2

Genes and Molecular Pathways of the Osteogenic Process

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1. Introduction

Bone tissue represents a specialized connective tissue, comprising metabolically active cells within a mineralized extracellular matrix, that are vital to the performance of its structural, mechanical and metabolic roles (Milat & Ng, 2009). The first descriptions of the bone formation process date back to two centuries ago and since then much effort has been spent by the scientific community to depict the complete scenario of the cellular and molecular events leading to ossification (Howship, 1815; Zaidi et al., 2007). Bone is formed through a complex process named osteogenesis, involving the proliferation of mesenchymal precursors, condensation of cells in closely interacting groups, differentiation and functional activation of bone cells, which finally leads to the deposition of an organic matrix and subsequent mineralization. Most bones are formed through the progressive growing and differentiation of condensed groups of mesenchymal cells into round chondrocytes that proliferate and secrete extracellular matrix (ECM): a cartilage mold is being formed. Thereafter, these cells stop proliferating, enlarge, modifiy the ECM composition by changing the type of collagen they secrete, and finally direct the mineralization of the new formed tissue. These hyperthrophic chondrocytes (HC) signal to adjacent perichondrial cells to stimulate their osteogenic differentiation and to promote vascular invasion. In this way, the primitive bone tissue, the primary "spongiosa", is formed starting from the center of the cartilage mold and proceeding to surrounding cells, following a longitudinal direction in long bones. Subsequently, osteoclasts derived from hematopoietic precursors of the bone marrow enter the cartilage mold and digest the ECM synthesized by the HC. These shortly undergo apoptosis at the border between cartilage and primary spongiosa, while HC at the extremities of the mold continue proliferating. Through the progressive proliferation and hypertrophy of growing numbers of chondrocytes, secondary sites of ossification are formed, and the process persist to engine the bone lengthening during post-natal life. Flat bones of the skull, conversely, undergo a direct "intramembranous" ossification process, as in this case mesenchymal cells directly differentiate into osteoblasts that start producing bone matrix.

The whole process requires a careful coordination of signals within cells, to drive proliferation, migration and differentiation in a chronologically and spatially organized fashion. It is therefore not so surprising that a wide number of molecules and cross-talking pathways drive this coordinated mechanisms through a complex network of interactions

which is at least partially known. The best known signaling pathways that orchestrate the osteogenic process involve molecules belonging to the wingless-int (WNT), the bone morphogenetic protein (BMP), the hedgehog (HH) and the fibroblast growth factor (FGF) families. During the last decades, new details on the control of bone mass remodeling and regeneration has been achieved, mainly thank to the rapidly growing body of knowledge regarding the genome structure, control and functioning. This chapter will provide an up-to-date depiction of the molecular networks involved in the osteogenic process, focusing on main genes and signaling pathways (schematically represented in Figure 1), whose integrity is required for the correct skeletal morphogenesis and patterning and for maintaining bone homeostasis. Particular attention will be devoted to list and dissect the human syndromes and disorders associated to genes belonging to the main osteogenic pathways, with regard to the skeletal phenotype.

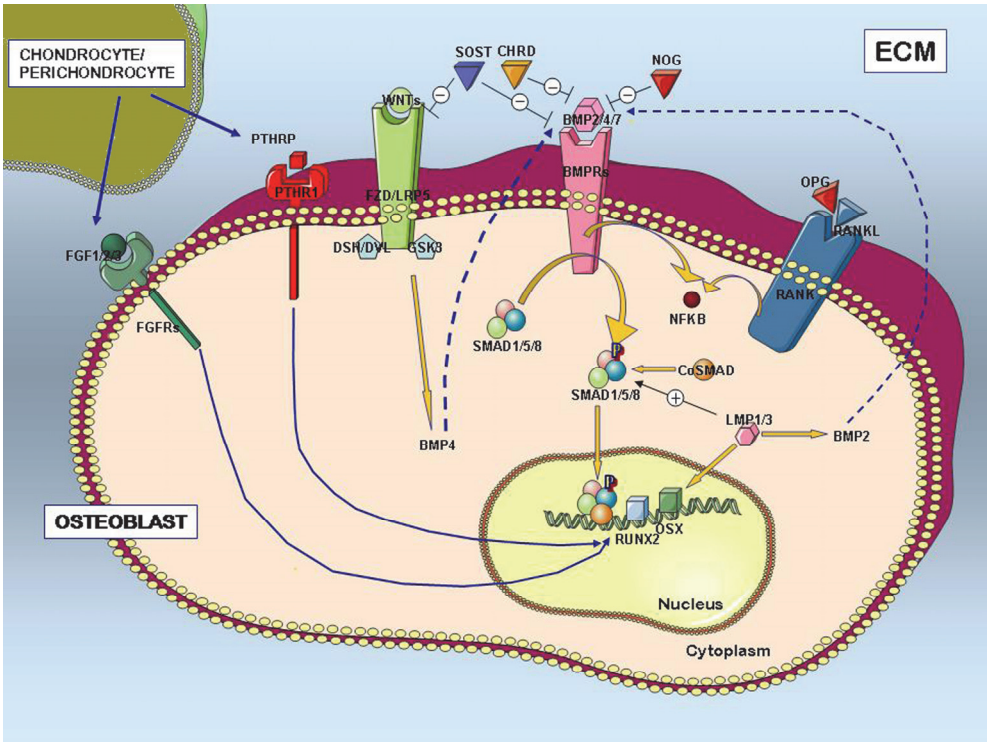

Fig. 1. The osteogenic network. The figure stigmatizes the main signaling pathways and reciprocal cross-talks acting during osteoblast differentiation. See text for details.

