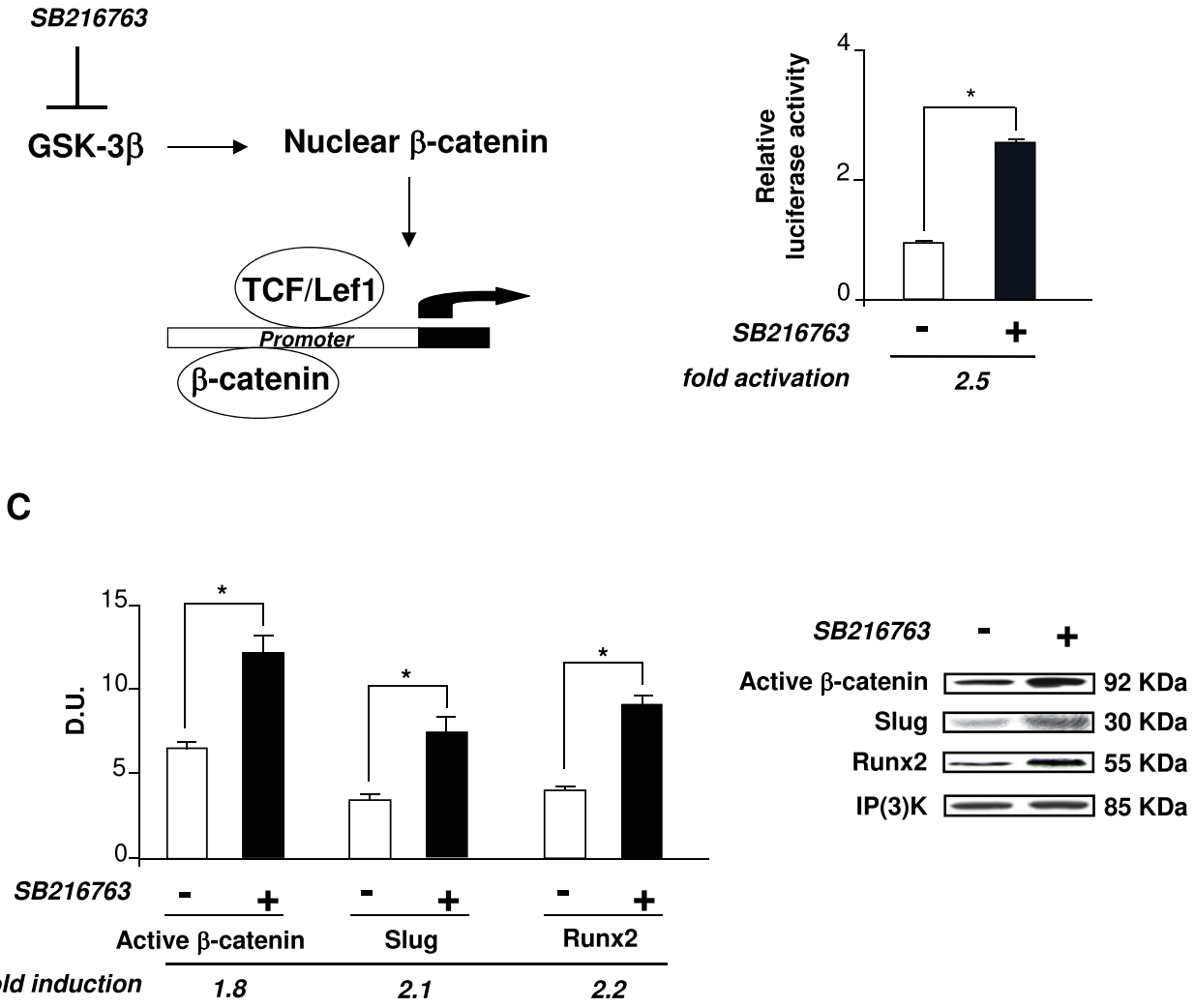

Figure 4 Treatment of hOBs with the glycogen synthase kinase (GSK-3β) inhibitor, SB216763. (A) A scheme of SB216763 action mechanism is reported (see the text for details). (B) Effect of SB216763 on the TOPflash reporter system. 24 h after transient transfection with the TOPflash plasmid, the cells were treated (+) or not (-) with SB216763 (10 μM) for 24 h prior to harvest. Luciferase activity was normalized to β-galactosidase activity in the same sample. The bars represent mean ± SEM. * = $p < 0.05$. (C) The levels of β-catenin expression, SLUG and RUNX2 were examined by Western blot in hOBs treated with SB216763 (10 μM) or with the only vehicle (-). The quantitative presentation of the protein levels was performed by densitometric analysis using Anti-IP(3)K as control. D.U. = densitometric units. A representative Western blot analysis with size markers (KDa) is reported. * = $p < 0.05$.

sequence regions containing these sites are all involved, even if at different levels, in the *in vivo* recruitment of Lef/Tcf factors, including Lef-1, TCF-1 and TCF-4, in human osteoblasts. The investigations on the only previously characterized TCF binding site (-859/-855), demonstrated its ability to recruit TCF-4 in SW480 human colon cancer [35], but not in Hec251 endometrial cancer cell line [34] where, on the contrary, SLUG expression seems to be under transcriptional control of β-catenin without the binding of Lef/Tcf factors at this site. Other studies in different experimental models provide evidence that Xenopus and mouse SLUG promoters are directly activated by β-catenin/TCF complexes through the binding sequences [32,33], and that SLUG promoter activity may be inhibited by dominant negative Tcf [42]. Combined with these reports, our results may lead to the hypothesis that, directly or indirectly, SLUG and Lef-1 are strictly correlated in many cellular events, including osteoblast differentiation, mediated by Wnt/β-catenin signalling. In addition, this is supported by our recent evidence demonstrating the requirement of SLUG for osteoblast maturation and the

decrease in Wnt/β-catenin signalling after SLUG knockdown [30]. This suggests a possible role of SLUG as effector of Wnt/β-catenin signalling.

Our findings confirm a relationship between SLUG and Wnt signalling showing that the increase in β-catenin levels, obtained by the suppression of GSK-3β activity with SB216763 inhibitor, induces a significative SLUG gene expression increase. β-catenin is known to associate with the Lef/Tcf transcription factor family and promote the expression of several genes through the recruitment of other factors to form a transcriptionally active complex [43,44]. Lef-1 is reported to have an important role in osteoblast maturation for its ability in the regulation of expression of genes involved in the stimulation of bone formation, such as RUNX2 and Col11a1 [45,46]. In addition, an age- and gender- dependent role for Lef-1 in regulating bone formation *in vivo* has recently been described [7]. The discovery that SLUG expression is upregulated during osteogenesis, is positively correlated with the expression of RUNX2 and Lef-1, and is under the control of Lef-1, corroborates the role of Lef/Tcf transcription factors in osteoblasts and highlights mechanisms by which Lef-1 may affect maturation and differentiation of these cells. Our results further support the hypothesis that SLUG may have a distinct role in normal human osteoblasts, and may be positively regulated by activity of canonical Wnt/β-catenin signalling pathway. Therefore, as far as bone tissue is concerned, SLUG should not be considered exclusively as a marker of malignancy and an attractive target for therapeutic modulation of bone metastasis and osteosarcoma invasiveness, as indirectly suggested by Guo et al. [31].

Considering the widespread expression of SLUG in all osteoblast samples analyzed, we cannot exclude that SLUG, which encodes an evolutionarily conserved antiapoptotic transcription factor, may confer a survival advantage in osteoblasts, as demonstrated for leukemic B cell progenitors [47].

In conclusion, although further studies are required to elucidate whether the two Lef-1 isoforms recently identified [6] may have distinguishable activities in determining the proper levels of SLUG expression, our study clearly shows that β-catenin/Lef-1 signalling is involved in the regulation of this gene in normal human osteoblasts. In addition, other factors may contribute to the SLUG gene regulation. At present, the relationship between other *cis*-regulatory elements in the SLUG promoter and osteoblast-inducing signals is completely unknown. The most likely candidates for this function are SLUG and RUNX2, which could associate with the E boxes and RUNX binding sites present in the promoter. Therefore, further work will be necessary to evaluate a potential transcription autoregulation and to elucidate the association between SLUG and RUNX2 expression. Our hypothesis is that SLUG might represent an interesting molecule for normal skeletogenesis acting inside the recently proposed [48] large signalosome in which inputs from Wnt/β-catenin/Lef-1 signalling, steroid receptors, BMPs, and kinases converge to induce differentiation of osteoblast precursors. With this in mind, we also speculate that study of the association between SLUG and some organizers of osteoblastic phenotype may improve the characterization of the human osteoblast differentiation stages. In particular, this may be relevant in approaches addressed to the discovery of new molecular targets to use in bone repair and regenerative medicine.

Conclusions

In this study we showed that transcription factor SLUG is expressed in both normal human osteoblasts and their mesenchymal precursors, and that Lef-1, a mediator of the Wnt/β-catenin signalling pathway, is recruited "*in vivo*" to its promoter acting as a positive transcriptional regulator. The relationship between SLUG and Wnt signalling has been confirmed demonstrating that increase in β-catenin levels induced a significative SLUG gene expression increase.

In conclusion, our findings reveal the biological functions of SLUG in the context of bone tissue showing that it is positively correlated with the osteogenesis, and highlights mechanisms by which Lef-1 may affect maturation and differentiation of osteoblasts.

Methods

Construction of reporter plasmid

Promoter region (+1 to -982 bp) of the human SLUG promoter was amplified by PCR from human genomic DNA using SLUG F genomic primer as sense primer and SLUG R genomic primer as antisense primer (Table 1). The PCR product was subcloned upstream of a firefly luciferase (LUC) gene in the promoter-less pGL3-Basic vector (Promega, Madison, WI) using MluI and BglII restriction sites, and the presence of the insert was confirmed by restriction digestion.

Cell culture, plasmids and transient transfection

Human primary osteoblasts were obtained from bone samples collected during nasal septum surgery, and were cultured as previously described [49]. Recruitment of subjects donating osteoblasts was in accordance with approved procedures, and informed consent was obtained from each patient. Briefly, the bone was cut into small pieces which were rinsed and then cultured in Eagle's MEM (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS) (CELBIO EuroClone, Milan, Italy), 2 mM glutamine, 100 units/ml

Table 1 Primers used in this study

Oligo name	*Primer sequences 5'-3'*
Primers for reporter construct	
Slug F	TGTCAAAAGTGTGAGAGAAT
Slug R	CTTGCCAGCGGGTCTGGC
Primers for ChIP	
Region 1 F	GAGGTTACCTCTCTTGAAAATACT
Region 1 R	GGAAGAAAGATCCAATCACA
Region 2 F	CCAGGCCAGATCCCAGGAGAGC
Region 2 R	GCCTCTGGTGTTAATGAGAGCCTA
Region 3 F	TGCCCCCCTTCTCTGCCAGAGTT
Region 3 R	TTCCGCGAAGCCAGGGGCAGCG

The sequence and the name of the forward (F) and reverse (R) primers for the construction of reporter plasmid and for Chromatin immunoprecipitation (ChIP) are reported.

penicillin, 100 μg/ml streptomycin, and 50 μg/ml ascorbate at 37°C in a humidified atmosphere of 5% CO_2. After about 5-7 days, outgrowth of bone cells from the bone chips commenced, and confluency in 9 cm^2 dishes was usually reached after 4-6 weeks. For the studies here presented, only first passage cells were used.

Mesenchymal stem cells were obtained from Wharton's Jelly of human umbilical cord after the mothers' consent and approval of the "Ethical committee of University of Ferrara and S.Anna Hospital ", and characterized as previously described [36].

The expression vector for full-length Lef-1 (K14-myc-hLEF1) was a gift from Elaine Fuchs and Rebecca C. Lancefield (Howard Hughes Medical Institute, The Rockfeller University, Lab. of Mammalian Cell Biology & Development, New York U.S.A.). The TCF reporter plasmid TOP FLASH was kindly provided by Rolf Kemler (Max Planck Institute, Heidelberg, Germany).

For transient transfection assays, 50000 cells/ml were seeded in 24 or 6 multiwell plates. After 24 h, cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and 0.5 μg of reporter construct where not specified.

SB216763 was purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA), and dissolved in DMSO.

Analysis of the osteoblast phenotype

For alkaline phophatase staining, prefixed mono-layered cells were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (Alkaline Phosphatase Leukocyte kit, Sigma). The presence of sites of ALP activity appeared as blue cytoplasmatic staining.

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma) in the cells cultured for up to 35 days in osteogenic medium consisting in DMEM, high-glucose, supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 mM dexamethasone and 50 mM ascorbate. In the committed cells, the osteogenic medium was changed every three days. The cells were then fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM AR-S (pH 4.2) for 10 min. at room temperature, washed five times with deionized water and incubated in PBS for 15 min. to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope.

Luciferase reporter gene assays

For experiments assessing activation of the SLUG promoter, 1 μg of reporter plasmid was cotransfected with 2.5 μg of expression vectors for Lef1 (K14-myc-hLEF1) and 0.25 μg of pCMV-Sport-βgal (Invitrogen). The cells were lysed 48 h after transfection using the reporter lysis buffer (Promega, Madison, WI). Luciferase and β-galactosidase activities were determined with luciferase and Beta-Glo assay systems respectively (Promega, Madison, WI). Their activities were normalized with respect to total protein amount.

Real-time RT-PCR analysis

For mRNA analysis total cellular RNA was extracted using Total RNA Isolation System (Promega) and cDNA synthesis was performed for 1 h at 42°C using 1 μg of total RNA as a template and 100 U of reverse transcriptase ImProm-II (Promega) as previously described [49]. The level of mRNA expression was analyzed by quantitative real-time PCR using the ABI Prism 7700 system (Applied Biosystems) and the following TaqMan MGB probes: 5' FAM-ATGATGAAAGGTGGGATACGAAAAG-TAMRA 3' for SLUG, 5' FAM-GAACCCAGAAGGCACAGACAGAAG-TAMRA 3' for RUNX2, 5' FAM-TCTAATCCAGAGTTTACCTTCCAGC-TAMRA 3' for SNAIL1, 5'FAM-GAGACGCAGAGAGAAATCAATGGTG-TAMRA 3' for SNAIL3, and 5' FAM-CATGTCCAGGTTTTCCCATCATATG-TAMRA 3' for Lef-1; GAPDH mRNA was used as an endogenous control (Applied Biosystems Inc, Foster City, CA, USA) and quantification was performed using a TaqMan assay. The mRNA levels of target genes were corrected for GAPDH mRNA levels. All PCR reactions were performed in triplicate for each sample and were repeated three times. All experimental data were expressed as the mean ± SEM.

Western Blot analysis

For Western Blot analysis, the cells were washed twice with ice-cold PBS and cell lysates were prepared as previously reported [50]. 25 μg of each sample was then

electrophoresed on a 12% SDS-polyacrylamide gel. The proteins were then transferred onto an Immobilon-P PVDF membrane (Millipore Corporation, 900 Middlesex Tpk Billerica, USA). After blocking with PBS-0.05% Tween 20 and 5% dried milk, the membrane was probed with the following antibodies: SLUG (L40C6) from Cells Signaling Technology Inc. (Danvers, CA, USA), RUNX2 (sc-10758) from Santa Cruz Biotechnology (Santa Cruz, CA), Lef-1 (L7901) from Sigma Aldrich (St Luis, MO, USA), IP3K (06-195) and Active-β-catenin from Upstate Biotechnology, Inc. (Lake Placid, NY). After washing with PBS-Tween, the membranes were incubated with peroxidase-conjugated anti-rabbit antibody (1:50000) or anti-mouse (1:2000) (Dako, 2600 Glostrup, Denmark) in 5% non-fat milk. Immunocomplexes were detected using Supersignal West Femto Substrate (Pierce). Anti-IP(3)K was used to confirm equal protein loading.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described [49] using the standard protocol supplied by Upstate Biotechnology (Lake Placid, NY) with their ChIP assay reagents.

The cells were cross-linked with 1% formaldehyde for 10 min at 37°C, washed in ice-cold PBS and resuspended in SDS lysis buffer for 10' on ice. Samples were sonicated, diluited 10-fold in diluition buffer supplemented with protease inhibitors and precleared with 80 μl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 5 μg of anti- Lef-1 (sc-8591), TCF-1 (sc-13025) and TCF-4 (sc-13027) (Santa Cruz Biotec, Ca, USA) overnight at 4°C. Immunocomplexes were mixed with 80 μl of DNA-coated protein A-agarose followed by incubation for 1 h at 4°C. Beads were collected and sequentially washed 5 times with 1 ml each of the following buffers: low salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH-8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-pH 8.1) and TE buffer. The immunocomplexes were eluted two times by adding a 250 μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$ and the cross-linking reactions were reversed by incubation at 65°C for 4 h. Further, the samples were digested with proteinase K (10 mg/ml) at 42°C for 1 h, DNA was recovered by phenol/chloroform extractions, ethanol precipitated using 1 μl of 20 mg/ml glycogen as the carrier, and resuspended in sterile water. For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). PCR was performed to analyze the presence of DNA precipitated by specific antibodies by using the primers reported in Table 1.

Each PCR reaction was performed with 10 μl of the bound DNA fraction or 2 μl of the input. The PCR was performed as follows: preincubation at 95°C for 5', 30 cycles of 1' denaturation at 95°C, 1' annealing at 62°C and 1 min at 72°C, with one final incubation at 72°C for 5'. No-antibody control was included in each experiment.

Statistical analysis

Data are presented as the mean ± SEM from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance and the Student's t-test. A P value < 0.05 was considered statistically significant.

Additional file 1: Detection of SLUG expression by quantitative RT-PCR in osteoblastic-like cell lines and hOB samples. The level of SLUG was examined by quantitative RT-PCR in U2OS, SaOS-2, Hobit, CAL72 osteoblastic-like cell lines and in eight hOB samples. MCF7 breast cancer cell line was used as negative control. The cDNA obtained from total RNA was subjected to quantitative TaqMan RT-PCR for SLUG transcript analysis. The experiments were carried out in triplicate, the expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. ΔΔCt method was used to value the gene expression; standard error of the mean (SEM) was calculated.
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Additional file 2: Treatment of osteoblastic-like cell lines with the glycogen synthase kinase (GSK-3β) inhibitor, SB216763. The levels of SLUG expression was examined by quantitative TaqMan RT-PCR in U2OS, SaOS-2, Hobit, CAL72 osteoblastic-like cell lines treated with SB216763 (10, 25 and 50 μM) or with the only vehicle (-), up to 3 days.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2199-11-13-S2.PPT]

Abbreviations

Lef-1: Lymphoid Enhancer binding Factor-1; hOBs: human osteoblasts; hMSCs: human mesenchymal stem cells; FBS: fetal bovine serum; ChIP: Chromatin Immunoprecipitation; HMG: high mobility group; RUNX2: Runt-related transcription factor 2; ALP: alkaline phosphatase.

Acknowledgements

This research was supported by grants from MIUR COFIN-2005, STAMINA project, Regione Emilia Romagna, Programma di Ricerca Regione Universita' 2007-2009, the Fondazione Cassa di Risparmio di Ferrara. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento. English revision of the article was carried out by dr. Elizabeth Jenkins.

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Authors' contributions

EL participated in the study design, cloned SLUG promoter, carried out the characterization of osteoblasts, ChIP assays, and experiments with SB216763. TF was responsible for the luciferase assays and helped the ChIP assays. ET performed the western blot assays, RT-PCR analysis and contributed to cell

culture experiments. LP was responsible for the isolation and characterization of mesenchymal stem cells from Wharton's Jelly. AP and SP collected bone samples during surgery interventions. RG contributed to data interpretation and provided useful suggestions. RP designed the studies, analyzed data and wrote the manuscript. All authors helped to draft the manuscript, and to read and approve the final version.

Received: 31 August 2009
Accepted: 3 February 2010 Published: 3 February 2010

References

1. Barker N: **The canonical Wnt/beta-catenin signalling pathway.** *Methods Mol Biol* 2008, **468**:5-15.
2. Westendorf JJ, Kahler RA, Schroeder TM: **Wnt signaling in osteoblasts and bone diseases.** *Gene* 2004, **341**:19-39.
3. Leucht P, Minears S, Ten Berge D, Nusse R, Helms JA: **Translating insights from development into regenerative medicine: the function of Wnts in bone biology.** *Semin Cell Dev Biol* 2008, **19(5)**:434-443.
4. Day TF, Guo X, Garrett-Beal L, Yang Y: **Wnt/beta-catenin signalling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis.** *Dev Cell* 2005, **8(5)**:739-50.
5. Matsuzaki E, Takahashi-Yanaga F, Miwa Y, Hirata M, Watanabe Y, Sato N, Morimoto S, Hirofuji T, Maeda K, Sasaguri T: **Differentiation-inducing factor-1 alters canonical Wnt signalling and suppresses alkaline phosphatase expression in osteoblast-like cell lines.** *J Bone Miner Res* 2006, **21(8)**:1307-16.
6. Hoeppner LH, Secreto F, Jensen ED, Li X, Kahler RA, Westendorf JJ: **RUNX2 and bone morphogenic protein 2 regulate the expression of an alternative Lef1 transcript during osteoblast maturation.** *J Cell Physiol* 2009, **221(2)**:480-89.
7. Noh T, Gabet Y, Cogan J, Shi Y, Tank A, Sasaki T, Criswell B, Dixon A, Lee C, Tam J, Kohler T, Segev E, Kockeritz L, Woodgett J, Müller R, Chai Y, Smith E, Bab I, Frenkel B: **Lef1 haploinsufficient mice display a low turnover and low bone mass phenotype in a gender- and age-specific manner.** *PLoS One* 2009, **4(5)**:e5438.
8. Nieto MA: **The snail superfamily of zinc-finger transcription factors.** *Nat Rev Mol Cell Biol* 2002, **3(3)**:155-66.
9. Hemavathy K, Ashraf SI, Ip YT: **Snail/SLUG family of repressors: slowly going into the fast lane of development and cancer.** *Gene* 2000, **257(1)**:1-12.
10. De Craene B, van Roy F, Berx G: **Unraveling signalling cascades for the Snail family of transcription factors.** *Cell Signal* 2005, **17(5)**:17535-47.
11. Hemavathy K, Guru SC, Harris J, Chen JD, Ip YT: **Human SLUG is a repressor that localizes to sites of active transcription.** *Mol Cell Biol* 2000, **20(14)**:5087-95.
12. Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F: **The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion.** *Mol Cell* 2001, **7(6)**:1267-78.
13. Przybylo JA, Radisky DC: **Matrix metalloproteinase-induced epithelial-mesenchymal transition: Tumor progression at Snail's pace.** *Int J Biochem Cell Biol* 2007, **39(6)**:1082-88.
14. Perez-Mancera PA, Gonzals-Herrero I, Perez-Caro M, Gutierrez-Cianca N, Flores T, Gutierrez-Adan A, Pintado B, Sánchez-Martín M, Sánchez-García I: **SLUG in cancer development.** *Oncogene* 2005, **24(19)**:3073-82.
15. Castro Alves C, Rosivatz E, Schott C, Hollweck R, Becker I, Sarbia M, Carneiro F, Becker KF: **SLUG is overexpressed in gastic carcinomas and may act synergistically with SIP1 and Snail in the down-regulation of E-cadherin.** *J Pathol* 2007, **211(5)**:507-15.
16. Hajra KM, Chen DY, Fearon ER: **The SLUG zinc-finger protein represses E-cadherin in breast cancer.** *Cancer Res* 2002, **62(6)**:1613-18.
17. Storci G, Sansone P, Trere D, Tavolari S, Taffurelli M, Ceccarelli C, Guarnieri T, Paterini P, Pariali M, Montanaro L, Santini D, Chieco P, Bonafé M: **The basal-like breast carcinoma phenotype is regulated by SLUG gene expression.** *J Pathol* 2008, **214(1)**:25-37.
18. Barrallo-Gimeno A, Nieto MA: **The Snail genes as inducers of cell movement and survival: Implications in development and cancer.** *Development* 2005, **132(14)**:3151-61.
19. Pérez-Losada J, Sánchez-Martín M, Rodríguez-García A, Sánchez ML, Orfao A, Flores T, Sánchez-García I: **Zinc-finger transcription factor Slug contributes to the function of the stem cell factor c-kit signaling pathway.** *Blood* 2002, **100(4)**:1274-86.
20. Pérez-Mancera PA, Bermejo-Rodríguez C, González-Herrero I, Herranz M, Flores T, Jiménez R, Sánchez-García I: **Adipose tissue mass is modulated by SLUG (SNAI2).** *Hum Mol Genet* 2007, **16(23)**:2972-86.
21. Carl TF, Dufton C, Hanken J, Klymkowsky MW: **Inibition of neural crest migration in Xenopus using antisense SLUG RNA.** *Dev Biol* 1999, **213(1)**:101-15.
22. LaBonne C, Bronner-Fraser M: **Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its susequent migration.** *Dev Biol* 2000, **221(1)**:195-205.
23. Cohen ME, Yin M, Paznekas WA, Schertzer M, Wood S, Jabs EW: **Human SLUG gene organization, expression, and chromosome map location on 8q.** *Genomics* 1998, **51(3)**:468-71.
24. Savagner P, Kusewitt DF, Carver EA, Magnino F, Choi C, Gridley T, Hudson LG: **Developmental transcription factor SLUG is required for effective re-epithelization by adult keratinocytes.** *J Cell Physiol* 2005, **202(3)**:858-66.
25. Yamamoto Y, Banas A, Murata S, Ishikawa M, Lim CR, Teratani T, Hatada I, Matsubara K, Kato T, Ochiya T: **A comparative analysis of the transcriptome and signal pathway in hepatic differentiation of human adipose mesenchymal stem cells.** *FEBS* 2008, **275(6)**:1260-73.
26. Basch ML, Bronner-Fraser M, Garcia-Castro M: **Specification of the neural crest occurs during gastrulation and requires Pax7.** *Nature* 2006, **441(7090)**:218-22.
27. Le Douarin NM, Dupin E, Ziller C: **Genetic and epigenetic control in neural crest development.** *Curr Opin Genet Dev* 1994, **4**:685-95.
28. Sarnat HB, Flores-Sarnat L: **Embriology of the neural crest: its inductive role in the neurocutaneous syndromes.** *J Chil Neurol* 2005, **20(8)**:637-43.
29. Sakai D, Suzuki T, Osumi N, Wakamatsu Y: **Cooperative action of Sox-9, Snail2 and PKA signaling in early neural crest development.** *Development* 2006, **133(7)**:1323-33.
30. Lambertini E, Lisignoli G, Torreggiani E, Manferdini C, Gabusi E, Franceschetti T, Penolazzi L, Gambari R, Facchini A, Piva R: **SLUG gene expression supports human osteoblast maturation.** *Cell Mol Life Sciences* 2009, **66(22)**:3641-53.
31. Guo Y, Zi X, Koontz Z, Kim A, Xie J, Gorlick R, Holcombe RF, Hoang BH: **Blocking Wnt/LRP5 signaling by a soluble receptor modulates the epithelial to mesenchymal transition and suppresses met and metalloproteinases in osteosarcoma SaOS-2 cells.** *J Orthop Res* 2007, **25(7)**:964-71.
32. Sakai D, Tanaka Y, Endo Y, Osumi N, Okamoto H, Wakamatsu Y: **Regulation of SLUG transcription in embryonic ectoderm by beta-catenin-Lef/Tcf and BMP-Smad signalling.** *Dev Growth Differ* 2005, **47(7)**:471-82.
33. Vallin J, Thuret R, Giacomello E, Faraldo MM, Thiery JP, Broders F: **Cloning and characterization of three Xenopus SLUG promoters reveal direct regulation by Lef/beta-catenin signalling.** *J Biol Chem* 2001, **276(32)**:30350-8.
34. Saegusa M, Hashimura M, Kuwata T, Okayasu I: **Requirement of the Akt/β-catenin pathway for uterine carcinosarcoma genesis, modulating E-cadherin expression through the transactivation of SLUG.** *Am J Pathol* 2009, **174(6)**:2107-15.
35. Hong CF, Chou YT, Lin YS, Wu CW: **MAD2B, a novel TCF4-binding protein, modulates TCF4-mediated epithelial-mesenchymal transdifferentiation.** *J Biol Chem* 2009, **284(29)**:19613-22.
36. Penolazzi L, Tavanti E, Vecchiatini R, Lambertini E, Vesce F, Gambari R, Mazzitelli S, Mancuso F, Luca G, Nastruzzi C, Piva R: **Encapsulation of mesenchymal stem cells from Wharton's jelly in alginate microbeads.** *Tissue Eng Part C Methods* 2010, **16(1)**:141-155.
37. Marie PJ: **Transcription factors controlling osteoblastogenesis.** *Arch Biochem Biophys* 2008, **473(2)**:98-105.
38. de Frutos CA, Dacquin R, Vega S, Jurdic P, Machuca-Gayet I, Nieto MA: **Snail1 controls bone mass by regulating Runx2 and VDR expression during osteoblast differentiation.** *EMBO J* 2009, **28(6)**:686-96.
39. Katoh M, Katoh M: **Identification and characterization of human SNAIL3 (SNAI3) gene in silico.** *Int J Mol Med* 2003, **11(3)**:383-8.
40. Cohen P, Frame S: **The renaissance of GSK3.** *Nat Rev Mol Cell Biol* 2001, **2(10)**:769-76.
41. Patel S, Doble B, Woodgett JR: **Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged word?.** *Biochem Soc Trans* 2004, **32(Pt 5)**:803-8.

42. Conacci-Sorrell M, Simcha I, Ben-Yedidia T, Blechman J, Savagner P, Ben-Ze'ev A: **Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, SLUG, and MAPK.** *J Cell Biol* 2003, **163(4)**:847-57.
43. Nusse R: **WNT targets. Repression and activation.** *Trends Genet* 1999, **15(1)**:1-3.
44. Case N, Ma M, Sen B, Xie Z, Gross TS, Rubin J: **Beta-catenin levels influence rapid mechanical responses in osteoblasts.** *J Biol Chem* 2008, **283(43)**:29196-205.
45. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB: **Canonical WNT signalling promotes osteogenesis by directly stimulating RUNX2 gene expression.** *J Biol Chem* 2005, **280(39)**:33132-40.
46. Kahler RA, Yingst SM, Hoeppner L, Jensen ED, Krawczak D, Oxford JT, Westendorf JJ: **Collagen 11a1 is indirectly activated by lymphocyte enhancer-binding facor 1 (Lef1) and negatively regulates osteoblast maturation.** *Matrix Biol* 2008, **27(4)**:330-8.
47. Inukai T, Inoue A, Kurosawa H, Goi K, Shinjyo T, Ozawa K, Mao M, Inaba T, Look AT: **SLUG a ces-1-Related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF Oncoprotein.** *Mol Cell* 1999, **4(3)**:343-52.
48. Kousteni S, Almeida M, Han L, Bellido T, Jilka RL, Manolagas SC: **Induction of osteoblast differentiation by selective activation of kinase-mediated actions of the estrogen receptor.** *Mol Cell Biol* 2007, **27(4)**:1516-30.
49. Penolazzi L, Zennaro M, Lambertini E, Tavanti E, Torreggiani E, Gambari R, Piva R: **Induction of estrogen receptor alpha expression with decoy oligonucleotide targeted to NFATc1 binding sites in osteoblasts.** *Mol Pharmacol* 2007, **71(6)**:1457-62.
50. Lambertini E, Tavanti E, Torreggiani E, Penolazzi L, Gambari R, Piva R: **ER alpha and AP-1 interact *in vivo* with a specific sequence of the F promoter of the human ER alpha gene in osteoblasts.** *J Cell Physiol* 2008, **216(1)**:101-10.

doi:10.1186/1471-2199-11-13
Cite this article as: Lambertini *et al.*: SLUG: a new target of lymphoid enhancer factor-1 in human osteoblasts. *BMC Molecular Biology* 2010 **11**:13.

MINERVA STOMATOL 2010;59:00-00

Effect of hydroxyapatite-based biomaterials on human osteoblast phenotype

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The present study evaluated human primary osteoblasts and two different osteoblast - like cell lines behaviour when cultured in presence of different hydroxyapatite-based (HA) biomaterials (SINTlife™ - FIN-CERAMICA S.p.a., Faenza, Italy; Bio-Oss™, Geistlich Biomaterials, Woulhusen, Switzerland; Biostite™ - GABA Vebas, San Giuliano Milanese, MI, Italy), focusing attention on the effect of HA/Biostite™ in terms of modulation of osteoblastic differentiation. Analysis were about adhesion, proliferation and mineralization activity. Runt-related transcription factor 2 (Runx2), Estrogen Receptor alpha (ERα) expression and alkaline phosphatase activity (ALP) were measured as osteoblastic differentiation markers. Determination of viable cells was done with MTT colorimetric assay. Scanning electron microscopy (SEM) analysis was performed on biomaterial-treated cells. All hydroxyapatite-based biomaterials didn't affect cells morphology and viability, whereas only presence of HA/Biostite™ improved cells adhesion, growth and differentiation. Adhesion and spreading of the primary cells on HA/Biostite™ were the same showed by two different osteoblast – like cell lines. These results have important implications for both tissue-engineered bone grafts and enhancement of HA implants performance, to develop new teeth's supporting structure therapies and replacement.

Key words: **Osteoblasts - Bone substitutes - Biocompatible materials.**

The development of an ideal material to stimulate a specific cellular response such that damaged tissues can be regenerated with full recovery of their biological function has long been a major goal in tissue engineering.[1]

Among bone substitutes, hydroxyapatite (HA) has been largely used as graft material and scaffold in the past decade.[2-4] HA is the major bone and dental mineral component and can promote faster bone regeneration and direct bonding to regenerated bone without intermediate connective tissue.[5] Different HA scaffolds showed excellent biocompatibility, bioactivity, and osteoconductivity and

Acknowledgments.—This work was supported in part by grants from STAMINA, and Fondazione Cassa di Risparmio di Ferrara. Elisabetta Lambertini is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

The authors declare that they have not conflict of interest.

Received on September 24, 2009.

Accepted for publication on February 15, 2010.

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have been developed as bone substitute both in bone grafting and dental devices.[6]

The cell compatibility with HA was mainly evaluated exposing osteoblasts to it. Cell adhesion, proliferation, spread and morphology, as well as different bone-specific characteristics of cell in presence of bone substitute have been investigated.[7]

Cell culture models are commonly used as "in vitro" approach to study cellular phenomena and to investigate tissue–biomaterial interactions.[8, 9] Different kind of human, rat or mouse osteoblast-like cells, all expressing osteoblast-specific proteins and producing mineralized nodules during "in vitro" maturation, have been generally considered a good model for osteoblast growth and differentiation. All these data provide evidence of possible achieving in bone regeneration, but current experiences and discrepancies present in literature suggest caution in drawing predictable and uniform conclusion from such "in vitro" models.[10] It is well known that the variability of cell sources and species can likely influence the downstream responses of different cells to the same biomaterial. In particular, the production of different modulating cofactors or the different expression of integrins and receptors on cellular membrane induce critical developmental differences between different species. These differences can influence outcomes in specific signaling cascades, cell adhesion and all complex interactions, that take place at osteoblast-biomaterial interface.[11] Moreover, in many different experiments osteosarcoma cell lines are used as osteoblast-like cells, and when the normal primary osteoblasts are employed the demonstration that they exibit authentic osteoblastic markers is usually not shown in results. Several evidences demonstrated that during osteoblast-biomaterial interaction the cells are able to discriminate not only chemical, physical, and structural properties of material, but also differences in surface roughness and topography.[12] Also these properties are variable and strongly influenced by the experimental model chosen.

Previously clinical effectiveness of HA/Biostite scaffolds, such as Biostite™ and Bio-Oss™ in the reconstruction of deep periodontal intraosseous defects was tested.[13] Both Biostite™ and Bio-Oss™ determined a clinically and statistically significant improvement of clinical conditions, in terms of clinical attachment gain, probing pocket depth reduction and radiographic defect depth reduction. However, no statistically significant differences among treatments were observed with regard to the treatment outcomes.

We investigated here the effects of 3 different hydroxyapatite biomaterials in cell culture systems, using different cellular models. Human primary osteoblasts (hOBs) and two osteosarcoma cell lines, SaOS-2 and Hobit, that exhibit morphological, immuno-histochemical and molecular characteristics of the osteoblastic lineage, were tested.

On the basis of previous considerations, we designed the present study in order to evaluate cells behaviour when cultured in presence of different hydroxyapatite-based biomaterials, focusing attention on the effect of HA/Biostite™ in terms of modulation of osteoblastic differentiation.

Cell adhesion, proliferation and mineralization activity were analyzed. Alkaline phosphatase activity (ALP) was evaluated, together with osteoblastic markers such as Runt-related transcription factor 2 (Runx2) and Estrogen Receptor alpha (ERα), that are transcription factors correlated with specific osteoblastic developing stage.[14, 15]

Materials and methods

Human osteoblast cells harvesting procedures

Autogenous bone particulate was collected from different human subject undergoing oral surgical procedures, after elevation of a mucoperiosteal flap. At the end of each surgery, before suture, an adequate amount (*i.e.*>500 mg) of autogenous bone graft was harvested from the surgical site using a bone scraper (Safescraper®, Meta C.G.M. Reggio Emilia, Italy) or a mini-bone scraper (Micross®, Meta C.G.M., Reggio Emilia, Italy), as described previously.[16-18] Harvesting procedures of autogenous bone were conduct-

ed in full accordance with the "Declaration of Helsinki" as adopted by the 18th World Medical Assembly in 1964 and revised in Edinburgh (2000) and the Good Clinical Practice guidelines. Before surgery, each subject provided a verbal informed consent.

Cell culture and biomaterials

SaOS-2 and Hobit osteosarcoma cell lines were maintained in D-MEM (Euroclone S.p.A., MILANO – ITALY), supplemented with 10% Fetal Bovine Serum (FBS) (Euroclone S.p.A., MILANO - ITALY) in a humidified atmosphere of 5% CO_2.

Autogenous cortical bone particulate was washed in sterile saline solution and incubated in T25 falcon Flasks containing D-MEM and HAM'S nutrient mixture F-12 (1:1) (Euroclone S.p.A., Milano – Italy), 20% FBS and antibiotics (Streptomycin 50 mg/ml and Penicillin 100 mg/ml) at 37 °C in an humidified atmosphere of 5% CO_2. Subcultures were obtained 20 days later. At day 20 after Trypsin-EDTA treatment all different cells were transferred to 24 or 6 well plates and maintained in a solution of D-MEM and HAM F12 medium (1:1) supplemented with 20% FBS and antibiotics. All the experiments were performed with cell cultures at the first passage in culture.

Human primary osteoblasts and osteosarcoma cell lines were exposed to the 3 different hydroxyapatite biomaterials (SINTlife™ - FIN-CERAMICA S.p.a., Faenza, Italy; Bio-Oss™, Geisthich Biomaterials, Woulhusen, Switzerland; Biostite™ - GABA Vebas, San Giuliano Milanese, MI, Italy) at different concentrations of 2.5, 5 and 10 mg/ml.

Cytotoxicity studies

The cytoxicity analysis was done on in vitro cultured hOBs and osteosarcoma cell lines (SaOS-2 and Hobit) exposed separately to the three hydroxyapatite-based biomaterials. For each biomaterial three different concentrations were tested, respectively 2.5, 5 and 10 mg/mL. Determination of viable cells was done with MTT colorimetric assay (thiazolyl blue). MTT assay is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals in the mitochondria of living cells.[19] MTT provides a quantitative determination of viable cells. After 72 hrs of treatments in triplicate, 200 µL of a solution of MTT (5 mg/ml) was added to each well of cells, and the plate was incubated for 2 hours at 37 °C. The medium was removed, and the MTT crystals were solubilized with 50% dimethylformammide (DMF). Spectrophotometric absorbance of each sample was measured at 570 nm.

SEM analysis

$20x10^3$ cells/cm^2 cells (hOBs, Hobit, SaOS-2) were plated and incubated for 4 days with the same amount of Bio-Oss™, SINTlife™ and Biostite™ (5 mg/ml). For SEM analysis samples were fixed in glutharaldeide 2.5% buffered solution and Osmium tetroxide 2% buffered solution, dehydrated and gold coated (Edward Sputter S150). For observation scanning electron microscope Cambridge S360 was used. Images were acquired at different magnification, as indicated for each one.

Alkaline phosphatase analysis

For alkaline phosphatase staining, the Alkaline phosphatase (ALP) Leukocyte kit (Sigma) was used. To perform the test, prefixed mono-layered cells were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (AMPD). The presence of sites of ALP activity appeared as blue cytoplasmatic staining.

ALP activity, with or without the presence of biomaterial, was evaluated in hOBs and cell lines by the hydrolysis of *p*-nitrophenylphosphate (PNPP), according to literature.[20] $20x10^3$ cells/cm^2 were plated and incubated with 5 mg/ml of HA/Biostite™; the enzime activity (expressed as U(nmol/min)/µg of protein) was evaluated at 6 and 21 days for cell lines and hOBs, respectively. One unit was defined as the amount of enzyme which hydrolyzed 1 nmol/PNPP per minute. Cell protein was determined according to the Lowry method.[21]

Alizarin Red staining

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (AR-S, Sigma); 20×10^3 cells/cm^2 were plated and incubated with 5 mg/mL of HA/Biostite™ for 10 and 40 days for cell lines and hOBs, respectively. After 10 and 40 days cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, and stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature. Next, cell preparations were washed five times with deionized water and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnification using a Leitz microscope.

Immunocytochemistry

Immunocytochemistry analysis was performed employing the streptavidin-biotin method using Ultraystain Polyvalent-HRP Immunostaining Kit (Ylem). Cells grown in chamber slides were fixed in cold 100% methanol, and permeabilized with 0.2% (vol/vol) Triton X-100 (Sigma) in TBS (Tris-buffered saline). Cells were incubated in 3% H_2O_2 and the endogenous peroxidase were blocked with Super Block reagent. Afterwards, the primary antibodies, a polyclonal antibody for Runx2 (clone M-70, rabbit anti-human, 1:1000 dilution—Santa Cruz Biotec) and ER((clone MC-20, rabbit anti-human, 1:500 dilution – Santa Cruz Biotec) were applied and incubated at 4°C overnight. Cells were then incubated at room temperature with anti-polyvalent Biotinylated Antibody (Ultraystain Polyvalent-HRP Immunostaining Kit–Ylem). After rinsing in TBS, Streptavidin HRP (Ultraystain Polyvalent-HRP Immunostaining Kit–Ylem) was applied, and then Substrate-chromogen mix (AEC Cromogeno kit–Ylem) was added. After washing, cells were mounted in glycerol/PBS 9:1 and observed using a Leitz microscope.

Statistical analysis

Data are presented as the mean ± SE from at least three independent experiments, where indicated. Statistical analysis was performed by one-way analysis of variance followed by the Student's t test. A P value <0.05 was considered statistically significant.

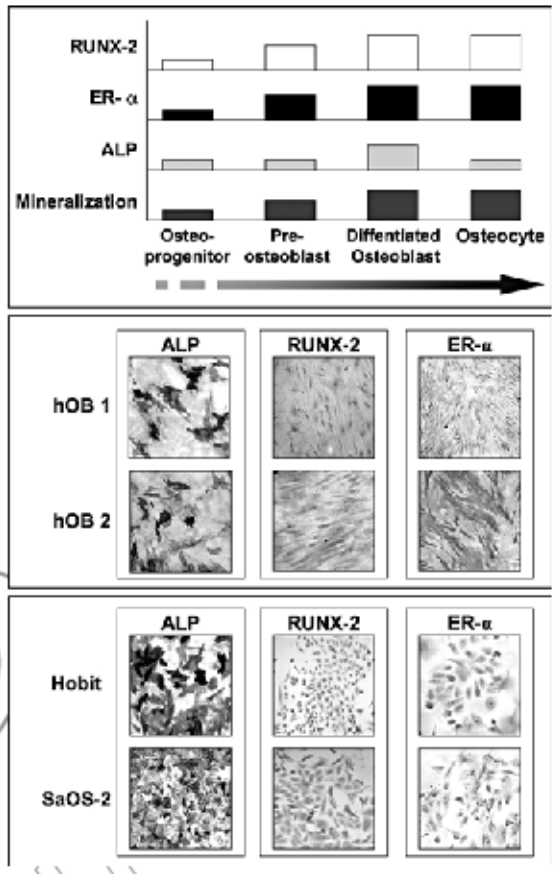

Figure 1.—Human primary osteoblasts and osteoblast-like cell lines; osteoblast markers expression. Human primary osteoblasts and cell lines (Hobit and SaOS-2) used in the experiments were characterized for specific markers expression: ERα and Runx-2 were determined testing the immunoreactivity with the specific antibodies; alkaline phosphatase (ALP) activity was evaluated by colorimetric assay. Positive staining was identified with light microscopy. Original magnification was 20 X. Data made possible to correlate markers levels to a specific osteoblastic developing stage. The variation of the levels of these markers during the osteoblastic development is schematically represented in the upper part of the figure.

Results

Osteoblast phenotype characterization

Before biomaterials exposure and in order to test osteoblastic phenotype, the cells were analyzed for their ALP activity, Runx2 and ERα expression. These osteoblastic markers

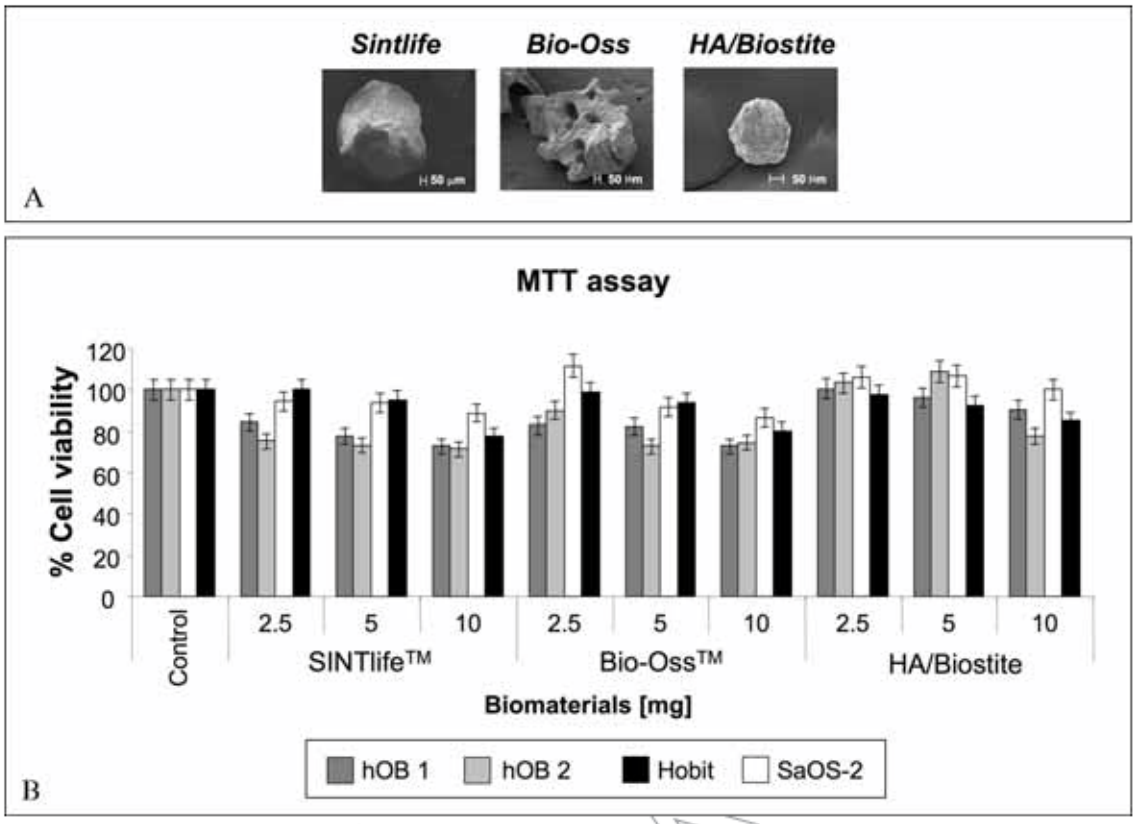

Figure 2.—Human primary osteoblasts and osteoblast-like cell lines viability test. A) SEM analysis of the three biomaterials before cells cultivation; B) human primary osteoblasts (hOB1, hOB2) and cell lines (Hobit and SaOS-2) were exposed to the 3 different hydroxyapatite biomaterial granules (SINTlife™, Bio-Oss™, Biostite™). Different concentrations (2.5 mg, 5 mg, and 10 mg/ml) were used. After 72 hours the amount of living cells was measured using MTT colorimetric assay.

are correlated with specific osteoblastic developing stage, as indicated in Figure 1, top scheme. ALP activity, an early osteoblastic marker, is associated with extracellular matrix maturation. Its level decreases with osteoblast differentiation, and it was analyzed by colorimetric reaction, as described in the Material and methods section and in literature before.[22] Runx2 and ERα are two important transcription factors that play a crucial role in osteoblast development and in maintainance of a functional osteoblastic phenotype.[23] Runx2 is an osteoblast-specific transcription factor required for the differentiation of mesenchymal progenitor cells towards the osteoblastic lineage. Its expression, as well as ERα, increased throughout the stages of osteoblast differentiation, and was evaluated by immunocytochemical analysis. As results from this analysis, a specific differentiation stage based on different levels of expression marker may be ascribed to each experimental model. All cells used in our experiments exibited osteoblastic phenotype, being Runx2 and ERα immunopositive and showing substantial ALP activity.

Cytotoxicity analysis

MTT analysis was performed after 72 hours on cells cultured directly exposed to the tested materials. Viability of the osteoblasts seeded onto the HA/Biostite™ was compared with that of the cells seeded onto other materials containinig HA (SINTlife™ and Bio-Oss™). The macroscopic morphology of the three different materials is shown in Figure 2A. The granules with concentrations of 2.5,

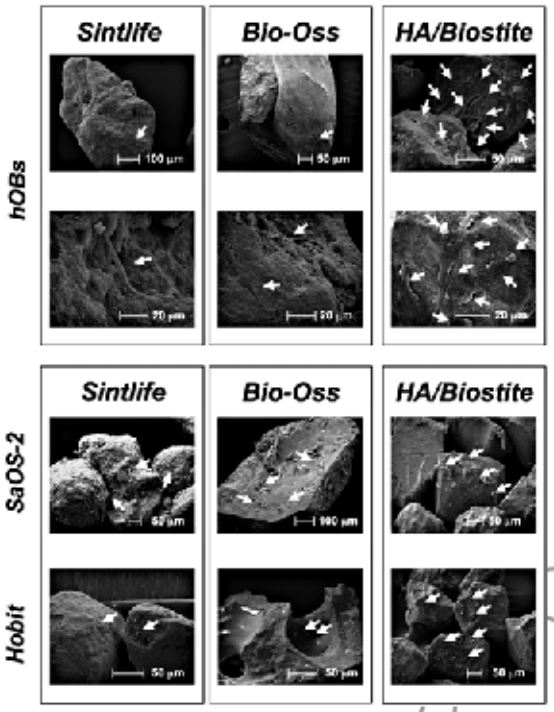

Figure 3.—SEM measurement. Scanning electron microscopic study of hOBs, SaOS-2 and Hobit cells cultivated for 4 days onto the three different hydroxyapatite surfaces. SEM images were at 200 X and at 800 X magnifications. The presence of osteoblasts is indicated by arrows.

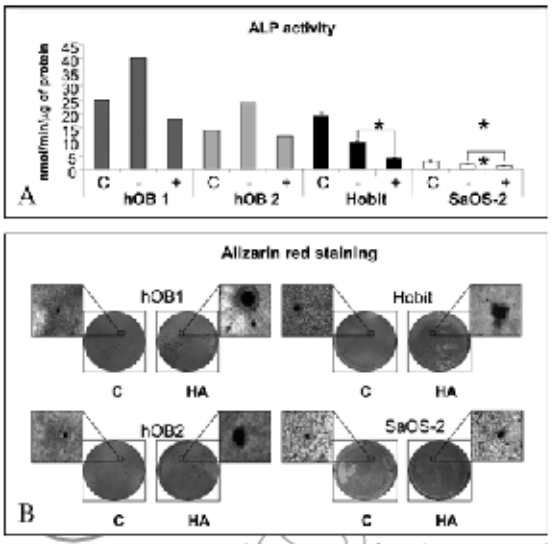

Figure 4.—ALP activity in the presence of HA/Biostite™ and mineralization staining. A) ALP activity was evaluated after incubation with 5 mg/ml of HA/Biostite™ at 6 days for cell lines (Hobit and SaOS-2) and 21 days for human primary osteoblasts (+); data are compared with control cells not seeded (-). ALP activity was also documented at baseline (C, before seeding each cells group onto biomaterial). For Hobit and SaOS-2 cells, data are mean of three different experiments. B) Alizarin red staining in the presence of HA/Biostite™. Calcium deposition was detected after incubation with 5 mg/ml of HA/Biostite™ at 10 days for cell lines (Hobit and SaOS-2) and at 40 days for human primary osteoblasts (HA); control cells not seeded are also showed (C). Magnification was 20 X in the upper inserts, and 2 X for each well. *P<0.01; **P<0.005).

5 and 10 mg/ml were dispersed into MEM medium with osteoblast cells. As shown in Figure 2B, HA/Biostite™ and the other two materials exhibited no appreciable cytotoxicity under our cell culture conditions (cells viability was higher than 75%).

Scanning electron microscopy analysis

In Figure 3 representative SEM fields show the adhesion ability of the cells after a culture period of 4-days on HA/Biostite™ surface in comparison with SINTlife™ and Bio-Oss™. This morphological analysis demonstrated that in general the cells did not spread well on all three analyzed biomaterials and did not create cell-to-cell contacts. Nevertheless, the cells seem to prefer HA/Biostite™. In particular, on HA/Biostite™ hOBs adhered to the surfaces similary to cell lines. These experiments have been repeated three times, with same results.

SEM observations correlated with the microscope observation of the proliferation rate demonstrating that the cells grew better on HA/Biostite™ in comparison with SINTlife™ and Bio-Oss™.

Effect of HA/Biostite™ on osteoblastic differentiation

Based on SEM analysis HA/Biostite™ was selected for further investigations on its effects on osteoblastic differentiation. At this purpose, ALP activity of osteoblast-like cell lines and hOBs cultured in combinations with the biomaterial was evaluated by the hydrolysis of p-nitrophenylphosphate (PNPP), at 21° and 6° day respectively, as described in the Material and methods section.

The results showed that, as expected, ALP activity basal level was different for each experimental model and that, in all cases

examined, the presence of HA/Biostite™ negatively affected the ALP activity (Figure 4A). Because ALP activity decreases with osteoblastic terminal differentiation, our data suggest that the presence of HA/Biostite™ could induce a differentiation stimulus. This was confirmed by a preliminary mineralization test (by Alizarin Red S staining) on osteosarcoma cell lines and hOBs, after 10 and 40 days respectively. The positive effect of HA/Biostite™ on the calcium deposition is represented in Figure 4B.

Discussion

The study showed that hOBs obtained from oral surgery can adhere, grow and differentiate *in vitro* in the presence of HA/Biostite™, a HA scaffold biomaterial. The adhesion and spreading of hOBs on HA/Biostite™ are comparable with that showed by osteosarcoma cell line SaOS-2 model system and the SV40 transfected human osteoblastic Hobit cell line, that are certainly favoured in these processes.[24, 25] This suggests that the same properties of HA/Biostite™ make it suitable as bone replacement system with human normal osteoblasts, and this represents an important prerequisite for any successful implant application.

We did not find any negative influence of HA/Biostite™ on cell morphology and viability. Nevertheless, HA/Biostite™ didn't allow high proliferation level, as happened on control plastic surface, confirming the observations made by many other researchers about the difficulty in setting up a very good matrix for bone microenviroment.

This study also showed that biopsy preparation and cell isolation technique based on the alveolar bone chips obtained with the protocol described, both methods are suitable to obtain hOBs for cell/biomaterial qualitative and quantitative tests.

ALP analysis, Runx2 and ERα expression revealed that cells extracted from autogenous cortical bone particulate exhibited a typical osteoblastic phenotype. This observation confirms previous studies, which identified the autogenous bone grafts from either intra or extra oral donor sites, as valid sources of vital osteoblastic lineage cells.[18, 26-28] Cortical bone particulate has been shown to contain high levels of vital cells and to have similar cell viability and cell capacity to respond to mitogenic and osteogenic stimulus, compared to other forms of bone graft (*i.e.* block, dust).[28, 29] Due to the above-mentioned characteristics, autogenous cortical bone particulate has been validated as a source of osteoblast-like cells for in vitro experimental models, including the present study. At present time, it is still not clear which harvesting procedure may provide the highest quantity of viable cells with optimal cost/benefit and risk/benefit ratios. In the present study, we selected the bone harvesting technique due to our previous positive clinical experties, the limited invasivity and postoperative morbidity of this procedure.[16-18]

Further studies are in progress in order to investigate the data that we have obtained and to yield a complete interpretation of our results in comparison with other experiences.[30-32] In particular, in order to better understand the positive influence of the HA/Biostite biomaterial on osteoblastic terminal differentiation, the molecular events of the osteoblast response to HA/Biostite™ and the expression of other osteoblastic markers will be studied in vitro as well as *in vivo*. It is difficult to state presently how our in vitro model can mimic what may happen in vivo at the interface biomaterial/tissue. Nevertheless, we hope that our observations could stimulate further researchers aimed to improve the in vitro system analysis for studying adhesion, growth and differentiation of human osteoblasts cultured in presence of specific biomaterials, and to develop suitable environment for osteogenic phenotype.

Finally, although HA appears safe in clinical trials, there are concerns relating to potential long-term unexplored effects on inflammation and bone resorption defects that are unlikely to emerge in short-term trials. Actually, as before shown, both Biostite™ and Bio-Oss™ determined a clinically and statistically significant improvement in terms of clinical attachment gain, probing depth

reduction and radiographic depth reduction. Our encouraging results in terms of experimental methodology offer the basis for further studies based on the analysis of inflammatory factors potentially released from human primary osteoblasts exposed for a long period to HA.

Conclusions

In this paper we showed an optimized experimental model for the evaluation of the potentialities of different biomaterials in the supporting for growth and differentiation of osteoblasts. Our results showed that human primary osteoblasts obtained from oral surgery could adhere, grow and differentiate in vitro in the presence of HA/Biostite™, but not in the presence of SINTlife™ and Bio-Oss™. These indications have important implications for both tissue engineer bone grafts, and the enhancement of the HA implants performance for oral- and tooth-supporting structures.

References

1. Wiedmann-Al-Ahmad M, Gutwald R, Gellrich NC, Hübner U, Schmelzeisen R. Search for ideal biomaterials to cultivate human osteoblast-like cells for reconstructive surgery. J Mat Science Mat Med 2005;16:57-66.
2. Oonishi H, Kushitani S, Yasukawa E, Iwaki H, Hench LL, Wilson J *et al*. Particulate bioglass compared with hydroxyapatite as a bone graft substitute. Clin Orthopedic Rel Res 1997;334:316-25.
3. Serre CM, Papillard M, Chavassieux P, Boivin G. In vitro induction of a calcifying matrix by biomaterials constituted of collagen and/or hydroxyapatite: an ultrastructural comparison of three types of biomaterials. Biomaterials 1993;14:97-106.
4. Boccaccini AR, Blaker JJ. Bioactive composite materials for tissue engineering scaffolds. Expert Review Medical Devices 2005;2:303-17.
5. Yoshikawa H, Myoui A. Bone tissue engineering with porous hydroxyapatite ceramics. J Artif Organs 2005;8:131-6.
6. Kasaj A, Röhrig B, Reichert C, Willershausen B. Clinical evaluation of anorganic bovine-derived hydroxyapatite matrix/cell-binding peptide (P-15) in the treatment of human infrabony defects. Clin Oral Investig 2008;12:241-7.
7. Herten M, Rothamel D, Schwarz F, Friesen K, Koegler G, Becker J. Surface- and nonsurface-dependent in vitro effects of bone substitutes on cell viability. Clin Oral Investig Doi 2008:10.1007/s00784-008-0214-8
8. Hunter A, Archer CW, Walker PS, Blunn GW. Attachment and proliferation of osteoblasts and fibroblasts on biomaterials for orthopaedic use. Biomaterials 1995;16:287-95.
9. Cooper LF, Masuda T, Yliheikkila PK, Felton DA. Generalizations regarding the process and phenomenon of osseointegration. Part II. In vitro studies. The Int J Oral Maxillofac Implants 1998;13:163-74.
10. Foster BL, Somerman MJ. Regenerating the periodontium: is there a magic formula? Orthod Craniofac Res 2005;8:285-91.
11. Bizios R. Mini-review: Osteoblasts: An in vitro model of bone-implant interactions. Biotechnol Bioeng 1994;43:582-5.
12. Ball M, Grant DM, Lo WJ, Scotchford CA. The effect of different surface morphology and roughness on osteoblast-like cells. J Biomed Mater Res A 2008;86:637-47.
13. Scabbia A, Trombelli L. A comparative study on the use of a HA/collagen/chondroitin sulphate biomaterial (Biostite) and a bovine-derived HA xenograft (Bio-Oss) in the treatment of deep intra-osseous defects. J Clin Periodontol 2004;31:348-55.
14. Camilleri S, McDonald F. Runx2 and dental development. Eur J Oral Science 2006;114:361-73.
15. Compston JE. Sex steroids and bone. Physiol Rev 2001;81:419-47.
16. Trombelli L, Annunziata M, Belardo S, Farina R, Scabbia A, Guida L. Autogenous bone graft in conjunction with enamel matrix derivative in the treatment of deep periodontal intraosseous defects: a report of 13 consecutively-treated patients. J Clin Periodontol 2006;33:69-75.
17. Guida L, Annunziata M, Belardo S, Farina R, Scabbia A, Trombelli L. Additional effect of autogenous cortical bone particulate in conjunction with enamel matrix derivative in the treatment of deep periodontal intraosseous defects. J Periodontol 2007;78:231-8.
18. Trombelli L, Farina R, Marzola A, Itro A, Calura G. GBR and Autogenous cortical bone particulate by bone scraper for alveolar ridge augmentation: a histologic report. Int J Oral Maxillofac Implants 2008;23:111-6.
19. Denizot F, Lang RJ. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Immunol Methods 1986;22:271-7.
20. Ibbotson KJ, Harrod J, Gowen M, D'Souza S, Smith DD, Winkler ME *et al*. Human recombinant transforming growth factor alpha stimulates bone resorption and inhibits formation in vitro. Proc Natl Acad Sci USA 1986;2228-32.
21. Waterborg JH, Matthews HR. The Lowry method for protein quantitation. Methods Mol Biol 1994;32:1-4.
22. Penolazzi L, Zennaro M, Lambertini E, Tavanti E, Torreggiani E, Gambari R, Piva R. Induction of estrogen receptor alpha expression with decoy oligonucleotide targeted to NFATc1 binding sites in osteoblasts. Mol Pharmacol 2007;71:1457-62.
23. Lambertini E, Penolazzi L, Tavanti E, Schincaglia GP, Zennaro M, Gambari R *et al*. Human estrogen receptor alpha gene is a target of Runx2 transcription factor in osteoblasts. Exp Cell Res 2007;313:1548-60.
24. Keeting PE, Rifas L, Harris SA, Colvard DS, Spelsberg TC, Peck WA *et al*. Evidence for interleukin-1 beta production by cultured normal human osteoblast-like cells. J Bone Mineral Res 1991;6:827-33.
25. Zhou Y, Mohan S, Linkhart TA, Baylink DJ, Strong DD. Retinoic acid regulates insulin-like growth factor-binding protein expression in human osteoblast cells. Endocrinology 1996;137:975-83.
26. Clausen C, Hermund NU, Donatsky O, Nielsen H. Characterization of human bone cells derived from the maxillary alveolar ridge. Clin Oral Implants Res 2006;17:533-40.
27. Chiriac G, Herten M, Schwarz F, Rothamel D, Becker

J. Autogenous bone chips: influence of a new piezoelectric device (Piezosurgery) on chip morphology, cell viability and differentiation. J Clin Periodontol 2005;32:994-9.
28. Springer IN, Terheyden H, Geiss S, Härle F, Hedderich J, Açil Y. Particulated bone grafts--effectiveness of bone cell supply. Clin Oral Implants Res 2004;15:205-12.
29. Gruber R, Baron M, Busenlechner D, Kandler B, Fuerst G, Watzek G. Proliferation and osteogenic differentiation of cells from cortical bone cylinders, bone particles from mill, and drilling dust. J Oral Maxillofac Surgery 2005;63:238-43.
30. Sibilla P, Sereni A, Aguiari G, Banzi M, Manzati E, Mischiati C *et al*. Effects of a hydroxyapatite-based biomaterial on gene expression in osteoblast-like cells. J Dent Res 2006;85:354-8.
31. Petrovic L, Schlegel AK, Schultze-Mosgau S, Wiltfang J. Different substitute biomaterials as potential scaffolds in tissue engineering. Int J Oral Maxillofac Implants 2006;21:225-31.
32. Brodie JC, Goldie E, Connel G, Merry J, Grant MH. Osteoblast interactions with calcium phosphate ceramics modified by coating with type I collagen. J Biomed Mater Res 2005;73:409-21.

Effetto di biomateriali a base di idrossiapatite sul fenotipo di osteoblasti umani

Lo sviluppo di un materiale ideale per indurre una specifica risposta cellulare al fine di rigenerare tessuti danneggiati col pieno recupero della loro funzione biologica è da molto tempo un obiettivo della ricerca nel campo dell'ingegneria tissutale [1].

Fra i sostituti di osso, l'idrossiapatite (HA) è usata come materiale da innesto già dalla decade passata [2-4]. L'idrossiapatite è la componente minerale principale di osso e denti, ed è in grado di promuovere la rigenerazione ossea in modo veloce e senza interposizione di tessuto connettivale [5]. Diversi tipi di scaffold di idrossiapatite hanno dimostrato un'eccellente biocompatibilità, e osteoconduttività, e vengono da tempo impiegati per la sostituzione sia dell'osso alveolare che dei tessuti dentari [6].

La biocompatibilità dell'idrossiapatite è stata valutata principalmente esponendo osteoblasti direttamente ad essa. Adesione cellulare, proliferazione, capacità di espansione e morfologia, così come i marcatori osso-specifici di cellule coltivate in presenza di sostituto dell'osso sono stati ampiamente investigati [7]. La maggior parte dei modelli sperimentali usati a questo scopo consistono in colture cellulari che vengono comunemente impiegate come approccio *in vitro* per studiare fenomeni cellulari e le proprietà stesse dei biomateriali in termini di interazioni tra il biomateriale e il tessuto vivente [8, 9]. Pertanto, diversi tipi di colture cellulari, umane o animali, con fenotipo osteoblastico, cioè tutte in grado di esprimere proteine osteoblasto-specifiche e produrre *in vitro* noduli mineralizzati durante la loro maturazione, sono generalmente considerate un buon modello per la crescita e il differenziamento osteoblastico. Le evidenze sperimentali finora raccolte indicano che la rigenerazione ossea *in vitro* è un evento non solo possibile, ma anche facilmente raggiungibile. I problemi insorgono per quanto riguarda la trasferibilità dei risultati *in vivo*. Dopo una fase di deciso ottimismo, osservazione discrepanti provenienti da diversi gruppi di ricerca suggeriscono cautela nel trarre conclusioni univoche dai modelli *in vitro* [10]. È noto infatti che la ariabilità di fonti cellulari e specie di derivazione può influenzare in modo significativo la cascata di eventi che modulano la risposta cellulare allo stesso biomateriale. In particolare, la produzione di cofattori che modulano l'espressione di integrine e recettori di membrana induce risposte molto diverse tra specie diverse. Queste differenze possono influenzare gli esiti di specifiche cascate del segnale, le caratteristiche di adesione cellulare, e tutte le interazioni complesse che hanno luogo all'interfaccia cellula-biomateriale [11]. Inoltre, in diversi esperimenti vengono impiegate linee cellulari derivate da osteosarcoma e non osteoblasti normali. Sono anche numerosi i casi in cui la definizione di osteoblasti primari non è accompagnata da una chiara evidenza comprovante il fenotipo osteoblastico del modello cellulare impiegato.

Molti dati dimostrarono che durante l'interazione cellula-biomateriale le cellule sono capaci non solo di discriminare proprietà chimiche, fisiche e strutturali del materiale, ma anche di apprezzare le differenze di superficie in termini di ruvidità di superficie e topografia [12]. Anche queste proprietà, estremamente rilevanti per la risposta *in vivo*, variano al variare del modello sperimentale impiegato.

L'efficacia clinica dei biomateriali a base di idrossiapatite, come Biostite™ e Bio-Oss™ , nella ricostruzione di difetti di intraossei parodontali è stata ampiamente documentata [13]. Biostite™ e Bio-Oss™ determinano un miglioramento statisticamente significativo delle condizioni cliniche, in termini di guadagno di attacco clinico, riduzione della profondità di sondaggio e riduzione della profondità del difetto in termini radiografici. Tuttavia non ci sono differenze statisticamente significative fra i diversi trattamenti in termini di risultati ottenuti.

In questo lavoro sono stati investigati gli effetti di 3 diversi tipi di biomateriale a base di idrossiapatite in diverse condizioni sperimentali e usando diversi modelli cellulari. Osteoblasti umani primari (hOBs) e

due linee cellulari di osteosarcoma umano, SaOS-2 e Hobit, che esibiscono un fenotipo osteoblastico-simile, sono state utilizzati come modelli sperimentali.

Sulla base delle considerazioni precedenti, lo studio presente ha avuto l'obiettivo di valutare il comportamento di queste cellule in presenza di diversi biomateriali (SINTlife™ - Pinna-CERAMICA S.p.a., Faenza, Italia; Bio-Oss™, Geistlich Biomaterials, Woulhusen, Svizzera; Biostite™ - GABA Vebas, San Giuliano Milanese, MI, Italia), concentrando l'attenzione sull'effetto di Biostite™ in termini di modulazione della differenziazione osteoblastica.

Sono state valutate l'adesione cellulare al biomateriale, la proliferazione cellulare e l'attività di mineralizzazione. Inoltre, sono stati analizzati parametri importanti per il differenziamento osteoblastico quali: l'attività di fosfatasi alcalina (ALP), i livelli di espressione dei fattori di trascrizione Runx-2 e recettore per l'estrogeno ER-alpha, correlandoli allo specifico stadio di differenziamento osteoblastico [14, 15].

Materiali e metodi

Procedure per la raccolta di cellule osteoblastiche primarie umane

Particolato di osso alveolare è stato raccolto da soggetti umani in corso di procedure chirurgiche orali, dopo l'elevazione di un lembo mucoperiostale. Alla fine di ogni chirurgia, prima della sutura, una quantità adeguata (più di 500 mg) di particolato di osso alveolare è stato raccolto con appositi strumenti (Safescraper®, Meta C.G.M., Reggio Emilia, Italia; Micross®, Meta C.G.M., Reggio Emilia, Italia), come descritto in letteratura [16-18]. Le procedure chirurgiche di raccolta del particolato osseo sono state condotte in piena concordanza con la "Dichiarazione di Helsinki" revisionata ad Edimburgo (2000) e le linee di buona condotta della Pratica Clinica. Prima di ogni intervento chirurgico, il paziente ha fornito il consenso verbale al prelievo.

Colture cellulari e biomateriali

Le linee cellulari di osteosarcoma SaOS-2 e Hobit sono state mantenute in coltura in terreno D-MEM (Euroclone S.p.A., MILANO-l'Italia), completo con 10% di Siero Bovino Fetale (FBS) (Euroclone S.p.A., MILANO - l'Italia) in atmosfera umidificata al 5% di CO_2.

Il particolato di osso alveolare è stato lavato con soluzione salina sterile, e successivamente posto in fiasche di coltura T25 (25 cm^2) in miscela di terreni D-MEM e HAM'S F-12 (Euroclone S.p.A., MILANO-l'Italia) (rapporto volume 1:1), in presenza di 20% di FBS ed antibiotici (Streptomicina 50 mg/ml e Penicillina 100 mg/ml) a 37 °C in un'atmosfera umidificata al 5% di CO_2. Sottocolture di osteoblasti primari umani sono state ottenute circa 20 giorni dopo. Al giorno 20 le cellule sono state distaccate, raccolte, contate e piastrate in piastre di coltura da 6 e 24 pozzetti alla concentrazione di 20×10^3 cellule per cm^2. Tutti gli esperimenti sono stati compiuti con cellule al primo passaggio in coltura.

Sia gli osteoblasti primari umani (hOBs) che le linee cellulari di osteosarcoma sono state messe a contatto con i tre biomateriali (SINTlife™ - Pinna-CERAMICA S.p.a., Faenza, Italia; Bio-Oss™, Geistlich Biomaterials, Woulhusen, Svizzera; Biostite™ - GABA Vebas, San Giuliano Milanese, MI, Italia) alle concentrazioni di 2.5, 5 e 10 mg/ml.

Studi di citotossicità

L'analisi di citotossicità è stata condotta in vitro sia per hOBs che per le linee cellulari di osteosarcoma SaOS-2 e Hobit; ciascun tipo di cellule è stato messo a contatto diretto con ciascuno dei tre biomateriali analizzati alle diverse concentrazioni di 2,5, 5 e 10 mg/ml. La determinazione di cellule vitali è stata fatta con il saggio colorimetrico MTT (thiazolyl blu). Il saggio MTT è basato sulla conversione del sale di tetrazolio giallo in cristalli di formazano dal colore viola da parte dei mitocondri di cellule vitali [19]. Il saggio colorimetrico MTT offre una determinazione quantitativa delle cellule vitali. Dopo 72 ore di trattamento in triplicato, 200 µl di soluzione MTT (5 mg/ml) sono stati aggiunti a ciascun pozzetto, e la piastra è stata incubata per 2 ore a 37 °C. Il terreno di coltura è stato quindi rimosso e i cristalli di formazano solubilizzati con dimetilformammide (DMF) al 50%. L'assorbimento di ogni campione è stato misurato a 570 nm tramite sprettrofotometro.

Analisi al SEM

20×10^3 cellule per cm^2 sono state piastrate ed incubate per 4 giorni con la stessa concentrazione di Bio-Oss™, SINTlife™ e Biostite™ (5 mg/ml) a 37 °C in un'atmosfera umidificata al 5% di CO_2. Per l'osservazione al SEM, dopo 4 giorni di incubazione i granuli di ciascun biomateriale sono stati fissati in soluzione tamponata di glutaraldeide al 2,5% e in soluzione tamponata al 2% di tetraossido di osmio; sono poi stati disidratati e rivestiti in oro (Edward Sputter S150). Per l'osservazione dei campioni è stato utilizzato un microscopio a scansione elettronica Cambridge S360. Le immagini sono state acquisite a diversi ingrandimenti.

Analisi di attività di fosfatasi alcalina

Per la colorazione immunoistochimica dell'enzima fosfatasi alcalina è stato utilizzato il kit Sigma per leucociti (Sigma Aldrich). Il saggio prevede l'utilizzo di un monostrato cellulare preventivamente fissato e incubato a temperatura ambiente con una soluzione contenente naphthol AS-BI e sale fast blue BB preparato al momento, tamponata a pH 9.5 con AMPD (2-amino-2-metile-1,3-propanediolo). L'attività di fosfatasi alcalina risulta evidente per la comparsa di macchie di colore blu a livello citoplasmatico.

L'attività di fosfatasi alcalina in presenza e non dei biomateriali è stata anche valutata per le cellule in modo quantitativo attraverso l'idrolisi di p-nitrofenilfosfato (PNPP) [20]. $20x10^3$ cellule/cm^2 sono stae piastrate ed incubate con 5 mg/ml di HA/Biostite™; l'attività enzimatica (espressa in termini di U(nmol/min)/μg) è stata valutata dopo 6 giorni nelle linee cellulari e dopo 21 giorni negli hOBs. La normalizzazione proteica è stata effettuata secondo il metodo di Lowry [21].

Colorazione con rosso alizarina

La deposizione di matrice mineralizzata è stata valutata con il metodo Alizarin Red (AR-S, Sigma); 20×10^3 cellule/cm^2 sono state piastrate ed incubate con 5 mg/ml di HA/Biostite™ per 10 (linee cellulari) e 40 giorni (hOBs). Dopo 10 e 40 giorni le cellule sono state fissate in etanolo al 70% per 1 h a temperatura ambiente, lavate con PBS e colorate con 40 mM AR-S (ph 4.2) per 10 minuti a temperatura ambiente. In seguito le preparazioni sono state lavate cinque volte con acqua deionizzata e e incubate in PBS per 15 minuti per eliminare la colorazione non specifica. La matrice colorata è stata osservata ad ingrandimenti diversi con microscopio ottico Leitz.

Immunocitochimica

L'analisi immunocitochimica è stata eseguita con il sistema Ultraystain Polyvalent-HRP Immunostaining Kit (Ylem). Le cellule cresciute in appositi vetrini sono state trattate con metanolo al 100%, e permeabilizzate con 0.2% (vol/vol) Triton X-100 (Sigma) in TBS (Tris-buffer salino). Le cellule sono state quindi incubate in 3% H_2O_2 e le perossidasi endogene sono state bloccate con il reagente Super Block. Successivamente, anticorpi primari policlonali per Runx2 (clone M-70, rabbit anti-human, diluizione 1:1000 – Santa Cruz Biotec) ed ERα (clone MC-20, rabbit anti-human, diluizione 1:500 – Santa Cruz Biotec) sono stati applicati alle cellule fissate e incubati a 4° C tutta la notte. Il giorno dopo gli stessi campioni a temperatura ambiente sono stati incubati con anti-polyvalent Biotinylated Antibody (Ultraystain Polyvalent-HRP Immunostaining Kit–Ylem). Dopo avere lavato con TBS, è stato impiegato il sistema Streptavidin HRP (Ultraystain Polyvalent-HRP Immunostaining Kit–Ylem) e quindi il sistema Substrate-chromogen mix (AEC Cromogeno kit–Ylem). Successivamente sono stati preparati vetrini per l'osservazione al microscopio ottico con glicerolo/PBS 9:1.

Analisi statistica

I dati presentati riportano valori medi ± SE ottenuti da almeno tre esperimenti indipendenti, dove indicato. L'analisi statistica di varianza è stata compiuta con tecnica "one-way" seguita da Student's t test. Un valore di $P<0.05$ è stato considerato statisticamente significativo.

Risultati

Caratterizzazione del fenotipo osteoblastico

Prima dell'esposizione ai biomateriali le cellule sono state caratterizzate per il loro fenotipo osteoblastico, in termini di attività di fosfatasi alcalina, espressione di Runx2 ed ERα. Come indicato in Figura 1, questi marcatori sono correlati con uno specifico stadio di differenziamento cellulare. L'attività di fosfatasi alcalina, un marcatore osteoblastico precoce è associata con la maturazione della matrice extracellulare e il suo livello, analizzato mediante reazione colorimetrica, diminuisce con il differenziamento cellulare, in accordo con la letteratura [22]. Runx2 ed ERα sono due importanti fattori di trascrizione che hanno un ruolo cruciale nello sviluppo degli osteoblasti e nel mantenimento di un fenotipo osteoblastico funzionale [23]. Runx2 è un fattore di di trascrizione osteoblasto-specifico richiesto per il differenziamento di cellule progenitrici verso il fenotipo osteoblastico. La sua espressione, così come quella di ERα, aumenta durante il differenziamento cellulare e l'analisi immunocitochimica rivela come ciascun modello cellulare impiegato presentio uno specifico pattern di espressione dei diversi marcatori correlabile con uno specifico stadio di differenziamento osteoblastico. Pertanto, tutte le cellule usate negli esperimenti hanno dimostrato il loro fenotipo osteoblastico, essendo positive per l'espressione di Runx2 ed ERα e per l'attività di fosfatasi alcalina.

Analisi di citotossicità

L'analisi di MIT è stata eseguita dopo 72 ore su cellule messe direttamente in coltura con i materiali esaminati. La vitalità dell'osteoblasti piastrati su HA/Biostite™ è stata comparata con quella delle cellule a contatto con gli altri materiali HA (SINTlife™ e Bio-Oss™). La morfologia macroscopica dei tre diversi materiali è mostrata in Figura 2. I granuli (con concentrazioni di 2.5, 5 e 10 mg/ml) sono stati dispersi direttamente nel terreno di coltura. Come mostra la figura Figura 2B, HA/Biostite™ e gli altri due materiali non causavano effetti citotossici apprezzabili in queste condizioni sperimentali (la vitalità cellulare era in tutti i casi superiore al 75%).

Analisi al microscopio a scansione elettronica (SEM)

In Figura 3 le immagini acquisite al SEM mostrano l'abilità di adesione delle cellule dopo un periodo di cultura di 4 giorni su HA/Biostite™ rispetto a SINTlife™ e Bio-Oss™. Questa analisi dimostra che le cellule faticano a proliferare e a creare contatti cellula-cellula sui tre biomateriali. Ciononostante, le cellule sembrano preferire HA/Biostite™. In particolare, hOBs su HA/Biostite™ aderiscono ai biomateriali in modo simile a quello presentato dalle linee cellulari di osteosarcoma. Questi esperimenti sono stati ripetuti tre volte, con gli stessi risultati.

Le osservazioni al SEM correlano con l'osservazione al microscopio ottico relativa alla proliferazione, che dimostra che le cellule crescono meglio su HA/Biostite™ rispetto a SINTlife™ e Bio-Oss™.

Effetto di HA/Biostite™ sul differenziamento osteoblastico

Sulla base delle analisi al SEM HA/Biostite™ è stato selezionato per ulteriori indagini sui suoi effetti sul differenziamento osteoblastico. A questo scopo, l'attività di fosfatasi alcalina di linee cellulari e di hOBs in combinazione con il biomateriale è stata valutata al 6° e al 21° giorno rispettivamente, come descritto nella sezione Materiali e metodi.

I risultati mostrano che, come atteso, l'attività di fosfatasi alcalina basale è diversa per ogni modello sperimentale e che, in tutti i casi esaminati, la presenza di HA/Biostite™ influenza negativamente l'attività di fosfatasi alcalina stessa (Figura 4). Poichè l'attività di fosfatasi alcalina decresce con il sopraggiungere della fase finale di maturazione osteoblastica, i nostri dati suggeriscono che la presenza di HA/Biostite™ possa essere uno stimolo al differenziamento. Questo è stato confermato da una prova di mineralizzazione preliminare (da Alizarin Red S) su linee cellulari di osteosarcoma e su hOBs, dopo 10 e 40 giorni rispettivamente. L'effetto positivo di HA/Biostite™ sulla deposizione di calcio è rappresentato in Figura 4.

Discussione

Lo studio ha dimostrato come hOBs ottenuti da chirurgia orale possano aderire, crescere e modificarsi *in vitro* in presenza di HA/Biostite™, un biomateriale a base di HA equina. L'adesione e lo "spreading" di hOBs su HA/Biostite™ è comparabile con quello mostrato dalle linea cellulare di osteosarcoma SaOS-2 e la linea cellulare Hobit trasfettata con SV40, che sono sicuramente favorite in questi processi [24, 25]. Questo suggerisce che le stesse proprietà di HA/Biostite™ lo rendono appropriato come sistema di sostituzione di osso con osteoblasti umani normali, e questo rappresenta un importante requisito indispensabile per il successo di qualunque impianto o innesto.

Non è stata riscontratata alcuna influenza negativa di HA/Biostite™ sulla morfologia e la vitalità cellulare. Ciononostante, HA/Biostite™ non favorisce la proliferazione, come invece accade per le cellule coltivate sulla plastica, confermando le osservazioni fatte da molti altri ricercatori sulla difficoltà nell'allestimento in vitro di una matrice utile per il microambiente osseo.

Questo studio ha inoltre dimostrato che la tecnica impiegata per la biopsia e l'isolamento delle cellule osteoblastiche da frammenti di osso alveolare con il protocollo descritto, è appropriata per ottenere hOBs per prove qualitative e quantitative di interazione cellule/biomateriali.

L'analisi dei marcatori osteoblastici (fosfatasi alcalina, Runx2 e ERα) hanno dimostrato che le cellule estratte da particulato dell'osso corticale umano esibiscono un fenotipo osteoblastico autentico. Questa osservazione conferma studi precedenti i quali hanno identificato gli innesti di osso con provenienza intra o extra orale come fonti valide di cellule vitali della linea osteoblastica [18, 26-28]. Il particolato dell'osso corticale è stato dimostrato essere un contenitore effettivo di cellule vitali, in grado a loro volta di rispondere a stimoli mitogeni e osteogenici in modo analogo ad altri modelli di innesto osseo (innesto a blocco, polvere ...) [28, 29]. A causa delle caratteristiche sopra riportate il particolato dell'osso corticale è stato convalidato come una fonte valida di cellule vitali della linea osteoblastica utili per la realizzazione di modelli sperimentali in vitro, incluso lo studio presente. Attualmente non è ancora chiaro quale sia la tecnica di prelievo di tessuto osseo che contenga la più alta percentuale di cellule vitali, considerando anche i rapporti ottimali in termini di costi/benefici. In questo studio la tecnica per la raccolta di osso è stata selezionata in base alla precedente esperienza clinica positiva, la limitata invasività e la scarsa morbilità postoperatoria [16-18].

Ulteriori studi sono in corso per indagare i dati raccolti e interpretare in modo completo i risultati ottenuti a confronto con altre esperienze [30-32]. In particolare, per capire meglio l'influenza positiva del biomateriale HA/Biostite sul differenziamento osteoblastico e sull'instaurarsi di eventi molecolari che mediano la risposta di cellulare al biomateriale HA/Biostite™, l'espressione di altri marcatori del differenziamento osteoblastico saranno studiati in vitro così come in vivo. È difficile affermare al momento come e quanto il nostro modello in vitro mimi quello che può accadere in vivo all'interfaccia biomateriale/tessuto vivente. Ciononostante, auspichiamo che le nostre osservazioni possano incentivare i ricercatori a migliorare i modelli in vitro per l'analisi di adesione, crescita e differenziamento di osteoblasti umani in presenza di biomateriali, e sviluppare un ambiente appropriato per l'induzione al fenotipo osteogenico.

Inoltre, benchè HA si dimostri sicuro per l'applicazione clinica, ci sono preoccupazioni relative a potenziali effetti inesplorati a lungo termine su infiammazione e difetti di riassorbimento, la cui manifestazione è improbabile in prove a breve termine. Ad oggi è stato dimostrato come Biostite™ e Bio-Oss™ determinino clinicamente un miglioramento statisticamente significativo delle condizioni patologiche, in termini di guadagno di attacco clinico, diminuzione di profondità di sondaggio clinico e radiografico. I nostri risultati in termini di metodologia sperimentale incoraggiano, a nostro parere, ulteriori studi basati sull'analisi di fattori infiammatori potenzialmente rilasciati da osteoblasti primari ed esposti a lungo termine a biomateriali a base di HA.

Conclusioni

È stato illustrato e ottimizzato un modello sperimentale per la valutazione delle potenzialità di biomateriali diversi nel supportare la crescita e la differenziazione degli osteoblasti. I risultati hanno dimostrato come osteoblasti umani primari ottenuti in corso di chirurgia orale sono in grado di aderire, crescere e differenziare *in vitro* in presenza di HA/Biostite™, ma non analogamente in presenza di SINTlife™ e Bio-Oss™. Queste indicazioni hanno importanti implicazioni per il miglioramento dei modelli di innesto osseo, ed il miglioramento della performance di impianti a base di HA nella sostituzione di tessuti e strutture mineralizzate di supporto all'elemento dentario.

Riassunto

Il presente studio ha valutato il comportamento di osteoblasti umani primari e di due linee cellulari di osteosarcoma umano in presenza di diversi biomateriali a base di idrossiapatite (HA) (SINTlife™- PiranaCERAMICA S.p.a., Faenza, Italia; Bio-Oss™, Geistlich Biomaterials, Woulhusen, Svizzera; Biostite™ - GABA Vebas, San Giuliano Milanese, MI, Italia) concentrando l'attenzione sull'effetto di HA/Biostite™ in termini di modulazione del differenziamento delle cellule in senso osteoblastico. L'analisi è stata rivolta ad analizzare le caratteristiche di adesione, proliferazione e mineralizzazione dei diversi tipi cellulari. Come marcatori del differenziamento in senso osteoblastico sono stati stati presi in considerazione i livelli di espressione del fattore di trascrizione Runx-2, del recettore per l'estrogeno ER-alpha e l'attività dell'enzima fosfatasi alcalina (ALP). La vitalità delle cellule, valutata mediante il metodo MTT, è stata monitorata nel corso degli esperimenti eseguiti in presenza dei diversi biomateriali. E' stata inoltre eseguita sui diversi tipi di biomateriali l'analisi al microscopio a scansione elettronica (SEM) allo scopo di valutare la quantità delle cellule aderenti. Morfologia e vitalità cellulare non sono state influenzate negativamente da nessuno dei biomateriali esaminati. In particolare, è stato rilevato un incremento in termini di adesione, crescita e differenziamento per le cellule cresciute in presenza di HA/Biostite™, paragonabile a quello osservato per le cellule delle linee di osteosarcoma. Questi risultati hanno importanti implicazioni nell'ambito della medicina rigenerativa, considerando le prospettive di impianto in vivo di scaffold a base di idrossiapatite e il loro impiego per la sostituzione dei tessuti e le strutture di sostegno degli elementi dentari.

Parole chiave: Osteoblasti - Sostituti ossei - Materiali biocompatibili.

ORIGINAL CONTRIBUTION

Rhinology, 48, 000, 2010

Osteogenic potential of cells derived from nasal septum*

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SUMMARY

Background: *The research addressed to detect new molecular targets in the development of therapeutic strategies aimed to repair bone tissues.*
The ***aim of this study*** *was to determine the potential osteogenic activity of bone cells from the nasal septum and their use to perform accurate molecular analysis from a single sample.*
Methodology: *The cells, after nasal septum surgery, were subjected to gene silencing, Reverse Transcriptase - Polymerase Chain reactions, immunocytochemistry and chromatin immunoprecipitation.*
Results: *Cells from the nasal septum can give rise to mature osteoblasts that express osteogenic markers (ALP, Runx2, Slug) and are able to mineralize. We demonstrated that Runx2, a transcription factor critical in early osteospecific differentiation, interacts in vivo with the promoter of the SLUG gene, a marker of osteoblast maturation.*
Conclusions: *We demonstrated that nasal septum-derived osteoblasts represent an interesting alternative source for bone forming cells, and a promising material to be utilized in bone cellular therapy.*

Key words: human primary osteoblasts, nasal septum, chromatin immunoprecipitation, bone repair

INTRODUCTION

There is currently no ideal approach for the treatment of craniofacial defects. Such defects arise from trauma, injuries, removal of bone tissue as a result of cancer, and developmental abnormalities. They often lead to massive destruction of the facial skeleton [1-4]. Tissue engineering, which is based on the use of a combination of appropriate cells, growth factors, engineering methods, and suitable carrier scaffolds to improve or replace biological functions, promises to offer new therapeutic chances for the repair of damaged bone [4-6]. Bone tissue engineering currently uses either growth factors on a variety of carriers or osteogenic cells seeded onto an even larger number of different materials [4-7]. In the craniofacial area, despite a decade of experimental evaluation, ambiguous results have been obtained in the reconstruction of bony defects, and a large number of unresolved questions remain in the head and neck area [7-17]. This is correlated with evidence that surgical repair of such defects, as well as three-dimensional enlargement of structures in the facial skeleton are remarkable difficult and unpredictable. Particularly, complex bony defects are difficult to reconstruct accurately, and complications in the area of reconstruction are common. It has been demonstrated that many unsatisfactory results are correlated with poor cell survival rate, and inferior bone specific cellular activity after transplantation has to be considered as reason of occurance due to premature differentiation and subsequent growth inhibition [18]. In addition, the extremely high dosages of growth factors that are required to successfully regenerate bone in a clinically relevant dimension is to some extent controversial. Therefore, because biomolecules, as well as cells, both embryonic and adult tissue derived, play a critical role in this field of research, a multidisciplinary approach is absolutely required for a successful outcome.

Based on of these considerations, we investigated the characteristics of bone cells from the nasal septum in terms of potential osteogenic activity and experimental models to perform an accurate molecular analysis for detection of new therapeutic targets.

*Received for publication: April 8, 2010; accepted: June 16, 2010

DOI:10.4193/Rhino10.087

We demonstrate here that these cells represent an interesting alternative source for bone forming cells. They can be an ideal, unique, easily accessible and no controversial source for osteoblasts, due to the simple collection procedure during nasal septum surgery once bone tissue is routinely discharged. The ability to proliferate and to differentiate into mature osteoblasts at the same time makes the use of these cells particularly attractive both for the research addressed to new molecular targets detection, and the development of therapeutic strategies aimed to repair and replace damaged tissues. In addition, we demonstrated that, from a single sample, it is possible to obtain sufficient material to perform several molecular analyses including gene expression studies by Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR), DNA-protein interactions assays by chromatin immunoprecipitation (ChIP), and immunocytochemical experiments.

MATERIALS AND METHODS

Isolation and culture of osteoblasts

Human craniofacial osteoblast cells were obtained from bone samples collected during nasal septum surgery. Recruitment of subjects donating osteoblasts was in accordance with approved procedures, and informed consent was obtained from each patient. Bone samples were collected from 81 patients (30F/51M) that underwent septoplasty surgery, FESS or both procedures (for 8 cases it was possible to collect osteoblasts both from septum and ethmoid bone) from June 2006 to February 2010 at the ENT Department of the University Hospital of Ferrara. This type of surgery is routinely and worldwide performed in an ENT Surgery Unit. It usually consists in the exeresis of the lower part of the osseous nasal septum (this portion has been used in this study). This sampling is very handily and costless. The average age of patients was 43 years (range: 22-75). Ten out 81 samples were selected and dedicated to the studies here presented. Briefly, the bone samples, about 1 cm^2 in size, were cut into small fragments that were washed several times in phosphate-buffered saline (PBS) to remove blood cells and debris with a final wash in culture medium. Fragments were then collected in culture flasks containing 6 ml of 1:1 mixture of DMEM/Ham's F12 (Sigma Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS) (CELBIO EuroClone, Milan, Italy), 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml ascorbate at 37°C in a humidified atmosphere of 5% CO_2. After about 5-7 days, outgrowth of bone cells from the bone chips commenced, and confluency in 25 cm^2 dishes was usually reached after 3-4 weeks [19]. For the studies here presented, only first and second passage cells were used.

Flow cytometric analysis

The cells from all 10 samples were analysed for expression of surface marker molecules, by direct immunofluorescent staining. Briefly, cell pellets were resuspended in PBS and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- conjugated mouse anti-human antibodies Stro1, CD45-PE, and CD90-FITC (DakoCytomation, Dako, Denmark), for 15 min at 4°C. Monoclonal antibodies with no specificity were used as negative control. Antibody treated cells were then washed with PBS and spinned down. For each sample, the cell pellet was resuspended in 400 µl of PBS and the fluorescence of 20,000 cells was analysed using FACS Scan flow cytometer (Becton Dickinson, New Jersey, USA) and CellQuest software (Becton Dickinson European HQ, Erembodegem Aalst, Belgium). Flow cytometric analysis was repeated three times for each sample to allow SEM calculation.

Analysis of the osteoblast phenotype

For alkaline phophatase staining, prefixed mono-layered cells from all 10 samples were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1.3-propanediol (Alkaline Phosphatase Leukocyte kit, Sigma). The presence of sites of ALP activity appeared as blue cytoplasmatic staining.

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma) in the cells cultured for up to 35 days in osteogenic medium consisting in DMEM, high-glucose, supplemented with 10% FBS, 10 mM b-glycerophosphate, 0.1 mM dexamethasone and 50 mM ascorbate. In the committed cells, the osteogenic medium was changed every 3 days. The cells were then fixed in 10% formaldehyde for 15 min at room temperature, washed with deionized water, stained with 40 mM AR-S (pH 4.2) for 20 min at room temperature, and washed 5 times with deionized water to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope.

Immunocytochemistry

Immunocytochemistry analysis was performed on all 10 samples using an ImPRESS Universal Reagent Kit (Vector Laboratories, Inc. Burlingame, CA, USA). About 10^4 cells were seeded in 4-well chamber slides, led to adhere for 48 hrs, fixed in cold 100% methanol and permeabilised with 0.2% (vol/vol) Triton X-100 (Sigma Aldrich) in TBS (Tris-buffered saline). Cells were incubated in 0.3% H_2O_2 and the endogenous peroxidase was blocked with ready-to-use (2.5%) normal horse blocking serum (ImPRESS Reagent Kit, Vector Laboratories). Afterwards, the primary antibodies, two polyclonal antibodies against human Runx2 (M-70; 1:100 dilution) and human Slug (H-140; 1:100 dilution) (Santa Cruz, Biotechnology, CA, USA), respectively, were applied and incubated at 4°C overnight. Cells were then incubated at room temperature with ImmPRESS reagent (ImPRESS Reagent Kit) for 30 min. After rinsing in TBS, substrate–chromogen mix (ImmPACT DAB, Vector Laboratories). After washing, cells were mounted in glycerol/PBS 9:1 and observed using a Leitz microscope.

Small interfering RNA (siRNA) transfection

BLOCK-iT Fluorescent Oligo (Invitrogen, Carlsbad, CA, USA) was used to assess the transfection efficiency of siRNA. It is a fluorescein-labelled, double-stranded RNA duplex with the same length, charge and configuration as standard siRNA. The sequence of the BLOCK-iT Fluorescent Oligo is not homologous to any known gene, ensuring against nonspecific cellular events caused by the introduction of the oligonucleotide into the cells. Human osteoblasts were seeded under the same experimental conditions and transfected with 30 nM of BLOCK-iT Oligo. After 24 hours, images of transfected cells were recorded using a digital imaging system based on a fluorescence microscope (model Axiovert 200L; Carl Zeiss MicroImaging, Inc.) equipped with a back-illuminated CCD camera (Roper Scientific), excitation and emission filterwheels (Sutter Instrument Company), and piezoelectric motoring of the z stage (Physik Instrumente, GmbH & Co). The data were acquired and processed using the MetaFluor analyzing program (Universal Imaging Corp).

Stealth RNAi duplexes and corresponding Stealth control were synthesized by Invitrogen Life Technologies. Stealth RNAi compounds are 25 mer dsRNA containing proprietary chemical modifications that enhance nuclease stability and reduce off-target effects. The previously described siRNA/Slug2 targeting human Slug was used [20]. Twenty four hrs before siRNA transfection, hOBs were seeded in triplicate at a density of 16 x 103/cm^2 in DMEM with 10% FBS. Cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies) according to the manufacturers' instructions. Transfected cells were incubated for 6 days at 37°C before gene silencing analysis. As a negative control for the siRNA treatment, Medium GC Stealth RNAi Negative Control Duplex (Invitrogen) was used. Knockdown of Slug expression was verified by Real-Time RT-PCR.

Real-time quantitative RT-PCR

Cells from three wells were harvested and total RNA was extracted using an RNeasy Mini Kit (Qiagen GMBH, Hilden, Germany) according to the manufacturer's instruction and as previously described [20]. Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA), TaqMan technology, and the Assays-On-Demand kit for human Slug, Runx2, and BSP. The mRNA levels of target genes were corrected for GAPDH mRNA levels (endogenous control). All PCR reactions were performed in triplicate for each sample and were repeated three times. All experimental data were expressed as the mean ± S.E.M.

Western blotting

For western blot analysis, the cells were washed twice with ice-cold PBS and cell lysates were prepared as previously reported [19]. Then, 10 µg of each sample was electrophoresed through a 12% SDS-polyacrylamide gel. The proteins were then transferred onto an Immobilon-P PVDF membrane (Millipore, Billerica, MA, USA). After blocking with PBS - 0.05% Tween 20 and 5% dried milk, the membrane was probed with the following antibodies: Slug (L40C6) from Cells Signaling Technology (Danvers, CA, USA), and IP3 K (06-195) from Upstate Biotechnology (Lake Placid, NY, USA). After washing with PBS-Tween, the membranes were incubated with peroxidase-conjugated anti-rabbit antibody (1:50000) or anti-mouse (1:2000) (Dako) in 5% non-fat milk. Immunocomplexes were detected using Supersignal West Femto Substrate (Pierce, Rockford, IL, USA). Anti-IP(3)K was used to confirm equal protein loading.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described using the standard protocol supplied by Upstate Biotechnology, Inc. with their ChIP assay reagents [21]. The cells were cross-linked with 1% formaldehyde for 10 min at 37°C, washed in ice-cold PBS and suspended in SDS lysis buffer for 10 min on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors and precleared with 80 ml of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 5 µg of anti-Runx2, (sc-10758) (Santa Cruz Biotec), overnight at 4°C. Immunocomplexes were mixed with 80 ml of DNA-coated protein A-agarose followed by incubation for 1 hr at 4°C. Beads were collected and sequentially washed 5 times with 1 ml each of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH-8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-pH 8.1) and TE buffer. The immunocomplexes were eluted two times by adding a 250 ml aliquot of a freshly prepared solution of 1% SDS, 0.1M $NaHCO_3$ and the cross-linking reactions were reversed by incubation at 65°C for 4h. Further, the samples were digested with proteinase K (10 mg/ml) at 42°C for 1hr, DNA was recovered by phenol/chloroform extractions, ethanol precipitated using 1 ml of 20 mg/ml glycogen as the carrier, and suspended in sterile water. For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). PCR was performed to analyse the presence of DNA precipitated by Runx2 specific antibody, and by using specific primers to amplify a fragment of the Slug gene promoter.

Each PCR reaction was performed with 5µl of the bound DNA fraction or 2 µl of the input. The PCR was performed as follows: preincubation at 95°C for 5 min, 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 62°C and 1 min at 72°C, with one final incubation at 72°C for 5 min. No-antibody control was included in each experiment.

Statistical analysis

Data are presented as the mean ± SEM calculated from at least three independent experiments performed on material obtained from all patient analysed. Statistical analysis was performed by one-way analysis of variance and the Student's t-test. A p-value < 0.05 was considered statistically significant.

RESULTS

Isolation and characterization of human primary osteoblasts

Human primary osteoblast cultures (hOBs) were generated from bone chips removed from nasal septum, and were subjected to the experimental procedure schematized in Figure 1. From a group of 81 patients, 10 samples have been selected for the analysis here described, essentially based on their ability to proliferate and differentiate. The appearance of isolated human osteoblastic cells was examined and we found cell morphology to be consistent with what has been reported in the literature (Figure 1) [22]. It was observed that immediately following isolation, there was limited cellular migration from the trabecular bone chips at day 1. After about 7 days, outgrowth of bone cells from the bone chips commenced, and confluency in T25 flask was usually reached after 3-4 weeks. The cellular morphology was spindle-shape as well as circular for some osteoblastic cells. On examination of the cells at day 21, there was a substantial increase in the number of primary cells that had migrated from the bone chips, increasingly further away from the bone chips.

The immunophenotypical profile of the cells from all 10 samples was determined by flow cytometric analysis The results indicate that most of the cells (98.12%) expressed cell surface marker CD90 (Thy-1) (Figure 2A) that is detected in the early stage of osteoblast differentiation and declines as osteoblasts differentiate into osteocytes [23]. The hematopoietic marker CD45 was not detectable on these cells (Figure 2A), indicating that cells were not contaminated with cells of hematopoietic origin. In addition, flow cytometry showed that the cells did not express STRO1 (Figure 2A), suggesting that they are mostly mature osteoblasts [24]. We then analysed the phenotypic markers by immunocytochemical staining. The cells from all 10 samples were highly positive for both Runx2, a typical osteogenic marker, and Slug, a zinc finger transcription factor involved in epithelial-mesenchymal transition and recently correlated with osteoblast maturation (Figure 2B) [20,25]. A significant component of the bone extracellular matrix, the bone sialoprotein (BSP), was detected at mRNA level by real time RT-PCR. These data confirm the level of maturation of the cells giving a definite indication of their commitment.

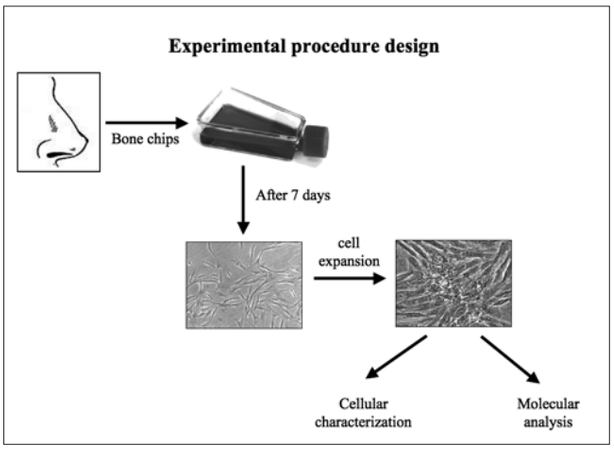

Figure 1. Isolation and use of human primary osteoblasts (hOBs). Human primary osteoblasts were obtained from bone chips removed from nasal septum. Bone was cut in small pieces which were rinsed and then cultured in T25 flask. About 7 days after isolation, outgrowth of bone cells from bone chips began. As shown in this figure, cells present a spindle-shape morphology. Once hOBs reached confluency, they were used for cellular and molecular analysis.

Next, the cells were characterized for their osteogenic capacity in terms of alkaline phosphatase (ALP) activity and mineral deposition (Figure 2C). The presence of sites of ALP activity was detected in all samples. In addition, Alizarin Red staining showed that all samples analysed exhibited an evident extracellular matrix mineralization after 14 days of culture under osteogenic conditions (a representative sample is shown in Figure 2C).

Effect of a specific gene knockdown

To analyse the potential to modify cellular phenotype in consequence of nucleic acid based drug treatments, 4 of the 10 samples were transfected with a double-stranded fluorescent dye-labeled oligonucleotide and the DNA uptake has been monitored for increasing length of time (2-24 hrs). After 24 hrs, the fluorescent molecules were widely distributed into the nuclei of most of the cells (Figure 3A), and the transfection efficiency was estimated about 80%. Automated cell culture observation by using the BioStation CT-LA image analysis system confirmed the results (data not shown).

To determine the effect of a specific oligonucleotide treatment, the cells were then transfected with a siRNA against Slug, a transcription factor that we previously demonstrated correlated with osteoblastic phenotype [20]. The transfection significantly decreased the mRNA and protein levels of Slug, compared with those in cells transfected with scramble siRNA (Figure 3B). Concomitantly, the ability to deposit mineral matrix of the cells decreased significantly, demonstrating that the cells clearly respond to modulation of their osteoblast phenotype (Figure 3B).

Runx2 interacts "in vivo" with the promoter of SLUG gene

To study the feasibility of other molecular investigations, 4 of the 10 samples were subjected to chromatin immunoprecipitation (ChIP) analysis, an "in vivo" transcriptional regulation assay. We set up sufficiently sensitive ChIP conditions to allow

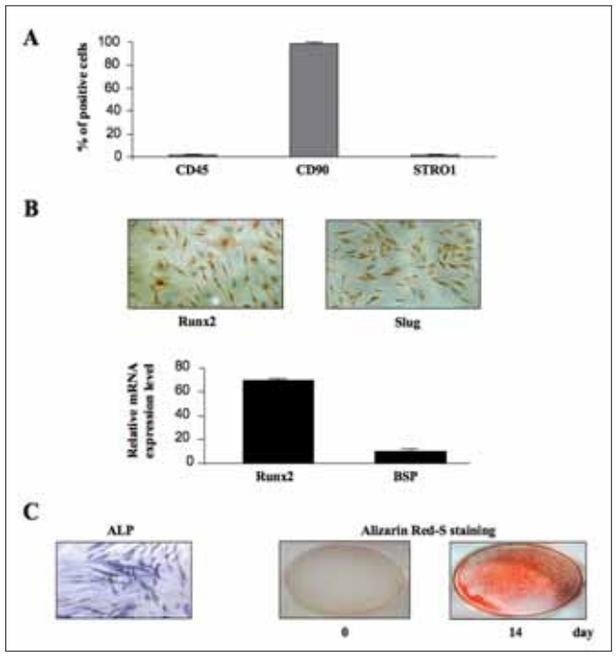

Figure 2. Phenotypical characterization of human primary osteoblasts (hOBs). A) The characterization of ten hOBs has been performed by flow cytometric analysis of CD45, CD90 and STRO-1 phenotypical markers. Data were expressed as % positive cells ± SEM, repeated three times for each sample. B) hOBs samples were subjected to immunocytochemical analysis for Runx2 and Slug phenotypical markers. A representative sample is shown (X10 magnification). The level of Runx2 and BSP expression was examined by quantitative RT-PCR in ten hOB samples. The experiments were carried out in triplicate, the expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels. $\Delta\Delta$Ct method was used to value the gene expression; SEM was calculated. C) The cells were treated with β-glycerophosphate, ascorbic acid, and dexamethasone and formation of extracellular matrix was valuated. Mineral formation was examined by Alizarin Red-S staining after 14 days of osteogenic induction. The authentic osteoblast phenotype was confirmed in hOBs by staining for alkaline phosphatase (ALP) activity.

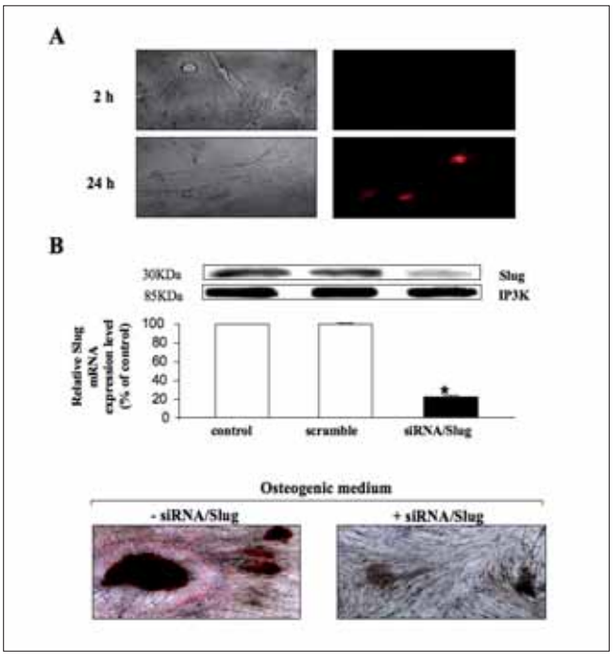

Figure 3. Transfection efficiency and effect of Slug knockdown during osteogenic differentiation. A) Cells were transfected with 30nM of a double-stranded fluorescent dye-labeled oligonucleotide. The uptake of oligo fluorescent siRNA was analysed in hOBs by fluorescence microscopy. Two and 24 hrs after the transfection, cells were washed and examined under phase contrast (left panels) or fluorescent light using a fluorescent filter (right panels). B) hOBs were treated for 6 days with siRNA/Slug2 30 nM or a scramble siRNA as negative control. At the top, representative western blot of siRNA/Slug2 treated cells shows a specific decrease of endogenous Slug protein level. Size markers are reported (KDa). IP3K was used as a loading control. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug2 over control ratio. Results represent means ± SEM of 4 hOB samples (*$p < 0.05$). After siRNA/Slug treatment, the cells cultured in osteogenic medium and analysed for the deposition of calcium salts by Alizarin Red-S staining. At day 14, more mineralized nodules were observed in untreated cells respect to silenced cells.

the analysis of primary cells. At this purpose, the involvement of a bone-specific transcription factor, Runx2, in the transcriptional regulation of SLUG, was tested. Using a program for predicting transcription factor binding sites (TFSEARCH, www.cbrc.jp/research/db/TFSEARCH.html), two potential cis elements for Runx2 were found in the promoter of the human SLUG gene at -537 and -476 (Figure 4). Their functionality was investigated by ChIP assay, analysing the "in vivo" association between Runx2 and the SLUG promoter. The cells were exposed to formaldehyde to cross-link proteins and DNA, and sonicated to fragment the chromatin. Specific antibody against Runx2 was used to immunoprecipitate the protein – DNA complexes, and Runx2 recruitment was assessed by using the specific set of primers reported in Table 1. The presence of the promoter specific DNA region before immunoprecipitation was confirmed by PCR (input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate a specific PCR product spanning the putative Runx2 binding site in the SLUG promoter gene. As shown in Figure 4, we demonstrated that the SLUG gene displays a specific reg-

Table 1. PCR primers used for chromatin immunoprecipitation assay (ChIP).

Regions	Primer sequences	Product size (bp)
Region 1	Forward F1: 5'-ATATAGGCTCTCATTAACAC-3'	218
	Reverse R1: 5'-AGTATTTTCAAGAGAGGTAA-3'	
Region 2	Forward F2: 5'-GAAATGGAGTGAAAAGCAAG-3'	175
	Reverse R2: 5'-TTGCAGGAGAGAGGAAAATA-3'	

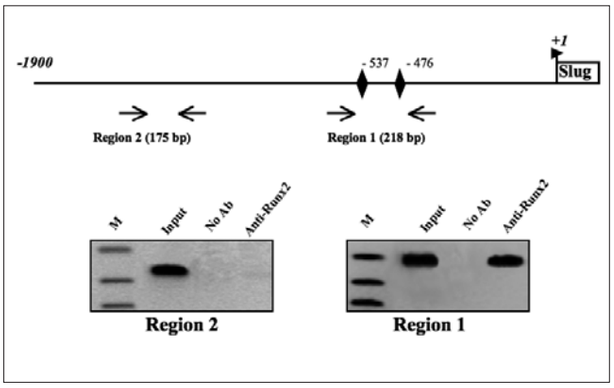

Figure 4. In vivo recruitment of Runx2 transcription factor on human Slug promoter. The Slug promoter region under investigation is reported (+1/-1900). The positions of the putative Runx2 consensus binding sites are enclosed by black diamonds. Recruitment of Runx2 transcription factor to the human Slug promoter is demonstrated by 'in vivo' ChIP binding assays. Protein-DNA complexes were formaldehyde-cross-linked in hOBs in vivo. Chromatin fragments from these cells were subjected to immunoprecipitation with antibody against Runx2. After cross-link reversal, the coimmunoprecipitated DNA was amplified by PCR using the reported primers, which positions are indicated with arrows. Region 2, lacking Runx2 binding sites, represents a negative control of ChIP experiments. PCR fragments were resolved through 1.5% agarose gels. No Ab represents a negative control. Input represents a positive control using the starting material (0.2%) prior to immunoprecipitation. The molecular weights of PCR fragments are shown in parentheses. M: molecular weight marker.

ulatory response in osteoblasts with the recruitment of a bone-specific transcription factor, Runx2, that is required for terminal differentiation of osteoblasts. As expected, no recruitment of this bone-specific transcription factor was observed in a sequence of SLUG promoter, (region 2), lacking of Runx2 binding sites (Figure 4) or in MCF-7 breast cancer cells (data not shown).

These ChIP experiments strengthen the role of Slug in osteoblasts showing that it is a Runx2 target gene, and demonstrate the feasibility of this analysis in human primary osteoblasts obtained from nasal septum.

DISCUSSION

The tissue engineering supported by cell therapy and scaffolds with advanced carrier technology for growth factors offers important opportunity for bone tissue repair and regeneration. It may be based on different approaches that combine the collaboration between surgeons, scientists and engineers.

Because the formation of bone tissue is based mainly on the action of osteoblasts, these cells are of special relevance [26]. Formation of bone involves a complex pattern of cellular events initiated by proliferation and differentiation of mesenchymal precursor cells into bone-forming cells and finally resulting in the mineralization of the extracellular matrix. Therefore, the ultimate aim of skeletal tissue regeneration is mineral deposition in a scaffold with a pore system for growth of suitable cells, such stem cells or precursor cells that may be implanted or seeded into the scaffolds capable of supporting three-dimensional tissue formation [4-7,18,27,28]. Various scaffolds have already been developed for bone tissue engineering applications. However, the properties of cellular component are considered particularly determining factors.

Reconstructive strategies of the craniofacial district, naso-orbito-ethmoidal fractures, whether isolated or as a component of complex facial fractures, are always some of the most challenging skeletal facial injuries to treat [1-3,8-17,29-32]. Regeneration of bone tissue is also required after frequent maxillary or mandible reabsorption due to tumour resections or tissue necrosis. The successful facial plastic surgery approaches and the development of more appropriate and effective treatments are based on knowledge of bone biology including cellular and molecular aspects of bone development, formation and repair. It is important to underline that several clinical studies on congenital defects of nasal septum and the findings on experimental animals after nasal septum ablation demonstrate that the septum acts as a growth center thrusting the midface bones downwards and forwards from the cranial base.

Therefore, a search for the ideal cells to employ in facial skeleton regenerative medicine, brought us to consider the potential use of bone cells obtained from nasal septum for autologous reconstruction techniques. In fact, the recovery and propagation facility of these cells make them both a good alternative to bone marrow osteogenic progenitors from iliac crest, and qualified differentiated cells that could be directly applied to the bone defect in combination with suitable scaffolds. It is important to underline that the lower part of the osseous nasal septum that we used in this study is part of the maxilla, and it has been previously demonstrated that the use of osteoblasts of maxillary origin is safe [33]. We demonstrated in the present study that cells derived from explants of nasal septum are easy to obtain, can be grown up in culture, express markers of osteoprogenitor cells, and are committed towards maturation because do not express STRO1. In fact, previous studies have shown that the STRO1 antigen defines a MSC precursor subpopulation, is present in osteogenic precursor cells isolated from human bone marrow, is maintained in immature, pre-osteoblastic phenotype, and is progressively lost by mature osteoblasts [24].

Under appropriate treatment, the cells can be induced to osteogenic differentiation giving rise to mature osteoblasts characterized by the expression of osteogenic markers, and ability to deposit mineralized matrix. These evidences indicate that these cells may represent a very promising material to be utilized in bone cellular therapy.

At the same time, we demonstrated that, from a single sample, it is possible to obtain a sufficient quantity of cells to perform several molecular analysis including RT-PCR, ChIP, and

immunocytochemical experiments. The information that can be obtained from the molecular approaches described here are worthy of interest because biochemical signal cascades that alter expression of genes or activation of proteins play critical role in tissue engineering field. Many of these signals, only in part known, include FGF, WNT, TGF-β, BMP, EGF, HIF, Notch, PTH, and integrins –mediated pathways, and are correlated with changes in specific activities of bone cells and may be also considered potential therapeutical targets [25,34]. Thus, it is very important, where feasible, to perform a direct molecular analysis on the same cells that are then to be used for tissue engineering.

All together, our findings may be considered a preliminary statement for clinical applications in the head and neck area aimed to develop an ex vivo model for induction of ectopic bone formation using autologous committed precursors cells.

ACKNOWLEDGEMENTS

This research was supported by grants from Regione Emilia Romagna, Programma di Ricerca Regione Universita' 2007–2009. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

REFERENCES

1. Kuriakose MA, Shnayder Y, DeLacure MD. Reconstruction of segmental mandibular defects by distraction osteogenesis for mandibular reconstruction. Head & Neck 2003; 25: 816-824.
2. Cordeiro PG. Frontiers in free flap reconstruction in the head and neck. J Surg Oncol. 2008; 97: 669-673.
3. Nussenbaum B, Rutherford RB, Krebsbach PH. Bone regeneration in cranial defects previously treated with radiation. Laryngoscope 2005: 115: 1170-1177.
4. Panetta NJ, Gupta DM, Longaker MT. Bone Regeneration and Repair. Curr Stem Cell Res Ther. 2010; 5: 122-128.
5. Porter JR, Ruckh TT, Popat KC. Bone tissue engineering: a review in bone biomimetics and drug delivery strategies. Biotechnol Prog. 2009; 25: 1539-1560.
6. Bueno EM, Glowacki J. Cell-free and cell-based approaches for bone regeneration. Nat Rev Rheumatol. 2009; 5: 685-697.
7. Grayson WL, Fröhlich M, Yeager K, et al. Regenerative medicine special feature: engineering anatomically shaped human bone grafts. Proc Natl Acad Sci USA. 2010; 107: 3299-3304.
8. Goessler UR, Stern-Straeter J, Riedel K, et al. Tissue engineering in head and neck reconstructive surgery: what type of tissue do we need? Eur Arch Otorhinolaryngol. 2007; 264: 1343-1356.
9. Bücheler M, Haisch A. Tissue engineering in otorhinolaryngology. DNA Cell Biol. 2003; 22: 549-564.
10. Malard O, Espitalier F, Bordure P, et al. Biomaterials for tissue reconstruction and bone substitution of the ear, nose and throat, face and neck. Expert Rev Med Devices 2007; 4: 729-739.
11. Pellegrini G, Seol YJ, Gruber R, et al. Pre-clinical models for oral and periodontal reconstructive therapies. J Dent Res. 2009; 88: 1065-1076.
12. Scheller EL, Krebsbach PH, Kohn DH. Tissue engineering: state of the art in oral rehabilitation. J Oral Rehabil. 2009: 36: 368-389.
13. Moioli EK, Clark PA, Xin X, et al. Matrices and scaffolds for drug delivery in dental, oral and craniofacial tissue engineering. Adv Drug Deliv Rev. 2007; 59: 308-324.
14. Gerressen M, Hermanns-Sachweh B, Riediger D, et al. Purely cancellous vs. corticocancellous bone in sinus floor augmentation with autogenous iliac crest: a prospective clinical trial. Clin Oral Implants Res. 2009; 20: 109-115.
15. Richmon JD, Sage AB, Shelton E, et al. Effect of growth factors on cell proliferation, matrix deposition, and morphology of human nasal septal chondrocytes cultured in monolayer. Laryngoscope 2005; 115: 1553-1560.
16. Rotter N, Haisch A, Bücheler M. Cartilage and bone tissue engineering for reconstructive head and neck surgery. Eur Arch Otorhinolaryngol. 2005; 262: 539-545.
17. Zaky SH, Cancedda R. Engineering craniofacial structures: facing the challenge. J Dent Res. 2009; 88: 1077-1091.
18. Khan SN, Cammisa, FP, Sandhu HS, et al. The biology of bone grafting. J Am Acad Orthop Surg. 2005; 13: 77-86.
19. Lambertini E, Franceschetti T, Torreggiani E, et al. Slug: a new target of lymphoid enhancer factor-1 in human osteoblasts. BMC Mol Biol. 2010; 11: 13.
20. Lambertini E, Lisignoli G, Torreggiani E, et al. Slug gene expression supports human osteoblast maturation. Cell Mol Life Sci. 2009; 66: 3641-3653.
21. Lambertini E, Tavanti E, Torreggiani E, et al. ERalpha and AP-1 interact in vivo with a specific sequence of the F promoter of the human ERalpha gene in osteoblasts. J Cell Physiol. 2008; 216: 101-110.
22. Robey PG, Termine JD. Human bone cells in vitro. Calcif Tissue Int. 1985; 37: 453-460.
23. Chen XD, Qian HY, Neff L, et al. Thy-1 antigen expression by cells in the osteoblast lineage. J Bone Miner Res. 1999; 14: 362-375.
24. Byers RJ, Brown J, Brandwood C, et al. Osteoblastic differentiation and mRNA analysis of STRO-1-positive human bone marrow stromal cells using primary in vitro culture and poly (A) PCR. J Pathol. 1999; 187: 374-381.
25. Soltanoff CS, Yang S, Chen W, et al. Signaling networks that control the lineage commitment and differentiation of bone cells. Crit Rev Eukaryot Gene Expr. 2009; 19: 1-46.
26. Lian JB, Stein GS. Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. Crit Rev Oral Biol Med. 1992; 3: 269-305.
27. Sundelacruz S, Kaplan DL. Stem cell- and scaffold-based tissue engineering approaches to osteochondral regenerative medicine. Semin Cell Dev Biol. 2009; 20: 646-655.
28. Penolazzi L, Tavanti E, Vecchiatini R, et al. Encapsulation of mesenchymal stem cells from Wharton's jelly in alginate microbeads. Tissue Eng Part C Methods. 2010; 16: 141-155.
29. Risbud MV, Shapiro IM. Stem cells in craniofacial and dental tissue engineering. Orthod Craniofac Res. 2005; 8: 54-59.
30. Scheller EL, Krebsbach PH, Kohn DH. Tissue engineering: state of the art in oral rehabilitation. J Oral Rehabil. 2009: 36: 368-389.
31. Scheller EL, Krebsbach PH. Gene therapy: design and prospects for craniofacial regeneration. J Dent Res. 2009; 88: 585-596.
32. Shanti RM, Li WJ, Nesti LJ, et al. Adult mesenchymal stem cells: biological properties, characteristics, and applications in maxillofacial surgery. J Oral Maxillofac Surg. 2007; 65: 1640-1647.
33. Doglioli P, Scortecci G. Characterization of endosteal osteoblasts isolated from human maxilla and mandible: an experimental system for biocompatibility tests. Cytotechnology 1991; 7: 39-48.
34. Lian JB, Javed A, Zaidi SK, et al. Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors. Crit Rev Eukaryot Gene Expr. 2004; 14: 1-41.

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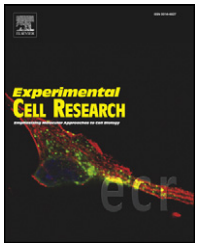

Research Article

Slug contributes to the regulation of CXCL12 expression in human osteoblasts

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ARTICLE INFORMATION

Article Chronology:
Received 4 November 2010
Revised version received
10 December 2010
Accepted 12 December 2010

Keywords:
CXCL12 chemokine
Slug
Human osteoblasts
Small interfering RNA
Gene regulation
Chromatin immunoprecipitation

ABSTRACT

CXCL12/CXCR4 chemokine/receptor axis signaling has recently been found to play an important role in the remodeling of bone tissue, but little is known about the molecular mechanisms that are involved. The present study shows that CXCL12 is present at high levels both in human mesenchymal stem cells (hMSCs) and primary osteoblasts (hOBs). When osteogenesis was induced, CXCL12 expression was strictly confined to mineralized nodules. To investigate what mechanisms contribute to the maintenance of a correct expression of CXCL12 in bone cellular context, we analyzed the relationship between CXCL12 and Slug, a transcription factor recently associated with osteoblast maturation. By gene silencing and chromatin immunoprecipitation assay, we showed that both proteins are required for the mineralization process and CXCL12 is transcriptionally and functionally regulated by Slug, which is recruited at specific sites to its gene promoter in vivo.

These findings showed for the first time a positive correlation between CXCL12 signaling and Slug activity, thus corroborating the role of these two proteins in bone cellular context and suggesting a new potential target for bone tissue repair and regeneration.

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doi:10.1016/j.yexcr.2010.12.011

Introduction

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), belongs to the CXC chemokine family and was identified according to NH2-terminal-cysteine motifs [1,2].

During the last decade, accumulating data have supported the role of CXCL12/CXCR4 axis signaling in the development, differentiation and repair of bone tissue. CXCL12 is mainly produced in the bone tissue by immature OBs lining the bone endosteum, MSCs and endothelial cells [3,4]. A continuous expression of CXCL12 has also been found in regenerating bone OBs as well as radioablated bone marrow and proliferating OBs [3,5,6]. Furthermore, CXCL12 has also been found to have a regulatory role in the osteogenic differentiation of murine MSCs [7].

Collectively, accumulated evidence suggests that constitutive and induced expression of CXCL12 is tightly regulated in different cells. Nevertheless, the molecular mechanisms involved in directing the correct levels of this chemokine remain unclear. Recently, a functional characterization of the CXCL12 gene promoter has been performed [8,9] and potential binding sites for transcriptional regulators including Sp1, AP2, HNF-3, NF-X3, glucocorticoid receptor, NFAT, c-myb, c/EPBβ and bHLH have been identified. In addition, several authors have observed a cell-specific inductivity of CXCL12 promoter activity [8] in response to anti-mitotic agents, γ irradiation, cytokines, cell confluence and transiently hypoxic microenvironments [3,8–11]. The important role of CXCL12/CXCR4 signaling in tumorigenesis was recently confirmed in another neoplastic context such as pancreatic cancer, in which the abrogation of CXCR4 inhibited invasion-related genes and the invasive ability of cancer cells [12]. Moreover, it has been highlighted that CXCL12/CXCR4 signaling finely modulates craniofacial development and both their knock-down and over-expression result in cranial neural crest cell migration defects and ectopic craniofacial cartilage formation [13].

Transcription factors play a critical role in cell–cell communication, which occurs in neural crest cell migration and epithelial mesenchymal transition (EMT) correlated with cancer invasion[12]. Slug, also called Snail2, is a member of a superfamily of zinc-finger transcription factors that play a central role in the patterning of vertebrate embryos [14]. Slug is implicated in the induction of EMT at specific stages of normal development and tumor progression, by acting as a transcriptional repressor of the gene-encoding components of cell–cell adhesive complexes in the epithelia [14–16]. Slug and its family members have also important roles in other processes, including the protection of cells from programmed cell death, the regulation of cytoskeletal elements [17] and the migration of neural crest cells [18]. We recently showed that Slug is required for OB maturation, being involved in the transcriptional control of OB-specific genes including Runx-2, a determinant transcription factor for osteoblastogenesis [19].

To provide critical new insights into the mechanisms underlying the OB phenotype that might lead to better control of the differentiation of these cells we studied a possible link between Slug and the CXCL12 chemokine. By expression analysis, gene silencing and chromatin immunoprecipitation assays we show, for the first time, that Slug activity and CXCL12 signaling are two strictly correlated phenomena in bone cellular context. The presence of Slug is required for CXCL12 expression and these two proteins are both co-expressed in the mineralized nodules. Our findings corroborate the role of Slug and CXCL12 in osteogenesis and may be relevant in approaches aimed at discovering new molecular targets to use in bone repair regenerative medicine.

Materials and methods

Isolation and culture of hOBs

HOBs were obtained from trabecular bone located in the inner portion of the tibial plateau of patients undergoing total knee replacement. Briefly, bone chips were removed from the tibial plateau, collected in a V-glass tube containing 1.5 ml of 1:1 mixture of DMEM/Ham's F12K no calcium (Gibco, Invitrogen Corporation, Paisley, Scotland, UK) and supplemented with 15% FBS, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 25 μg/ml ascorbic acid (Sigma, St Louis, MO, USA), 4 mM glutamine (Sigma) according to the methods previously described [20].

Isolation and culture of hMSCs

HMSCs were isolated from bone marrow iliac crest aspirates using Ficoll-Hypaque density gradient (1.077 g/ml) (Sigma, St Louis, MO, USA), as previously reported [21]. Briefly, nucleated cells were collected at the interface, washed twice, suspended in α-MEM supplemented with 15% FBS and penicillin G (Sigma), counted and plated at a concentration of 2×10^6cell/T150 flask. After 48 h non-adherent cells were removed and the adherent MSCs expanded in vitro.

Osteogenesis of MSCs ($N=6$) was induced 24 h after seeding, by incubating cells in α-MEM medium supplemented with 100 μM ascorbic acid, 2 mM β-glycerophosphate and 100 nM dexamethasone (all purchased from Sigma) for 4 weeks and analyzed after 24 h (day 0) and at days 14, 21 and 28. The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (Sigma) as previously reported [19].

Characterization of hOBs and hMSCs by flow cytometry

Both hOBs ($N=10$) and hMSCs ($N=10$) (at passage 2) were fixed in 4% paraformaldehyde and incubated at 4 °C for 30 min with 5 μg/ml of the following monoclonal antibodies: anti-human-CD3, -CD34, -CD45 (all purchased from Dako Cytomation, Glostrup, Denmark), -CD90, -CD146 (Becton Dickinson, Mountain View, CA), -CD105 (produced from the hybridoma cell line, clone SH2, ATCC, Rockville, MD), diluted in PBS supplemented with 2% BSA and 0.1% sodium azide. The cells were washed twice and incubated with polyclonal rabbit anti-mouse immunoglobulins/FITC conjugate (DakoCytomation) at 4 °C for 30 min. After two final washes, the cells were analyzed using a FACStar plus Cytometer (Becton Dickinson). For isotype control, FITC-coupled non-specific mouse IgG was substituted for the primary antibody. Alkaline phosphatase activity was analyzed using alkaline phosphatase leukocyte kit (Sigma) as previously described [19].

Real-time RT-PCR

hOBs ($N=14$) and hMSCs ($N=13$) were harvested from three wells and total RNA was extracted using an RNeasy Mini Kit (Qiagen GMBH, Hilden, GM) according to the manufacturer's instructions and as previously described [22]. Real-time PCR was

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carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA). TaqMan technology, the Assays-On-Demand kit for human Slug and CXCL12 were used. The mRNA levels of target genes were corrected for GAPDH mRNA levels (endogenous control). All PCR reactions were performed in triplicate for each sample and were repeated three times. All experimental data were expressed as the mean ± s.e.m.

CD11 positive cells were also evaluated for CXCL12 expression as cells expressing low level of CXCL12 in physiological conditions.

Secretion of CXCL12 chemokine

Supernatants from both osteogenic-induced hMSCs (at days 0, 14, 21 and 28) and hOBs ($N=10$) (after 24 h of culture) were collected. CXCL12 production was evaluated by a specific immunoassay standardized in our laboratory using antibody pairs matched for CXCL12 (R&D Systems, Minneapolis, MN, USA). Sensitivity of the assay was 15 pg/ml. Data were expressed as measured pg/mlCXCL12/μg of total proteins ± s.e.m.

Immunocytochemical analysis of CXCL12 chemokine

For immunocytochemical analysis of osteogenic-induced hMSCs (at days 0, 14 and 28) the cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. Cells were then incubated with anti-human-CXCL12 (R&D Systems) or -Slug (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h, washed twice with TRIS buffered saline (TBS) pH 7.2 and then incubated with undiluted affinity purified goat anti-mouse immunoglobulins conjugated with alkaline phosphatase labeled polymer (Envision kit, Dako, Glostrup, Denmark) or goat anti-rabbit-HRP (DakoCytomation) at room temperature for 30 min. An alkaline phosphatase reaction using new fucsin as a substrate or a peroxidase reaction using 3,3' diaminobenzidine as substrate was performed. Negative staining control experiments were performed either by omitting the primary antibody or using a control isotype-matched antibody. Slides were counterstained with haematoxylin and evaluated in a brightfield microscope.

Small interfering RNA (siRNA) transfection

Three sets of Stealth RNAi duplexes and corresponding Stealth control were synthesized by Invitrogen Life Technologies (Carlsbad, CA, USA). Stealth RNAi compounds were 25 mer dsRNA containing proprietary chemical modifications that enhance nuclease stability and reduce off-target effects.

The following Stealth RNAi sequences were used:

siRNA/Slug1 sense: 5'-CCGUAUCUCUAUGAGAGUUACUCCA-3';
antisense: 5'-UGGAGUAACUCUCAUAGAGAUACGG-3';
siRNA/Slug2 sense: 5'-CCCUGGUUGCUUCAAGGACACAUUA-3';
antisense: 5'-UAAUGUGUCCUUGAAGCAACCAGGG-3';
siRNA/Slug3 sense: 5'-GGCUCAUCUGCAGACCCAUUCUGAU-3';
antisense: 5'-AUCAGAAUGGGUCUGCAGAUGAGCC-3'.

As previously found [19], the most effective fragments used for targeting human Slug were siRNA/Slug2.

Twenty-four hours before siRNA transfection, hOBs ($N=9$) were seeded in triplicate at a density of $16 \times 10^3/cm^2$ in DMEM with 10% FBS. The cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfected cells were incubated for 6 days at 37 °C before gene silencing analysis. As a negative control for the siRNA treatment, Medium GC Stealth RNAi Negative Control Duplex (Invitrogen) was used. Knockdown of Slug expression was verified by real-time RT-PCR.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out as previously described [22] using the standard protocol supplied by Upstate Biotechnology, Inc. (Lake Placid, NY) with their ChIP assay reagents. The hOBs ($N=3$) and hMSCs ($N=3$) were cross-linked with 1% formaldehyde for 10 min at 37 °C, washed in ice-cold PBS and suspended in SDS lysis buffer for 10 min on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors and precleared with 80 μl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 5 μg of anti- Slug (Santa Cruz Biotechnology, CA, USA), overnight at 4 °C. Immunocomplexes were mixed with 80 μl of DNA-coated protein A-agarose followed by incubation for 1 h at 4 °C. The beads were collected and sequentially washed five times with 1 ml each of the following buffers: low salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS,1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH-8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-pH 8.1) and TE buffer. The immunocomplexes were eluted two times by adding a 250 μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$ and the cross-linking reactions were reversed by incubation at 65 °C for 4 h. Then, the samples were digested with proteinase K (10 mg/ml) at 42 °C for 1 h, DNA was recovered by phenol/chloroform extractions, ethanol precipitated using 1 μl of 20 mg/ml glycogen as the carrier, and suspended in sterile water. For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). PCR was performed to analyze the presence of DNA precipitated by Slug specific antibody. Specific primers to amplify fragments of the CXCL12 gene promoter were:

Region 1 (243 bp fragment):

Forward F1: 5'-TACAGGCGAGGAAACTGAGGCT-3'
Reverse R1: 5'-GGACTGAATGAGAACCAATGAA-3'

Region 2 (292 bp fragment):

Forward F2: 5'-ATGCGTAATTACTTATGCTTAGC-3'
Reverse R2: 5'-TCAGGTGGGCAGCTGGACCTA-3'

Each PCR reaction was performed with 5 μl of the bound DNA fraction or 2 μl of the input. The PCR was performed as follows: pre-incubation at 95 °C for 5 min, 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 62 °C and 1 min at 72 °C, with one final incubation at 72 °C for 5 min. No-antibody control was included in each experiment.

Statistical analysis

Statistical analysis was performed using the non-parametric tests (Wilcoxon matched-pair test and Friedman ANOVA and Kendall's

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concordance non-parametric test) to evaluate if there was any tendency to increase or decrease along the time points analyzed. The Mann–Whitney *U* test was also used for comparing non-matched group of data. All values were expressed as the mean ± s.e.m. of five different experiments. The analyses were performed using CSS Statistica Statistical Software (Statsoft Inc., Tulsa, OK, USA). Values of $p < 0.05$ were considered significant.

Results

hMSC and hOB characterization

To characterize all samples used in this study, a panel of seven phenotypic markers were first analyzed in isolated hMSCs and hOBs. As shown in Fig. 1A, flow cytometric analysis showed that both hMSCs and hOBs were negative for typical hematopoietic markers such as CD3, CD34, CD45, whereas they were highly positive to CD90 and CD105. Interestingly, approximately 75% of hMSCs were positive to CD146, whereas hOBs were negative, as also described by other authors [23]. Moreover, alkaline phosphatase activity was found, as expected, both in hMSCs and in hOBs, at different levels (Fig. 1B). The osteogenic potential of hMSCs was then tested by inducing the cells to differentiate along the osteoblast lineage in osteogenic medium and demonstrating the positive staining for extracellular calcium deposition at days 14 and 28 (Fig. 1C).

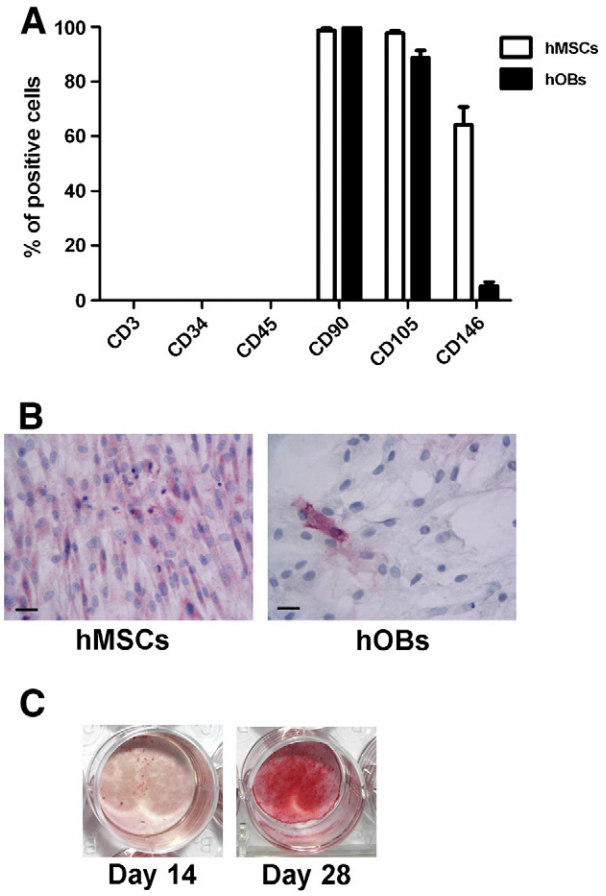

Fig. 1 – Characterization of hMSCs and hOBs. (A) Flow cytometry analysis of CD3, CD34, CD45, CD90, CD105, CD146. Data were expressed as % positive cells ± s.e.m. (B) Cytochemical activity of alkaline phosphatase in hMSCs and hOBs. Scale bar = 25 µm. (C) Alizarin red S staining of osteogenic-induced hMSCs at day 14 and day 28.

CXCL12 chemokine expression in hMSCs and hOBs

CXCL12 chemokine expression analysis was then performed on hMSCs and hOBs both at mRNA and protein level. The analysis was performed both in the cells at the uninduced basal condition, and in hMSCs induced to differentiate along the osteoblast lineage up to 28 days. As shown in Fig. 2A, we found high levels of CXCL12 mRNA in uninduced hMSCs and hOBs cells, whereas the exposure to osteogenic medium downregulated the expression of CXCL12 mRNA, producing a significant decrease of CXCL12 transcript at day 14 ($p < 0.05$). The level of CXCL12 mRNA detected at day 14 remained unchanged during osteogenesis up to day 28, and, was always higher than that found in physiological conditions in CD11b + cells (% GAPDH = 0.0446 ± 0.012) (data not shown). Fig. 2B showed that the level of CXCL12 secretion in the hMSCs induced to osteogenesis decreased, as determined by ELISA performed in cell supernatants. This analysis revealed that CXCL12 protein levels were consistently higher in uninduced versus induced hMSCs in agreement with results from mRNA analysis. However, it is interesting to note that the CXCL12 secretion continued to decrease during osteogenesis even if CXCL12 transcript levels remained unchanged throughout the time course relative to osteogenesis induction (from day 14 to day 28). This suggests that different mechanisms are required to determine the level of CXCL12 expression at mRNA and protein level. Furthermore, intracellular CXCL12 expression during osteogenic induction (from days 0 to 28) also generally decreased, as found by immunocytochemical analysis (Fig. 2C). Interestingly, the same analysis revealed that CXCL12 expression at day 28 was strictly confined to mineralized nodules (Fig. 2C), thus suggesting a potential role of this chemokine in cells destined to become osteoblasts. Concerning the CXCR4 receptor, we found that its expression was not modulated during osteogenic differentiation (data not shown).

Slug knockdown and CXCL12 expression

To provide additional evidence for the role of CXCL12 in the bone cellular context, we correlated the expression of this chemokine with Slug, a transcription factor that we recently showed to be required for osteoblast maturation [19]. As shown in Fig. 3A, Slug mRNA, analyzed by quantitative RT-PCR, increased during the process of osteogenic differentiation of hMSCs, was highly expressed in mature hOBs. This was confirmed also at protein level by immunocytochemical analysis, thus showing that the positive signal increased during osteogenic differentiation of hMSCs, both in the single cells and in the mineralized nodules (Fig. 3B).

A possible role for Slug in regulating CXCL12 expression was tested by knocking down the expression of Slug with a siRNA approach in hOBs. We blocked the endogenous production of Slug by treating hOBs for 6 days with a previously tested siRNA against Slug [19] and obtained a strong inhibition of Slug mRNA and protein, as revealed by quantitative RT-PCR and western blot analysis,

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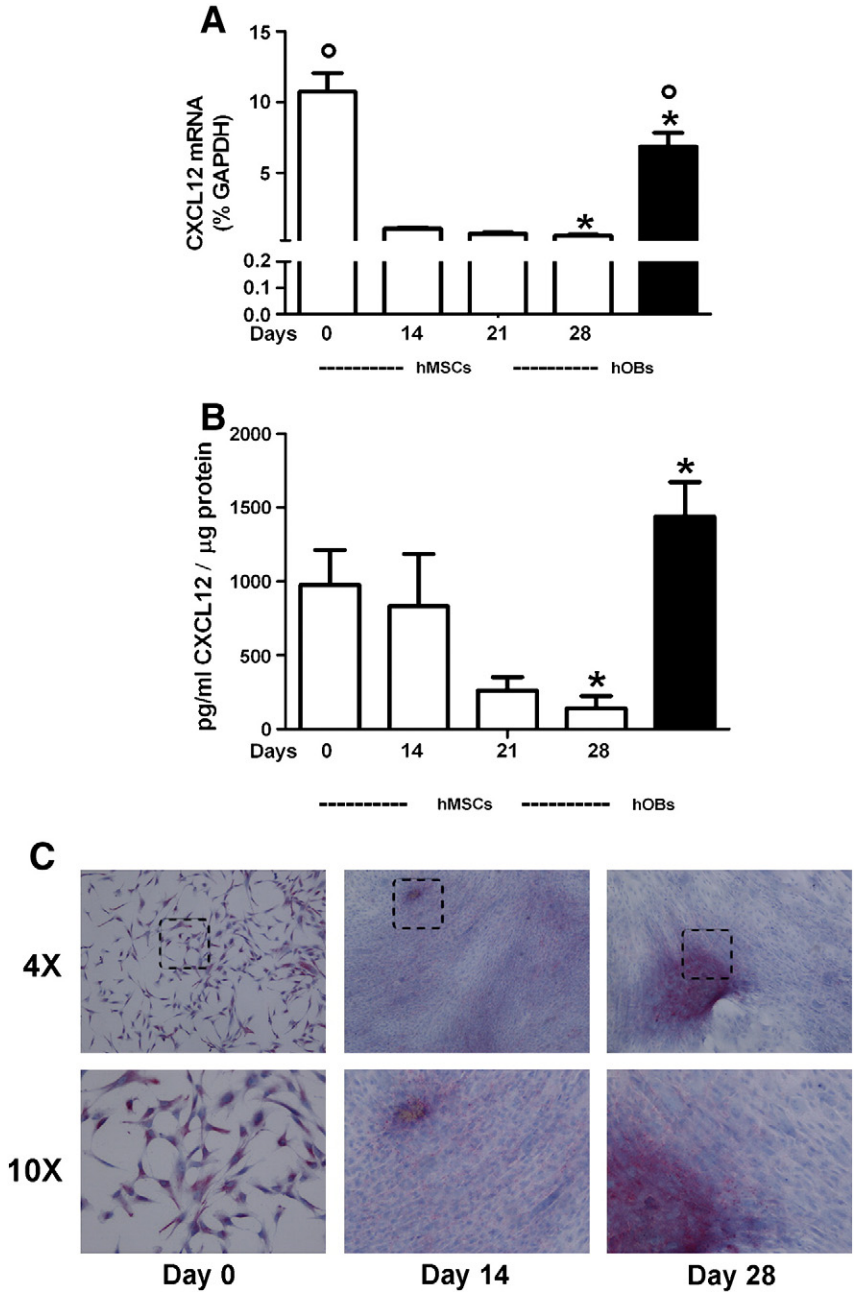

Fig. 2 – CXCL12 expression in osteogenic-induced hMSCs and hOBs. (A) Analysis of CXCL12 mRNA expression in both osteogenic-induced hMSCs (from day 0 to day 28) and hOBs. Data were expressed as % GAPDH ± s.e.m. °$p < 0.05$ hMSCs day 0 versus hOBs, *$p < 0.05$ hMSCs day 28 versus hOBs. (B) CXCL12 protein secreted levels in both osteogenic-induced hMSCs (from day 0 to day 28) and in hOBs. Data were expressed as pg/ml CXCL12/µg protein ± s.e.m. *$p < 0.05$ hMSCs day 28 versus hOBs. (C). Immunocytochemical analysis of CXCL12 on osteogenic-induced hMSCs at days 0, 14, 28. Magnification, 4× and 10×.

respectively (Fig. 4). In addition, as previously shown [19], Slug knockdown produced a decrease in mineralized matrix deposition (Fig. 4B). We chose hOBs as an experimental model because of their high CXCL12 and Slug contents, and their more homogeneous phenotype in comparison with that presented by hMSCs. As reported in Fig. 4C, Slug-silenced cells showed an increase in CXCL12 mRNA. Nevertheless, when CXCL12 was investigated at protein level, Slug-silenced cells showed a strong decrease of CXCL12 intracellular content, as demonstrated by immunocytochemical analysis (Fig. 4D). In addition, Slug-silenced cells released significantly lower levels of CXCL12 in comparison with control cells, as demonstrated by ELISA analysis (control cells: mean ± SE 936.61 ± 261.01; Slug-silenced cells: mean ± SE 703 ± 228.24) (Fig. 4E).

The absence of correlation between mRNA and protein levels of CXCL12 in Slug-silenced cells is in agreement with that observed in the MSCs induced to osteogenesis (see Fig. 2) and suggests that Slug is differently involved in determining CXCL12 expression at mRNA and protein level.

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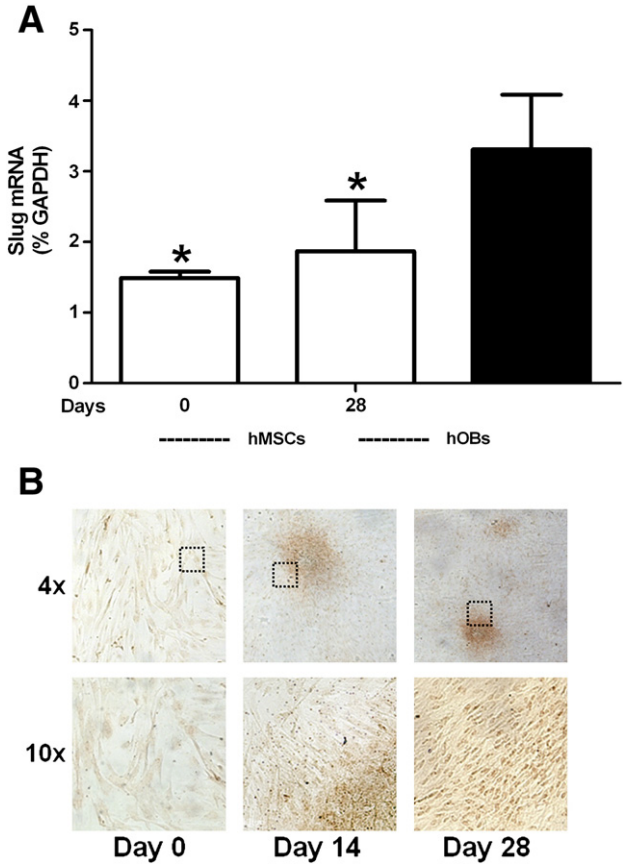

Fig. 3 – Slug expression in osteogenic-induced hMSCs and hOBs. (A) Evaluation of Slug mRNA expression in osteogenic-induced hMSCs (at days 0 and 28) and in hOBs. Data were expressed as % GAPDH ± s.e.m. *$p < 0.05$ hMSCs day 28 versus hOBs. (B) Immunocytochemical analysis of Slug on osteogenic-induced hMSCs at days 0, 14, 28. Magnification, 4× and 10×.

Slug interacts in vivo with the promoter of the CXCL12 gene

We then tested whether Slug participated directly in the control of CXCL12 gene transcription in hOBs. For this purpose, chromatin immunoprecipitation (ChIP) analysis was performed on the promoter of the CXCL12 gene. Using a program for predicting transcription factor binding sites (TFSEARCH, www.cbrc.jp/research/db/TFSEARCH.html), we found in the promoter of human CXCL12 gene two consensus sequences for Slug (5'-CANNTG-3'), at −509, and −1877 together with other non-canonical Slug binding sites, partially in agreement with those recently determined by Calonge et al. [9] (Fig. 5). We focused on the consensus E boxes at −509 and −1877. Their functionality was assessed by ChIP assay, by analyzing the in vivo association between Slug and the promoter sequences containing these two E boxes. hOBs and hMSCs were exposed to formaldehyde to cross-link proteins and DNA, and sonicated to fragment the chromatin. Specific antibody against Slug was used to immunoprecipitate the protein–DNA complexes, and Slug recruitment was assessed at the two different promoter regions by using the specific sets of primers as reported in Fig. 5. The presence of the promoter specific DNA region before immunoprecipitation was confirmed by PCR (input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate specific PCR products spanning the putative Slug binding sites from −2000 bp to +1 bp in the CXCL12 gene promoter. Slug occupancy was detected in regions 1 and 2 in hOBs and only in region 2 in hMSCs, thus suggesting a possible different role of these cis elements and different regulatory function of Slug in hOBs and hMSCs. These ChIP experiments show that CXCL12 is a novel Slug target gene in vivo.

Discussion

The role of CXCL12/CXCR4 chemokine/receptor signaling axis has been elucidated in many cellular contexts [8]. Concerning bone tissue, it has recently been suggested that this signaling is involved in the remodeling process [3,5,6], has a critical role in the recruitment of MSCs to the fracture site during skeletal repair [24], and is highly expressed and secreted by regenerating/proliferating osteoblasts after irradiation in mouse models [5]. Although a recent partial characterization of the gene promoter suggested that both the constitutive and induced expression of CXCL12 is finely regulated at transcriptional level [8,9], the molecular mechanisms involved in directing the correct levels of this chemokine remain unclear.

This study shows that CXCL12 is present at high levels both in hMSCs and hOBs uninduced basal condition. In agreement with previous studies [7,25,26], the expression of this chemokine is generally downregulated in hMSCs induced to differentiate toward the osteoblastic lineage, but mineralized nodules show a strong immunostaining for CXCL12, thus suggesting a potential role for this chemokine in cells destined to become osteoblasts. In fact, also recent reports [5,24], using in vivo models, demonstrated an elevated expression of this chemokine in active proliferating osteoblasts lining the bone trabeculae, so corroborating its role in functional activity of these cells. To investigate what mechanisms contribute to the maintenance of a correct balance of CXCL12 in bone cellular context, we analyzed for the first time the relationship between CXCL12 and Slug, a transcription factor recently associated with osteoblast maturation [19]. Interestingly, we found that Slug is highly expressed in the mineralized nodules and is specifically recruited to CXCL12 gene promoter in vivo, as shown by chromatin immunoprecipitation assays. These findings, suggesting an involvement of Slug and

Fig. 4 – Evaluation of Slug knockdown effect. (A) siRNA efficiency evaluation on Slug gene and protein knockdown. hOBs were transfected with siRNA against Slug or a non-relevant siRNA (scr). Slug expression was determined both at protein and mRNA level, and revealed by western blot and quantitative RT-PCR analysis, respectively. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug over control ratio. Results represent means ± SEM of nine hOBs samples. (B) Alizarin Red S staining of osteogenic-induced hMSCs both in control (−) and in siRNA/Slug2 silenced (+) hOBs. (C) Evaluation of Slug and CXCL12 mRNA expression both in control (−) and in siRNA/Slug2 silenced (+) hOBs. Data were expressed as relative mRNA expression levels (% GAPDH) ± s.e.m. *$p < 0.05$. (D) Immunocytochemical analysis of CXCL12 in control (−) and in siRNA/Slug2 silenced (+) hOBs. Scale bar = 25 μm. (E) CXCL12 protein secreted levels in control (−) and in siRNA/Slug2 silenced (+) hOBs. Data were expressed as pg/ml CXCL12/μg protein ± s.e.m. *$p < 0.05$.

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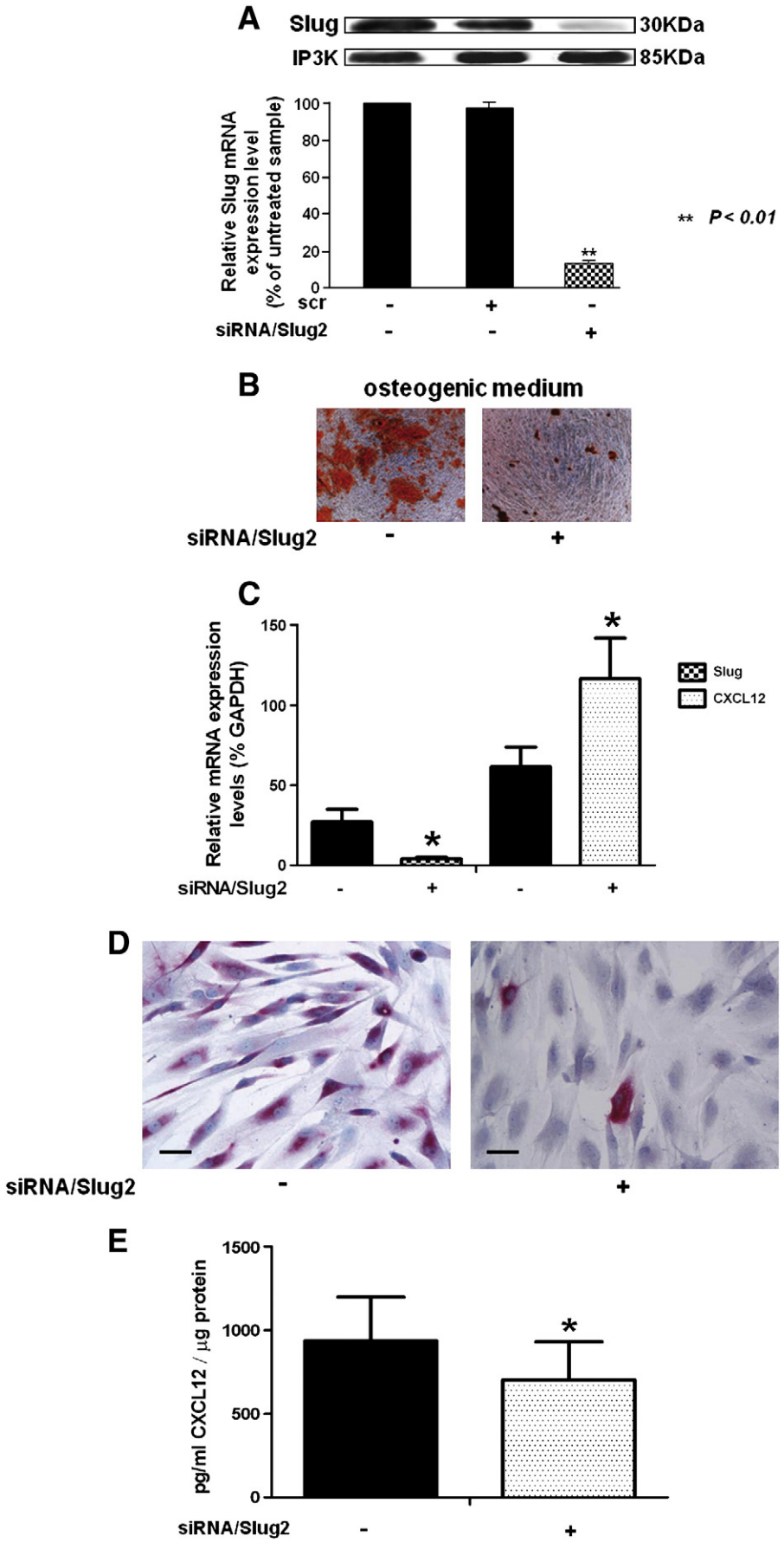

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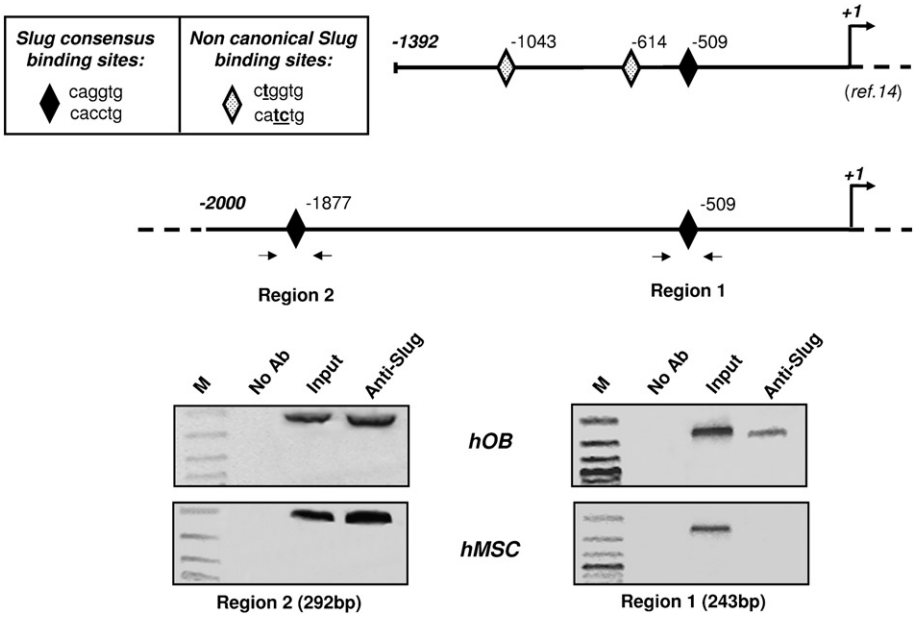

Fig. 5 – Slug binds human CXCL12 promoter in vivo. The CXCL12 promoter region under investigation is shown (+1/−2000). The positions of the putative Slug consensus binding sites are enclosed by black diamonds and are compared with those recently investigated by others [9]; non-canonical binding sites (hatched diamonds) are included. The arrows indicate the positions of PCR primers used in chromatin immunoprecipitation (ChIP) experiments. Recruitment of Slug transcription factor to the human CXCL12 promoter is shown by in vivo ChIP binding assays. Soluble chromatin was prepared from hOBs and hMSCs and immunoprecipitated with the specific antibody against Slug. The association of the transcription factor on the regions 1 and 2 of the human CXCL12 promoter was analyzed by PCR generating the reported DNA fragments. No Ab represents a negative control. Input represents a positive control using the starting material (0.2%) prior to immunoprecipitation. Representative agarose gels are shown. M: molecular weight marker.

CXCL12 in mineralized matrix deposition, were confirmed by Slug silencing experiments performed in hOBs, since they express both proteins at high level.

For this reason, it is important to underline that mature OBs are committed cells representing the last cellular stage before terminally differentiated osteocytes. In line with other authors [23,27] hOBs, compared to MSCs, expressed a low percentage of CD146, which is a typical marker of self-renewing, clonogenic osteoprogenitor cells in the bone marrow, and a low expression of alkaline phosphatase, thus confirming the different stage of maturation of these cells. In fact, hOBs have a peculiar gene expression profile, which is different from that shown by MSCs induced to differentiate towards OBs [28]. In addition, it has been shown [28] that cytokine expression is influenced by the osteogenic compartment of MSCs and defines the response to in vitro stimulatory treatment. Therefore, it is not surprising that OBs expressed higher mRNA and protein levels for CXCL12 than MSCs.

We found that Slug knockdown in hOBs induced a consistent decrease of CXCL12 protein even if the corresponding mRNA increased. One explanation for this apparent discrepancy found for mRNA and protein expression of CXCL12 in Slug-silenced cells may be the presence of numerous potential Slug targets in the cell and, consequently, the wide spectrum of effects generated by its silencing. Therefore, our findings suggest possible different roles for Slug and consequently at least two different main effects of Slug knockdown: a) Slug may act as a repressor of CXCL12 gene transcription and its silencing removes the negative control by increasing CXCL12 mRNA levels, b) Slug may have an indirect positive impact on components of specific pathways correlated with expression, stability secretion and activity of CXCL12 and its silencing results in a decrease in CXCL12 protein. Therefore, our data suggest that high levels of CXCL12 mRNA are not sufficient to assure high levels of CXCL12 protein level that is evidently correlated with other factors and mechanisms including Slug-mediated events. Nevertheless, since the remarkable final phenotypic effect mediated by Slug silencing is the decrease in CXCL12 protein expression and secretion, it may be concluded that the presence of Slug is required for CXCL12 protein expression, thus supporting the hypothesis that both proteins are crucial for osteoblast phenotype.

The analysis of this large and complex hypothesis spectrum is not the object of our study and remains to be explored in the future; however, the evidence that Slug suppression and consequent CXCL12 decrease induced a dramatic phenotypical change in hOBs with a loss of their mineralization ability, indicates that both Slug and CXCL12 are required for the osteoblastic phenotype.

It is important to underline that the potential strict correlation between CXCL12 signaling and Slug activity has been shown in other experimental models that can be related to bone. For example, CXCL12 and Slug are both involved in neural crest cell (NCC) migration. NCCs are multipotent cells that migrate along defined pathways throughout the embryo in a Slug-dependent manner, giving rise to many diverse cell types including craniofacial skeletal elements [13]. It is well known that aberrant migration of NCCs results in a wide variety of congenital birth defects including craniofacial skeletal abnormalities [13]. Moreover, recent evidence suggests that both knockdown and an over-expression of CXCL12 and its receptor leads to migration and

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important craniofacial defects for patterning the craniofacial skeleton [13]. Further evidence for the need for a correct balance of CXCL12 signaling and the correlation between Slug and CXCL12 comes from a recent paper demonstrating that elevated CXCL12 and CXCR4 expression was correlated to advanced cancer stage and metastasis [29,30]. Interestingly, in CXCR4-silenced pancreatic cancer cells Slug expression is inhibited [12].

Other factors and mechanisms may surely contribute to CXCL12 gene regulation. At present, the relationship between other cis-regulatory elements in the CXCL12 promoter and hOBs-inducing signals is completely unknown. The most likely candidates for this function are bone-related transcription factors Lef-1 and Runx2 whose putative binding sites are present in the CXCL12 gene promoter.

In conclusion, although further studies are required to elucidate in detail the exact function of Slug in regulating CXCL12/CXCR4 axis, not only in osteoblasts but also during the osteogenic differentiation of their mesenchymal precursors, our study clearly shows that Slug activity and CXCL12 signaling are two strictly correlated phenomena in bone cellular context, and may be relevant in approaches aimed at discovering new molecular targets to use in bone repair regenerative medicine. Our hypothesis is that Slug together with CXCL12 might be one of the components of the recently proposed [31] large signalosome in which inputs from Wnt/β-catenin/Lef-1 signaling, steroid receptors, BMPs, and kinases converge to induce osteoblast differentiation and maturation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by grants from Regione Emilia Romagna, Programma di Ricerca Regione Università 2007–2009. E.L. is a recipient of a fellowship from the Fondazione Cassa di Risparmio di Cento.

REFERENCES

[1] C.R. MacKay, Chemokines: immunology's high impact factors, Nat. Immunol. 2 (2001) 95–101.
[2] B. Moser, P. Loetscher, Lymphocyte traffic control by chemokines, Nat. Immunol. 2 (2001) 123–128.
[3] T. Ponomaryov, A. Peled, I. Petit, R.S. Taichman, L. Habler, J. Sandbank, F. Arenzana-Seisdedos, A. Magerus, A. Caruz, N. Fujii, A. Nagler, M. Lahav, M. Szyper-Kravitz, D. Zipori, T. Lapidot, Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function, J. Clin. Invest. 106 (2000) 1331–1339.
[4] G. Lisignoli, S. Toneguzzi, A. Piacentini, S. Cristino, F. Grassi, C. Cavallo, A. Facchini, CXCL12 (SDF-1) and CXCL13 (BCA-1) chemokines significantly induce proliferation and collagen type I expression in osteoblasts from osteoarthritis patients, J. Cell Physiol. 206 (2006) 78–85.
[5] M. Dominici, V. Rasini, R. Bussolari, X. Chen, T.J. Hofmann, C. Spano, D. Bernabei, E. Veronesi, F. Bertoni, P. Paolucci, P. Conte, E.M. Horwitz, Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation, Blood 114 (2009) 2333–2343.
[6] S. Otsuru, K. Tamai, T. Yamazaki, H. Yoshikawa, Y. Kaneda, Circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site by the CXCR4/stromal cell-derived factor-1 pathway, Stem Cells 26 (2008) 223–234.
[7] W. Zhu, O. Boachie-Adjei, B.A. Rawlins, B. Frenkel, A.L. Boskey, L.B. Ivashkiv, C.P. Blobel, A novel regulatory role for stromal-derived factor-1 signaling in bone morphogenic protein-2 osteogenic differentiation of mesenchymal C2C12 cells, J. Biol. Chem. 282 (2007) 18676–18685.
[8] C. Garcia-Moruja, J.M. Alonso-Lobo, P. Rueda, C. Torres, N. Gonzalez, M. Bermejo, F. Luque, F. Arenzana-Seisdedos, J. Alcami, A. Caruz, Functional characterization of SDF-1 proximal promoter, J. Mol. Biol. 348 (2005) 43–62.
[9] E. Calonge, J.M. Alonso-Lobo, C. Escandon, N. Gonzalez, M. Bermejo, B. Santiago, L. Mestre, J.L. Pablos, A. Caruz, J. Alcami, c/EBPbeta is a major regulatory element driving transcriptional activation of the CXCL12 promoter, J. Mol. Biol. 396 (2010) 463–472.
[10] D. Simons, G. Grieb, M. Hristov, N. Pallua, C. Weber, J. Bernhagen, G. Steffens, Hypoxia-induced endothelial secretion of macrophage migration inhibitory factor and role in endothelial progenitor cell recruitment, J. Cell Mol. Med. (2010), doi:10.1111/j.1582-4934.2010.01041.x.
[11] L. Chen, S. Xu, X. Zeng, J. Li, W. Yin, Y. Chen, Z. Shao, W. Jin, c-myb activates CXCL12 transcription in T47D and MCF7 breast cancer cells, Acta Biochim. Biophys. Sin. 42 (2010) 1–7.
[12] Z. Wang, Q. Ma, Q. Liu, H. Yu, L. Zhao, S. Shen, J. Yao, Blockade of SDF-1/CXCR4 signalling inhibits pancreatic cancer progression in vitro via inactivation of canonical Wnt pathway, Br. J. Cancer 99 (2008) 1695–1703.
[13] E.C. Olesnicky Killian, D.A. Birkholz, K.B. Artinger, A role for chemokine signaling in neural crest cell migration and craniofacial development, Dev. Biol. 333 (2009) 161–172.
[14] M.A. Nieto, The snail superfamily of zinc-finger transcription factors, Nat. Rev. Mol. Cell Biol. 3 (2002) 155–166.
[15] M. Conacci-Sorrell, I. Simcha, T. Ben-Yedidia, J. Blechman, P. Savagner, A. Ben-Ze'ev, Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK, J. Cell Biol. 163 (2003) 847–857.
[16] K.M. Hajra, D.Y. Chen, E.R. Fearon, The SLUG zinc-finger protein represses E-cadherin in breast cancer, Cancer Res. 62 (2002) 1613–1618.
[17] A. Barrallo-Gimeno, M.A. Nieto, The Snail genes as inducers of cell movement and survival: implications in development and cancer, Development 132 (2005) 3151–3161.
[18] D. Sakai, T. Suzuki, N. Osumi, Y. Wakamatsu, Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development, Development 133 (2006) 1323–1333.
[19] E. Lambertini, G. Lisignoli, E. Torreggiani, C. Manferdini, E. Gabusi, T. Franceschetti, L. Penolazzi, R. Gambari, A. Facchini, R. Piva, Slug gene expression supports human osteoblast maturation, Cell Mol. Life Sci. 66 (2009) 3641–3653.
[20] P.G. Robey, J.D. Termine, Human bone cells in vitro, Calcif. Tissue Int. 37 (1985) 453–460.
[21] G. Lisignoli, S. Cristino, A. Piacentini, S. Toneguzzi, F. Grassi, C. Cavallo, N. Zini, L. Solimando, N.M. Maraldi, A. Facchini, Cellular and molecular events during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold, Biomaterials 26 (2005) 5677–5686.
[22] E. Lambertini, E. Tavanti, E. Torreggiani, L. Penolazzi, R. Gambari, R. Piva, ERalpha and AP-1 interact in vivo with a specific sequence of the F promoter of the human ERalpha gene in osteoblasts, J. Cell Physiol. 216 (2008) 101–110.
[23] B. Sacchetti, A. Funari, S. Michienzi, S. Di Cesare, S. Piersanti, I. Saggio, E. Tagliafico, S. Ferrari, P.G. Robey, M. Riminucci, P. Bianco, Self-renewing osteoprogenitors in bone marrow sinusoids can

organize a hematopoietic microenvironment, Cell 131 (2007) 324–336.
[24] T. Kitaori, H. Ito, E.M. Schwarz, R. Tsutsumi, H. Yoshitomi, S. Oishi, M. Nakano, N. Fujii, T. Nagasawa, T. Nakamura, Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model, Arthritis Rheum. 60 (2009) 813–823.
[25] H.L. Aguila, D.W. Rowe, Skeletal development, bone remodeling, and hematopoiesis, Immunol. Rev. 208 (2005) 7–18.
[26] N. Hosogane, Z. Huang, B.A. Rawlins, X. Liu, O. Boachie-Adjei, A.L. Boskey, W. Zhu, Stromal derived factor-1 regulates bone morphogenetic protein 2-induced osteogenic differentiation of primary mesenchymal stem cells, Int. J. Biochem. Cell Biol. 42 (2010) 1132–1141.
[27] L. Song, N.J. Young, N.E. Webb, R.S. Tuan, Origin and characterization of multipotential mesenchymal stem cells derived from adult human trabecular bone, Stem Cells Dev. 14 (2005) 712–721.
[28] I. Shur, F. Lokiec, I. Bleiberg, D. Benayahu, Differential gene expression of cultured human osteoblasts, J. Cell Biochem. 83 (2001) 547–553.
[29] J. Schulze, J. Albers, A. Baranowsky, J. Keller, A. Spiro, T. Streichert, J. Zustin, M. Amling, T. Schinke, Osteolytic prostate cancer cells induce the expression of specific cytokines in bone-forming osteoblasts through a Stat3/5-dependent mechanism, Bone 46 (2010) 524–533.
[30] Y.X. Sun, A. Schneider, Y. Jung, J. Wang, J. Dai, J. Wang, K. Cook, N.I. Osman, A.J. Koh-Paige, H. Shim, K.J. Pienta, E.T. Keller, L.K. McCauley, R.S. Taichman, Skeletal localization and neutralization of the SDF-1 (CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites in vivo, J. Bone Miner. Res. 20 (2005) 318–329.
[31] S. Kousteni, M. Almeida, L. Han, T. Bellido, R.L. Jilka, S.C. Manolagas, Induction of osteoblast differentiation by selective activation of kinase-mediated actions of the estrogen receptor, Mol. Cell Biol. 27 (2007) 1516–1530.

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Transcription factor decoy against NFATc1 in human primary osteoblasts

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DOI: 10.3892/ijmm_xxxxxxxx

Abstract. The present study describes, for the first time, the removal of the nuclear factor of activated T cells cytoplasmic 1 (NFATc1) by a decoy approach in human primary osteoblasts (hOBs). hOBs with different NFATc1 expression levels were used. The functionality of endogenous NFAT proteins in our experimental model was analyzed by monitoring the transcriptional activity on a luciferase reporter construct driven by three copies of an NFAT response element (pNFAT-TA-luc). Cell treatment with the decoy against NFATc1 resulted in a significant increase in the expression of osteoblastic markers, including ERα and ColXV. On the contrary, the expression of Runx2, which is known to not be transcriptionally regulated by NFATc1, was not altered, indicating the specificity of the decoy effect. To our knowledge, this is the first time that transcription factor decoy has been successful in hOBs to allow the investigation of the role of NFATc1 in an experimental model that, compared to the use of cell lines, more closely resembles an *in vivo* model. In addition, by using chromatin immunoprecipitation we found that *in vivo* NFATc1 is recruited on the ColXV gene promoter. The specific role of NFATc1 in osteoblast differentiation is not well understood, however, our findings reinforce the action of NFATc1 in the transcriptional program of osteoblasts, also supporting the therapeutic potential for the proper manipulation of NFATc1-mediated events in different bone disorders. At the same time, our data add important information on the regulation of the expression of ColXV, which only recently has been proposed as an osteoblastic marker.

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Key words: nuclear factor of activated T cells, collagen type XV, transcription factor decoy, human primary osteoblasts, chromatin immunoprecipitation

Introduction

Modulation of the activities of bone-forming osteoblasts and bone-resorbing osteoclasts is one of the major therapeutic objectives in a variety of bone tissue diseases (1,2). As a therapeutic target for modulation of bone remodeling, the nuclear factor of activated T cells (NFAT) has recently received considerable attention, even if many reports describe the importance of the NFAT-mediated signaling in the control of both bone resorption and bone formation with contradictory findings (3-8).

NFAT proteins comprise a family of transcription factors (NFAT 1-5) that, after calcium/calcineurin-dependent dephosphorylation, are activated and regulate the expression of many genes involved in a wide range of cellular processes (9-11). NFAT proteins are capable of forming strong cooperative complexes with related transcription factors such as NF-κB proteins, and unrelated binding partners such as GATA, Maf, Oct, PPAR-γ, and members of the AP-1 family on composite cis elements (1,12-15).

To gain insight into the role of NFAT proteins in the bone, and in the search for novel NFAT target genes in osteoblasts, we analyzed the effect of the inhibition of NFATc1 function, through a specific decoy approach, in human primary osteoblasts (hOBs). The transcription factor decoy (TFD) strategy is a kind of gene silencing approach aimed at eliminating or attenuating the activity of a specific transcription factor (16-23). TFD is based on the cellular transfection of double-stranded DNA oligonucleotides (ODNs) mimicking the *cis*-element that is recognized by the transcription factor, and transcription factor binding on endogenous target genes is quickly and effectively blocked. Therefore, the decoy approach is a powerful tool for interfering with the activity of a specific transcription factor and for modulating transcription of its target genes. To our knowledge, this is the first time that TFD has been successfully used in hOBs to investigate the role of NFATc1 in an experimental model which compared to the use of cell lines is more closely resembles an *in vivo* model.

Our previous study identified NFATc1 as negative regulator of the estrogen receptor α (ERα) gene transcription in

the SaOS-2 osteosarcoma cell line (24). Considering that ERα plays an important positive role in the control of proliferation, differentiation, and survival of osteoblasts (25), our previous data (24) led us to more thoroughly investigate the effect of NFATc1 inhibition in hOBs, an experimental model more easily associated with an *in vivo* context.

Materials and methods

Isolation and culture of osteoblasts. Bone tissues were harvested from ten elderly patients undergoing total knee replacement for osteoarthritis (mean age 70±9). The study was approved by the local Ethics Committee and informed consent was obtained from each patient.

Trabecular bone was obtained from the inner portion of the tibial plateau. Bone chips were removed from the tibial plateau, collected in a V-glass tube containing 1.5 ml of α-MEM supplemented with 15% FBS, 25 mg/ml ascorbic acid and penicillin G (Sigma, St. Louis, MO, USA) (referred as α-MEM medium).

Transient transfections and the luciferase assay. Transient transfections were performed on cells grown to 60% confluency, in 24-well plates with α-MEM medium plus 10% FBS, using Lipofectamine reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were transfected with a total of 1 μg/ml of ODN-decoy complexed with 3 μg of Lipofectamine 24 h after the seeding (first administration); on day 4, the cells were further transfected with the same DNA amount until day 7, when the cells were harvested.

For transfection experiments with pNFAT-TA-luc or empty vectors, the cells were plated in 24-well plates, maintained in α-MEM medium with 10% FBS, in the presence of different amounts of $CaCl_2$ (0.5, 1.3, 2.6 mM) and in the presence of 0.5 μM ionomycin and 100 μg/ml PMA (Sigma). After 24 h the cells were transiently transfected using Lipofectamine reagent (Invitrogen Corp.) with 5 mg of plasmid vector. After overnight incubation, the medium was removed and fresh medium was added. After another 24 h, the cells were harvested, lysed, and assayed for luciferase activity. The luciferase activity was normalized by the total protein amount and by β-galactosidase values resulting from co-transfection of 0.25 μg of pCMVSport β-gal (Invitrogen Corp.). β-galactosidase activity was measured by using the Beta-Glo Assay System (Promega, WI, USA).

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was carried out as previously described (26) using the standard protocol supplied by Upstate Biotechnology (Lake Placid, NY) with their ChIP assay reagents. The cells were cross-linked with 1% formaldehyde for 10 min at 37˚C, washed in ice-cold PBS, and suspended in SDS lysis buffer for 10 min on ice. Samples were sonicated, diluted 10-fold in dilution buffer supplemented with protease inhibitors, and precleared with 80 μl of DNA-coated protein A-agarose; the supernatant was used directly for immunoprecipitation with 10 μg of anti-NFATc1, (H-110) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at 4˚C. Immunocomplexes were mixed with 80 μl of DNA-coated protein A-agarose followed by incubation for 1 h at 4˚C. Beads were collected and sequentially washed 3 times with 1 ml each of the following buffers, low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 mM LiCl, 1% Igepal CA630, 1% deoxycholic acid, 1 mM EDTA,10 mM Tris pH 8.1), and TE buffer. The immunocomplexes were eluted two times by adding a 250 μl aliquot of a freshly prepared solution of 1% SDS, 0.1 M $NaHCO_3$ and the cross-linking reactions were reversed by incubation at 65˚C for 4 h. The samples were then digested with proteinase K (10 mg/ml) at 45˚C for 2 h and DNA was purified by Qiaquick Spin Columns (Qiagen Inc., Germantown, MD). For PCR analysis, aliquots of chromatin before immunoprecipitation were saved (Input). PCR was performed to analyze the presence of DNA precipitated by the NFATc1-specific antibody, and by using specific primers (Table I) to amplify fragments of the collagen type XV (ColXV) gene promoter. Each PCR reaction was performed with 5 μl of the bound DNA fraction or 2 μl of the Input. The PCR was performed as follows, pre-incubation at 95˚C for 5 min, 30 cycles of 1 min denaturation at 95˚C, 1 min annealing at 52˚C and 1 min at 72˚C, with one final incubation at 72˚C for 5 min. A no-antibody control was included in each experiment.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from Jurkat cells were prepared as previously described (27). Double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase (GE Healthcare, Germany). For the gel shift assay 10 μg of nuclear proteins were pre-incubated for 5 min at room temperature with 1.2 μg of non-specific competitor DNA poly(dI-dC)-(dI-dC) (Pharmacia Corp., Bridgewater, NJ) and then 10,000 cpm of labeled oligonucleotides were added and incubated for another 30 min at room temperature. For the competition experiments a 50- and 100-fold excess of unlabeled oligonucleotides was incubated with the nuclear extracts for 15 min at room temperature and then incubated with the appropriate ^{32}P-labeled DNA probe. For supershift experiments, nuclear extracts were pre-incubated for 15 min at room temperature with 4 μg of the antibody against NFATc1 prior to their 30 min incubation with the labelled oligonucleotides at room temperature. Reactions were run on 6% polyacrylamide gels and electrophoresed at 150 V for 2 h in 0.5X TBE buffer. Gels were then dried and exposed to film at -80˚C.

Immunocytochemistry. Cells grown in chamber slides were fixed in 4% PFA for 20 min at room temperature and then hydrated with TBS 1% BSA for 5 min at room temperature. The slides were incubated with the monoclonal antibodies: anti-human-Runx2, anti-osteocalcin (OC; R&D Systems, Minneapolis, MN), anti-bone sialoprotein (BSP; Fisher Scientific, Pittsburg, PA, USA), anti-collagen type 1 (Col1A1; obtained from DSHB, Department of Biological Sciences, Iowa City, IA), anti-NFATc1 (Santa Cruz Biotechnology) and anti-ERα (Upstate Biotechnology), for 1 h at room temperature. The slides were washed three times with TBS 1% BSA and then sequentially incubated with multilinker biotinylated secondary antibody and alkaline phosphatase-conjugated

Table I. PCR primers used for the chromatin immunoprecipitation assay (ChIP) on the ColXV gene.

	Primer sequences	Product size (bp)
Forward F1	5'-TCCACTCCTGGGCATTCAAG-3'	314
Reverse R1	5'-GACGGAATACATTGGACGCT-3'	
Forward F2	5'-TTACTGGCAGGCAGCATGGCT-3'	310
Reverse R2	5'-CACAAACTAAGTCAAGGTGAGAT-3'	
Forward F3	5'-CACAGGGAATGTGAACAGATT-3'	370
Reverse R3	5'-AGAACGGGAACCAGACTAAA-3'	
Forward F4	5'-CATGTTCTTCTCCCCATATATC-3'	296
Reverse R4	5'-AACTCTGAAGACCTAAGAAAAACT-3'	

streptavidin (BioGenex kit, San Ramon, CA, USA) or goat anti-rabbit HRP (DakoCytomation), at room temperature for 20 min. The slides were developed using fast red as a substrate or a peroxidise reaction using 3,3'-diaminobenzidine as a substrate, counterstained with haematoxylin, mounted with glycerol jelly and and observed using a Leitz microscope. Negative and isotype matched controls were performed.

Mineralization assay. The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (ARS; Sigma) in the cells cultured for up to 21 days in osteogenic medium consisting of DMEM, high-glucose, supplemented with 10% FBS, 10 mM β-glycerophosphate, 0.1 mM dexamethasone, and 50 mM ascorbate (Sigma). In the committed cells, the osteogenic medium was changed every 3 days. The cells were then fixed in 70% ethanol for 1 h at room temperature, washed with PBS, stained with 40 mM ARS (pH 4.2) for 10 min at room temperature, washed five times with deionized water, and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnifications using a Leitz microscope.

Real-time quantitative RT-PCR. For RT-PCR analysis, total-RNA was isolated from hOBs using a total-RNA isolation system (RNeasy Plus Micro kit, Qiagen, Italy). Total-RNA (2 μg) was reverse transcribed with the ImProm-II RT System (Promega). The mRNA of the target genes was quantified by real-time PCR using the ABI Prism 7700 system and TaqMan probes for Runx-2, ERα and ColXV (Applied Biosystems, CA, USA). PCR was carried out in a final volume of 25 μl. After a 10 min pre-incubation at 95˚C (denaturation), 1 min at 60˚C (annealing/elongation) the mRNA levels were corrected for GAPDH mRNA levels (reference gene) and normalized to a calibrator sample (control cells).

Calcium measurements. Basal levels of the cytoplasmic Ca^{2+} concentration were measured in Fura-2-AM-loaded cells as previously described (28). Cells were grown on 24 mM coverslips in α-MEM medium supplemented with 1% FBS and loaded with 4-AM Fura-2-AM (added to KRB/Ca^{2+} solution + 1% FBS) at 37˚C for 30 min. The coverslip was washed and transferred to the thermostated stage of a Zeiss Axiovert 200 inverted microscope, equipped with a Sutter filterwheel and 340/380 excitation filters. The sample was excited at 340 and 380 nm using a random access monochromator (Photon Technology International, NJ, USA) and emission was detected using a 505/30 emission filter. Images were acquired every 3 sec by the BFT512 camera (Princeton Instruments, AZ, USA). Image acquisition was controlled by the MetaFluor 5.0 software (Universal Imaging Corp., PA, USA). Calibration was performed using the MetaFluor and the MetaFluor Analyst software, according to the equation $[Ca^{2+}] = K_d$ x (Sf2/Sb2) x (R - R_{min})/(R_{max} - R), where K_d = 224 nM. R_{max} and R_{min} were determined at the end of each experiment in KRB/Ca^{2+}/1 μM ionomycin, and KRB/1 mM EGTA/ ionomycin, respectively.

Statistical analysis. Data are presented as the mean ± SEM from at least three independent experiments, where indicated. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. A P-value <0.05 was considered statistically significant.

Results

Characterization of primary osteoblast cultures. hOBs were generated from bone chips removed from the tibial plateau as previously described (29) and characterized for the typical osteogenic markers. In Fig. 1A the analysis of a representative sample of the cells that we used is shown. The cells were highly positive for Runx2, Col1A1, BSP and OC, and exhibited an evident extracellular matrix mineralization after 21 days of culture under osteogenic conditions (ARS). In addition, the cells were positive for ERα, a transcription factor strictly associated with osteoblast differentiation, and for NFATc1, the target of our decoy treatment, as demonstrated by immunocytochemical analysis and quantitative RT-PCR (Fig. 1B).

NFATc1 binds to the decoy ODN molecule. We used the EMSA to analyze NFATc1 binding to the decoy ODN, which contains one NFAT motif (24). Proteins from the nuclear extract of Jurkat, a highly NFATc1 positive human T-leukemia cell line, efficiently bound to the decoy ODN probe, giving two major retarded complexes (Fig. 2). The labeled retarded complexes were found to be specifically out-competed by increasing concentrations of cold excess of decoy ODN, while they were unaffected by a 100-fold excess of an unrelated

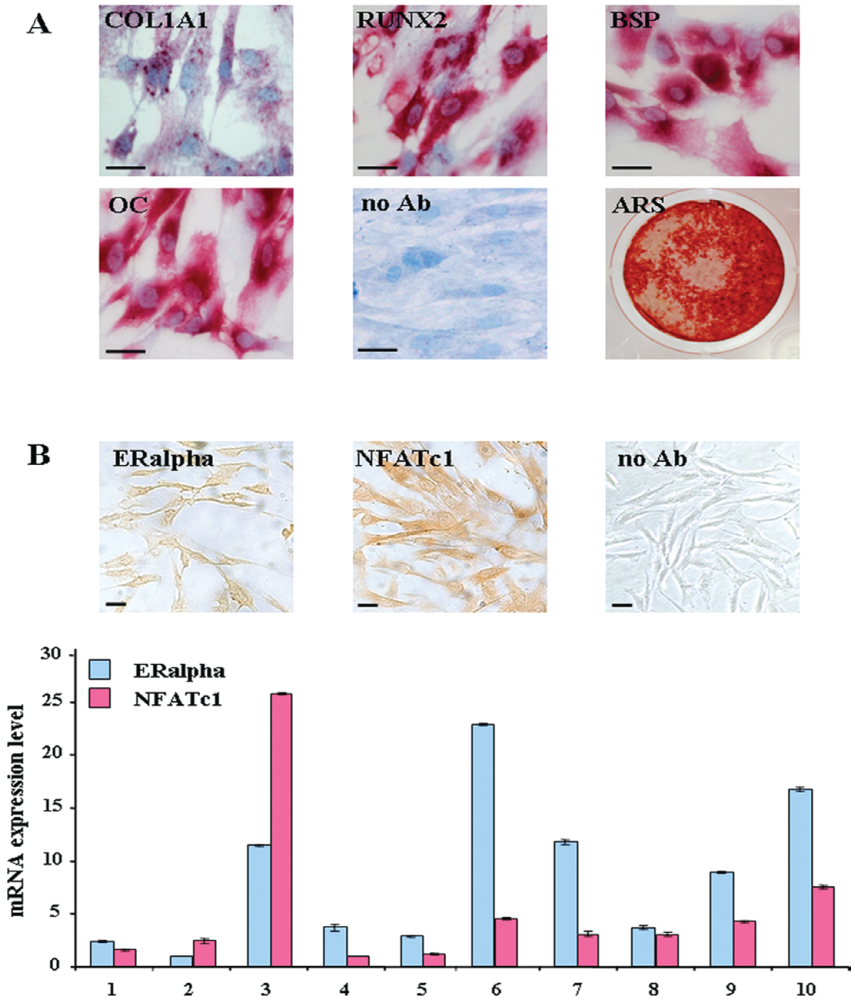

Figure 1. Phenotypical characterization of hOBs. (A) Ten hOB samples were subjected to immunocytochemical analysis for collagen type 1 (Col1A1), Runx2, bone sialoprotein (BSP) and osteocalcin (OC), and protein mineral matrix deposition was analyzed by Alizarin Red staining (ARS). The staining shows the local expression of the markers analyzed in a representative sample (x40 magnification). (B) The same samples were analyzed for ERα and NFATc1 by immunocytochemistry [a representative sample and a negative control (no Ab) are shown, x20 magnification; bar denotes, 20 μm], and quantitative RT-PCR. The mRNA expression levels were normalized on the basis of GAPDH expression and the ΔΔCt method was used to compare gene expression data; the means and standard error of the means (SEM) were calculated.

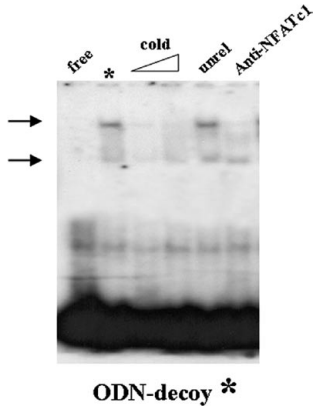

Figure 2. A gel shift experiment was performed with nuclear extracts from Jurkat cells in the presence of ^{32}P-labeled ODN-decoy oligonucleotide against NFATc1. Protein DNA complexes were resolved from the free probe on a non-denaturing 6% polyacrylamide gel. A representative gel is shown. The ^{32}P-labeled oligonucleotide was incubated alone (free), in combination with 10 μg of nuclear extracts (*), and in the presence of 50- and 100-fold molar excess of specific unlabeled oligonucleotide (cold), or unrelated oligonucleotide (unrel). An antibody against NFATc1 (4 μg) was also incorporated into the binding reaction (anti-NFATc1). The main retarded complexes are indicated by arrows.

oligonucleotide, demonstrating the specificity of these DNA protein interactions. The upper band was abolished when an anti-NFATc1 antibody was added, confirming that NFATc1 was present in this retarded complex.

Analysis of NFAT activity. The functionality of the endogenous NFAT proteins in our experimental model were analyzed by monitoring the transcriptional activity on a luciferase reporter construct driven by three copies of an NFAT response element (pNFAT-TA-luc). As shown in Fig. 3A, NFAT-driven luciferase activity in all transfected hOBs cultured in the presence of ionomycin and PMA, was remarkable and positively correlated with calcium levels, demonstrating the ability of endogenous-activated NFAT to bind the specific *cis*-acting elements in these cells. The presence of NFAT-driven luciferase activity was also found in hOBs cultured in medium without ionomycin and PMA in the absence of exogenous calcium (Fig. 3B), suggesting that the intracellular calcium may be sufficient to activate NFAT proteins. The calcium mobilization ability of hOBs was demonstrated through accurate measurements of intracellular calcium release in

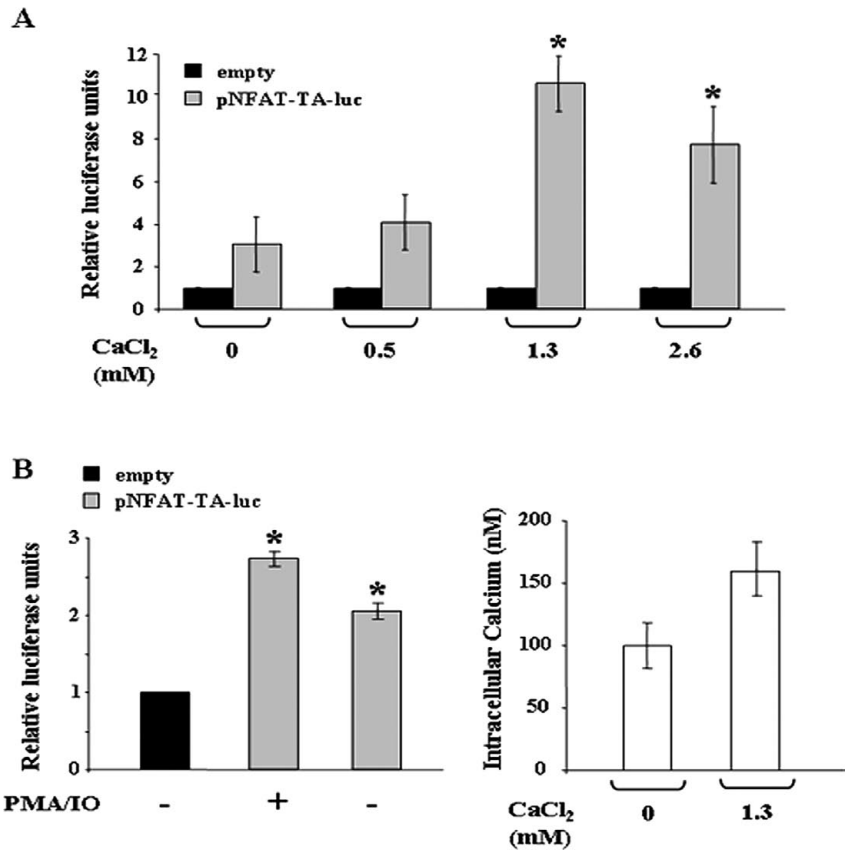

Figure 3. NFAT reporter gene assay. (A) Five different hOB samples were transiently transfected with pNFAT-TA-luc or empty vector in four different culture medium conditions, 0, 0.5, 1.3 and 2.6 mM $CaCl_2$, in the presence of 0.5 μM ionomycin and 100 μg/ml PMA. The results were normalized to the protein concentration and β-galactosidase activity for the transfection efficiency and represents the average of five different experiments performed in duplicate. The data are expressed as the mean ± SEM and indicated as fold-inductions over the empty vector. *P<0.05 vs. 0 mM. (B) The same protocol was applied for hOBs cultured in α-MEM 10% FBS, in the presence (+) or in the absence (-) of 0.5 mM ionomycin (IO) and 100 mg/ml PMA treatment before pNFAT-TA-luc transient transfection. *P<0.05 vs. empty vector. The basal intracellular Ca^{2+} concentrations (nM) in hOBs was measured (diagram at the right). Cells were grown in the absence (0) and in the presence of 1.3 mM $CaCl_2$ medium on coverslips, then loaded with Fura-2-AM and analyzed as reported in Materials and methods. The average and the SEM of all recorded data are reported.

the absence or presence of exogenous calcium was achieved through Fura-2-AM-loaded cells (Fig. 3B).

The effect of the decoy against NFATc1. The cells were plated at 60% confluence, and after 24 h a first administration of NFATc1 decoy ODN or scrambled ODN was performed. A second administration was applied on day 4 before cell harvesting on day 7 for the molecular analysis (scheme at the top of Fig. 4).

On the basis of the results reported in Fig. 3, we performed decoy experiments in hOBs cultured in α-MEM medium and left untreated with ionomycin and PMA. This allowed the estimation of the decoy effect at basal conditions in the absence of the calcium mobilization induced by the phorbol ester, PMA, and the ionomycin calcium ionophore.

We first examined the efficiency of hOBs treatment with the NFATc1 decoy ODN by analyzing the expression of the ERα gene by quantitative RT-PCR. In fact, we previously demonstrated that in different cell lines this decoy treatment induced a significative increase of ERα gene expression because NFATc1 acts as negative regulator of ERα gene transcription. As shown in Fig. 4, decoy against NFATc1, but not scramble oligonucleotide, increased the expression of ERα confirming the specific role of NFATc1. We then turned our attention to the expression pattern of Runx2, the master regulator of osteoblastogenesis that is not transcriptionally regulated by NFATc1 (5), and of ColXV, a recently investigated novel marker of osteoblast differentiation whose expression is regulated by exogenous calcium addition (30). The data are reported in Fig. 4. As expected, the functional inactivation of NFATc1 did not affect Runx2 expression indicating the specificity of the decoy effect. On the contrary, a significant increase of ColXV expression was found in the decoy-treated cells. Using a program for predicting transcription factor binding sites (TFSEARCH, www.cbrc.jp/research/db/TFSEARCH.html), we found that 5' regulatory regions of all three genes analyzed contain potential binding sites (5'-GGAAA-3'), for NFATc1. However, only ERα and ColXV but not Runx2 seem to be critical downstream targets of NFATc1.

NFATc1 interacts in vivo with the promoter of the ColXV gene. We then focused our attention on the poorly studied ColXV gene promoter by investigating *in vivo* NFATc1 recruitment by ChIP binding assays (Fig. 5). To this aim, we employed four hOB samples that were treated with PMA/ionomycin to ensure full NFAT induction. hOBs were exposed to formaldehyde to cross-link proteins and DNA,

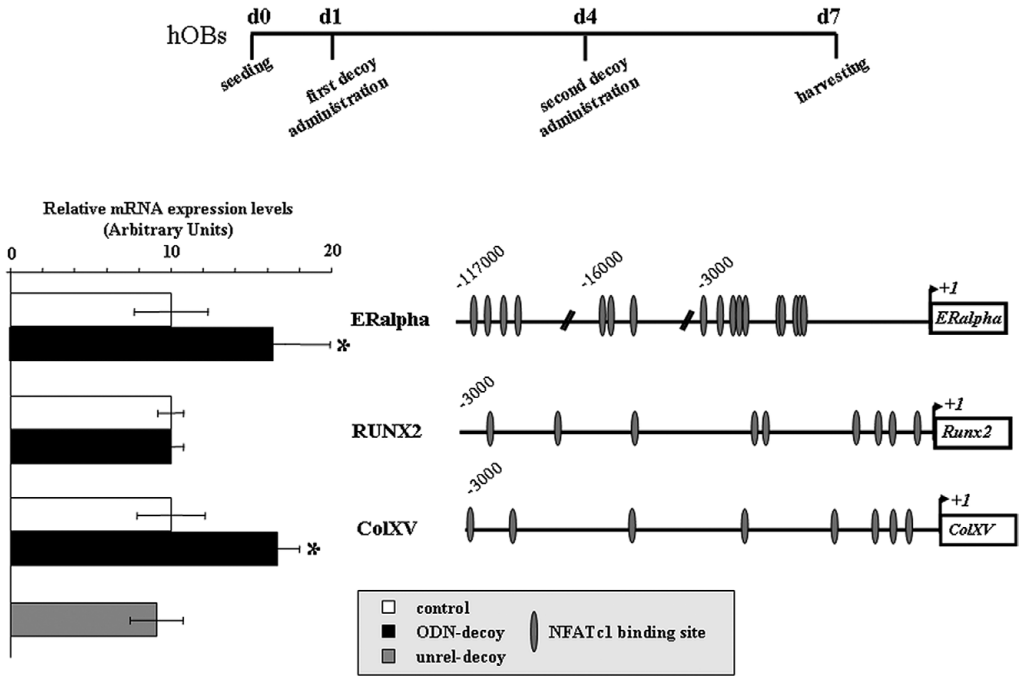

Figure 4. Analysis of ERα, Runx2 and ColXV expression by quantitative RT-PCR after decoy against NFATc1. Ten hOB samples were cultured up to 7 days with 1 mg/ml of ODN-decoy administered in two different steps, as indicated in the scheme above. The expression levels were normalized on the basis of GAPDH expression and results of the experiments are reported as relative mRNA expression levels over the control cells. Arbitrary units are used for the attribution of the fold inductions. The ΔΔCt method was used to compare gene expression data; means ± standard error of the mean (SEM) was calculated. The effect of unrelated oligonucleotides was also reported. *P<0.05. The localization of the potential binding sites for NFATc1 in the 5' regulatory regions of human ERα, Runx2 and ColXV genes are reported.

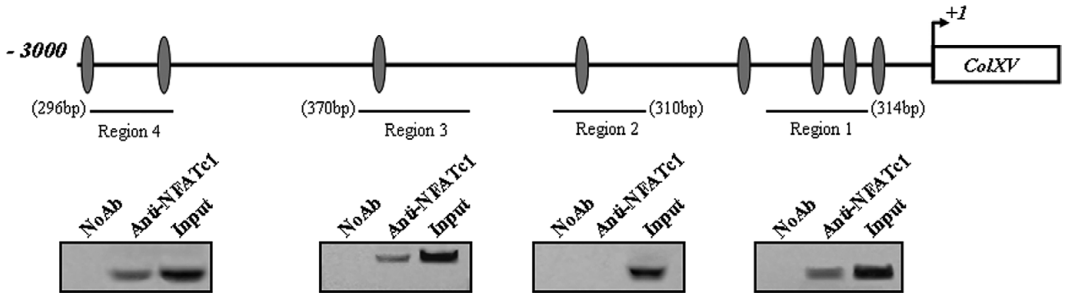

Figure 5. NFATc1 binds the human ColXV promoter *in vivo*. The ColXV promoter region under investigation is reported (+1 to -3000). The positions of the putative NFATc1 consensus binding sites are enclosed by gray ovals. Recruitment of the NFATc1 transcription factor to the human ColXV promoter was demonstrated by *in vivo* ChIP binding assays. Soluble chromatin was prepared from hOBs and immunoprecipitated with the specific antibody against NFATc1. The association of the transcription factor on the regions 1-4 of the human ColXV promoter was analyzed by PCR using primer pairs spanning the reported regions of ColXV promoter. The specific molecular weights of PCR fragments are shown in parentheses. No Ab, negative control; Input, a positive control using the starting material (0.2%) prior to the immunoprecipitation. Representative agarose gels are shown.

and sonicated to fragment the chromatin. A specific antibody against NFATc1 was used to immunoprecipitate the protein DNA complexes. The presence of the promoter-specific DNA region before immunoprecipitation was confirmed by PCR (Input). After immunoprecipitation, DNA was extracted from the beads and used as a template to generate specific PCR products spanning the putative NFATc1 binding sites from -3000 bp to +1 bp in the promoter of the ColXV gene. NFATc1 recruitment was assessed at the different promoter regions by using specific sets of primers (Table I). As shown in Fig. 5, NFATc1 occupancy was detected at the regions 1, 3 and 4, but not at region 2. This demonstrated that ColXV is a specific NFATc1 target gene in human osteoblasts and its transcriptional activation.

Discussion

To our knowledge, the results of the present study establish, for the first time, that removal of the NFATc1 transcription factor by a decoy approach in hOBs induces the expression of ERα and ColXV that are positively correlated with osteoblastic differentiation. These findings have a double meaning: on the one hand, they reinforce the role of this factor in the transcriptional program of osteoblasts, on the other they contribute important information on ColXV which only recently has been proposed as an osteoblastic marker (30). In addition, the present study in agreement with several published reports (17,18,21-23,31-33) supports the potential utility of the decoy strategy to treat specific diseases. It is

well known that synthetic double-stranded ODN as decoy cis elements block the binding of nuclear factors to promoter regions of targeted genes, resulting in the modulation of gene expression. The interest in this approach is supported by studies correlating the altered activity of transcription factors with specific diseases (34,35).

NFATc1 is referred to as the key transcription factor in osteoclastogenesis (36); however, several lines of evidence indicate that it regulates bone mass by functioning not only in osteoclasts but also in osteoblasts (6,7). In fact, it has recently been shown that NFATc1 contributes to the regulation of osteoblastic specific genes including Osterix (8) and Fra-2 (37), modulates the activity of ligands for osteoclast-associated receptors such as OSCAR (38) primarily produced by osteoblasts, and is involved in the transcriptional regulation of the ERα gene in the SaOS-2 osteosarcoma cell line (24).

Here we have demonstrated that hOBs efficiently respond to NFATc1 decoy treatment in terms of an increase in ERα gene expression. This evidence may be correlated with an increased potential for cell sensitivity to estrogens and consequently an improvement in osteoblast differentiation. Actually, the positive effect of estrogen on bone metabolism is well known (39). These considerations substantiate the interest in NFATc1 as a promising therapeutic target that could be employed for the development of anabolic agents for the treatment of bone loss. This conclusion is supported by the evidence that decoy-treated hOBs showed a significant increase in ColXV mRNA levels. Interestingly, the expression of ColXV, was recently demonstrated in human osteoblasts both *in vitro* and *in vivo* (30).

In addition, a direct involvement of this molecule in the mineralization process of mesenchymal stem cells induced to osteogenesis and in bone tissue remodelling has been suggested (30). 5'-Regulatory sequences of the ColXV gene promoter have been only partially characterized (40) and, until now, it is known that its expression is modulated by exogenous calcium induction. Our results demonstrate, for the first time, a specific *in vivo* interaction of NFATc1 with specific sequences of the ColXV gene promoter suggesting a direct involvement of NFATc1 during osteoblastogenesis. Therefore, even if further studies are required to investigate the molecular mechanism of action of NFATc1, the novelty of our findings provide insight into the regulation of the ColXV gene transcription, ascribing to NFATc1 a role of negative transcription factor.

Regarding the therapeutic potential of the decoy experimental approach for bone diseases, it is worth noting that NFATc1 is utilized in both bone-forming and bone-resorbing cells. Therefore, the decoy against NFATc1 in the bone microenviroment may be effective for treating bone diseases exerting positive and negative action on osteoblasts and osteoclasts, respectively. Based on these considerations, the decoy against NFATc1, working upstream of the transcription machinery, may prove to be a gene-targeted tool for treating skeletal disorders caused by excessive bone resorption, including osteoporosis and destructive bone metastasis. In addition, considering that NFATc1 cooperates with other transcription factors, the here described approach may help to shed light on upstream signal-transduction networks that may lead to altered transcription programs in bone cells. In particular, after translocation to the nucleus and DNA binding, NFATc1 protein facilitates the binding of AP-1 to NFAT-AP-1 binding sites whereupon the AP-1 components can mediate the induction of transcription (13). As AP-1 family members play a key role in the regulation of bone-specific genes and consequently in osteoblast differentiation (41), the interaction with NFATc1 is particularly relevant in the bone microenvironment. In this regard, it will be useful to further investigate the recruitment of the AP-1 family members at the ColXV gene promoter by ChIP analysis.

In conclusion, to the best of our knowledge this is the first time that TFD has been successful in hOBs allowing the investigation of the role of NFATc1 in an experimental model that compared to cell lines more closely resembles an *in vivo* model. In addition, our results provide new insight into the regulation of osteoblastic markers and strengthen the therapeutic potential of the decoy approach to treat skeletal disorders by properly manipulating NFATc1-mediated cellular events.

Acknowledgements

This research was supported by grants from MIUR-Cofin 2007; Regione Emilia Romagna, 'Programma di Ricerca Regione Universita' 2007-2009; the 'Fondazione Cassa di Risparmio di Padova e Rovigo'. E.L. is a recipient of a fellowship from the 'Fondazione Cassa di Risparmio di Ferrara'.

References

1. Rodan GA and Martin TJ: Therapeutic approaches to bone diseases. Science 289: 1508-1514, 2000.
2. Kwan MD, Slater BJ, Wan DC and Longaker MT: Cell-based therapies for skeletal regenerative medicine. Hum Mol Genet 17: 93-98, 2008.
3. Sitara D and Aliprantis AO: Transcriptional regulation of bone and joint remodeling by NFAT. Immunol Rev 233: 286-300, 2010.
4. Fromigué O, Haÿ E, Barbara A and Marie PJ: Essential role of nuclear factor of activated T cells (NFAT)-mediated Wnt signaling in osteoblast differentiation induced by strontium ranelate. J Biol Chem 285: 25251-25258, 2010.
5. Choo MK, Yeo H and Zayzafoon M: NFATc1 mediates HDAC-dependent transcriptional repression of osteocalcin expression during osteoblast differentiation. Bone 45: 579-589, 2009.
6. Stern PH: The calcineurin-NFAT pathway and bone: intriguing new findings. Mol Interv 6: 193-196, 2006.
7. Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G and Crabtree GR: Calcineurin/NFAT signaling in osteoblasts regulates bone mass. Dev Cell 10: 771-782, 2006.
8. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K and Takayanagi H: NFAT and Osterix cooperatively regulate bone formation. Nat Med 11: 880-885, 2005.
9. Hogan PG, Chen L, Nardone J and Rao A: Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17: 2205-2232, 2003.
10. Wu H, Peisley A, Graef IA and Crabtree GR: NFAT signaling and the invention of vertebrates. Trends Cell Biol 17: 251-260, 2007.
11. Im SH and Rao A: Activation and deactivation of gene expression by Ca^{2+}/calcineurin-NFAT-mediated signaling. Mol Cells 18: 1-9, 2004.
12. Bert AG, Johnson BV, Baxter EW and Cockerill PN: A modular enhancer is differentially regulated by GATA and NFAT elements that direct different tissue-specific patterns of nucleosome positioning and inducible chromatin remodeling. Mol Cell Biol 27: 2870-2885, 2007.
13. Macián F, López-Rodríguez C and Rao A: Partners in transcription: NFAT and AP-1. Oncogene 20: 2476-2489, 2001.

14. Yang G, Yu F, Fu H, Lu F, Huang B, Bai L, Zhao Z, Yao L and Lu Z: Identification of the distinct promoters for the two transcripts of apoptosis related protein 3 and their transcriptional regulation by NFAT and NFkappaB. Mol Cell Biochem 302: 187-194, 2007.
15. Yang XY, Wang LH, Chen T, Hodge DR, Resau JH, DaSilva L and Farrar WL: Activation of human T lymphocytes is inhibited by peroxisome proliferator-activated receptor gamma (PPARgamma) agonists. PPARgamma co-association with transcription factor NFAT. J Biol Chem 275: 4541-4544, 2000.
16. Borgatti M, Boyd DD, Lampronti I, Bianchi N, Fabbri E, Saviano M, Romanelli A, Pedone C and Gambari R: Decoy molecules based on PNA-DNA chimeras and targeting Sp1 transcription factors inhibit the activity of urokinase-type plasminogen activator receptor (uPAR) promoter. Oncol Res 15: 373-383, 2005.
17. Mann MJ and Dzau VJ: Therapeutic application of transcriptional factor decoy oligonucleotides. J Clin Invest 106: 1071-1075, 2000.
18. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A, Maeda K, Sawa Y, Kaneda Y, Higaki J and Ogihara T: *In vivo* transfection of cis element 'decoy' against nuclear factor-κB binding site prevents myocardial infarction. Nat Med 3: 894-899, 1997.
19. Piva R, Penolazzi L, Lambertini E, Giordano S and Gambari R: Induction of apoptosis of human primary osteoclasts treated with a transcription factor decoy mimicking a promoter region of estrogen receptor alpha. Apoptosis 66: 1079-1094, 2005.
20. Lui VW, Boehm AL, Koppikar P, Leeman RJ, Johnson D, Ogagan M, Childs E, Freilino M and Grandis JR: Antiproliferative mechanisms of a transcription factor decoy targeting signal transducer and activator of transcription (STAT) 3: the role of STAT1. Mol Pharmacol 71: 1435-1443, 2007.
21. Bezzerri V, Borgatti M, Nicolis E, Lampronti I, Dechecchi MC, Mancini I, Rizzotti P, Gambari R and Cabrini G: Transcription factor oligodeoxynucleotides to NF-kappaB inhibit transcription of IL-8 in bronchial cells. Am J Respir Cell Mol Biol 39: 86-96, 2008.
22. Moriyama I, Ishihara S, Rumi MA, Aziz MD, Mishima Y, Oshima N, Kadota C, Kadowaki Y, Amano Y and Kinoshita Y: Decoy oligodeoxynucleotide targeting activator protein-1 (AP-1) attenuates intestinal inflammation in murine experimental colitis. Lab Invest 88: 652-663, 2008.
23. Gambari R, Borgatti M, Bezzerri V, Nicolis E, Lampronti I, Dechecchi MC, Mancini I, Tamanini A and Cabrini G: Decoy oligodeoxyribonucleotides and peptide nucleic acids-DNA chimeras targeting nuclear factor kappa-B: Inhibition of IL-8 gene expression in cystic fibrosis cells infected with *Pseudomonas aeruginosa*. Biochem Pharmacol 80: 1887-1894, 2010.
24. Penolazzi L, Zennaro M, Lambertini E, Tavanti E, Torreggiani E, Gambari R and Piva R: Induction of estrogen receptor alpha expression with decoy oligonucleotide targeted to NFATc1 binding sites in osteoblasts. Mol Pharmacol 71: 1457-1462, 2007.
25. Deroo BJ and Korach KS: Estrogen receptors and human disease. J Clin Invest 116: 561-570, 2006.
26. Lambertini E, Lisignoli G, Torreggiani E, Manferdini C, Gabusi E, Franceschetti T, Penolazzi L, Gambari R, Facchini A and Piva R: Slug gene expression supports human osteoblast maturation. Cell Mol Life Sci 66: 3641-3653, 2009.
27. Dignam JD, Lebovitz RM and Roeder RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11: 1475-1489, 1983.
28. Manzati E, Aguiari G, Banzi M, Manzati M, Selvatici R, Falzarano S, Maestri I, Pinton P, Rizzuto R and del Senno L: The cytoplasmic C-terminus of polycystin-1 increases cell proliferation in kidney epithelial cells through serum-activated and Ca(2+)-dependent pathway(s). Exp Cell Res 304: 391-406, 2005.
29. Tonnarelli B, Manferdini C, Piacentini A, Codeluppi K, Zini N, Ghisu S, Facchini A and Lisignoli G: Surface-dependent modulation of proliferation, bone matrix molecules, and inflammatory factors in human osteoblasts. J Biomed Mater Res 89: 687-696, 2008.
30. Lisignoli G, Codeluppi K, Todoerti K, Manferdini C, Piacentini A, Zini N, Grassi F, Cattini L, Piva R, Rizzoli V, Facchini A, Giuliani N and Neri A: Gene array profile identifies collagen type XV as a novel human osteoblast-secreted matrix protein. J Cell Physiol 220: 401-409, 2009.
31. Han W and Liu GN: EGR-1 decoy ODNs inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia of balloon-injured arteries in rat. Arterioscler Thromb Vasc Biol 29: 1080-1086, 2009.
32. Aoki T, Kataoka H, Ishibashi R, Nozaki K, Morishita R and Hashimoto N: Reduced collagen biosynthesis is the hallmark of cerebral aneurysm: contribution of interleukin-1beta and nuclear factor-kappaB. Life Sci 86: 234-243, 2010.
33. Stadlbauer TH, Wagner AH, Hölschermann H, Fiedel S, Fingerhuth H, Tillmanns H, Bohle RM and Hecker M: AP-1 and STAT-1 decoy oligodeoxynucleotides attenuate transplant vasculopathy in rat cardiac allografts. Cardiovasc Res 79: 698-705, 2008.
34. Semenza GL: Transcription Factors and Human Disease. Oxford Monographs on Medical Genetics. 1st edition. Oxford University Press, New York, 1998.
35. Blancafort P, Segal DJ and Barbas CF III: Designing transcription factor architectures for drug discovery. Mol Pharmacol 66: 1361-1371, 2004.
36. Takayanagi H: The role of NFAT in osteoclast formation. Ann NY Acad Sci 1116: 227-237, 2007.
37. Zayzafoon M: Inhibition of NFAT increases osteoblast differentiation by increasing Fra-2 expression. J Musculoskelet Neuronal Interact 5: 347, 2005.
38. Kim JH, Kim K, Youn BU, Jin HM and Kim N: MHC class II transactivator negatively regulates RANKL-mediated osteoclast differentiation by downregulating NFATc1 and OSCAR. Cell Signal 22: 1341-1349, 2010.
39. Zallone A: Direct and indirect estrogen actions on osteoblasts and osteoclasts. Ann NY Acad Sci 1068: 173-179, 2006.
40. Eklund L, Muona A, Liétard J and Pihlajaniemi T: Structure of the mouse type XV collagen gene, Col15a1, comparison with the human COL15A1 gene and functional analysis of the promoters of both genes. Matrix Biol 19: 489-500, 2000.
41. Bozec A, Bakiri L, Jimenez M, Schinke T, Amling M and Wagner EF: Fra-2/AP-1 controls bone formation by regulating osteoblast differentiation and collagen production. J Cell Biol 190: 1093-1106, 2010.