2. Osteogenic genes and pathways

2.1 The Transforming Growth Factor- ß (TGF-ß) superfamily

The Transforming Growth Factor- β (TGF-β) superfamily incorporate over 30 multifunctional growth factors implicated in the regulation of a wide variety of biological

functions, such as proliferation, differentiation, migration, and apoptosis. The role of TGF-β ligands is context-dependent, being affected by diverse environmental features, including tissue and cell type, cell differentiation stage and level of expression of interacting genes. Also, the quantity and the quality, in terms of type of isoform, of the growth factor itself, along with its paracrine or autocrine effect, influence the downstream cascade. TGF-β superfamily members are grouped into 3 families, on the basis of sequence homologies and functional activities exploited through the activation of a specific signaling pathway: 1. the TGF-β/activin/nodal family; 2. the bone morphogenetic protein (BMP); 3. the growth differentiation factor (GDF)/Muellerian inhibiting substance (MIS) family. As a rule, all the molecules acting in the TGF-β signaling pathway are extremely conserved across species during evolution (Miyazono, 2000).

A fine regulation of the spatio-temporal expression and function of TGF-β pathway players occurs during embryonic development, leading to an astounding heterogeneity of cellular responses (Ripamonti et al, 2006). Signaling is initiated when binding of the ligand induces the assembly of a heteromeric complex of type I and type II serine/threonine kinase receptors (Cohen, 2006; Miyazono, 2000). The constitutively active type II receptor recruits and activates a type I receptor (also known as activin receptor-like kinase; ALK) by phosphorylating its cytoplasmic domain, inducing the assembly of two type I, and two type II receptors. This, in turn, results in the phosphorylation and activation of specific intracellular proteins, belonging to the SMAD (mothers against decapentaplegic (MAD) homologs) family, by the type I receptor (receptor-regulated Smads, R-Smads: Smad1–3, 5, and 8). The consequent activation of the Smad signaling cascade implies the formation of heterodimeric complexes with the common partner Smad (Co-Smad, Smad4), which translocates to the nucleus and recruits distinct transcription factors to regulate transcription. The transcriptional targets of this signaling pathway are represented by over 500 genes, which are regulated in a cell-specific, ligand dose–dependent manner (Cohen, 2006; Miyazono, 2000). Generally, Smad-2 and -3 transduce cellular responses downstream of the TGF- β and activin receptors, while Smad-1, -5 and -8 primarily mediate BMP signals (as further discussed). Signaling by the TGF- β family is carefully regulated at several levels, through either of the TGF-β ligand agonists and antagonists, which compete for receptor binding, or capture the ligand in the extracellular compartment. In addition, the different downstream effects of TGF-β signalling activation are due to the different combinations of receptors (seven type I receptors and five type II receptors are classified), leading to alternative ligand-specific composition of the receptor complex. Finally, diverse biological response to the same signaling pathway are also obtained through the recruitment of different accessory proteins (Kanaan RA & Kanaan LA, 2006). The vast majority of TGF-β superfamily members actively involved in bone formation belong to the most numerous family, represented by the bone morphogenetic proteins.

2.2 Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) were originally identified and named after their ability to induce ectopic bone formation (Urist, 1965). The BMP family comprises over 20 distinct highly conserved secreted proteins, further categorized into multiple subgroups according to functional and/or structural features (Miyazono et al., 2005; X. Wu et al., 2007). BMP functions cover several aspects of the cell differentiation program. In particular they induce the osteoblastic commitment of mesenchymal cells, inhibit their differentiation along the myoblastic and adipogenic lineage and increase osteoclastogenesis (Katagiri et al., 1994;

Okamoto et al., 2006; Pham et al., 2011; X. Wu et al., 2007). On the whole, BMPs play a pivotal role in skeletogenesis during all processes associated with limb development. Nonetheless, the biological activities of BMPs are not identical among members. In fact, despite their name, not all members of the BMP family are directly involved in skeletogenesis; they display distinct spatio-temporal expression patterns, with consequent diversified roles in the morphogenesis of even non-skeletal structures during embryo development (H. Chen et al., 2004; Cheng et al., 2003; Dudley et al., 1995; Jena et al., 1997; Luo et al., 1995; Mcpherron et al., 1999; Solloway et al., 1998; Zhao et al., 1996).
BMP2, BMP4 and BMP7 (also known as osteogenic protein-1, OP-1, see Figure 1) are the most extensively studied osteogenic BMPs, being involved in basic skeletal body patterning mechanisms (Bahamonde et al., 2001; Gimble et al., 1995; Ozkainak et al., 1990). *In vitro*, BMP-2, BMP-4, and BMP-7 can induce the differentiation of multipotent mesenchymal cells into both osteochondrogenic lineage cells and osteoblast precursor cells, suggesting their essential contribute to both direct and indirect ossification mechanisms occurring in vertebrates (Balint et al., 2003; Canalis et al., 2003; Yamaguchi et al., 2000). BMP2 and BMP4 are essential during embryonic development; mice deficient in BMP2 are not viable because of amnio-chorial defects and severe impairment of cardiac development. The BMP4-null mutation is lethal between 6.5 and 9.5 days of gestation because of the lack of mesodermal differentiation and patterning defects (X. Wu et al., 2007). During skeletal development, BMP2/4 are involved in the molecular regulation of condensation, the pivotal stage during which previously dispersed mesenchymal progenitors proliferate, migrate and aggregate to form a growing cell mass (Hall et al., 1995). The molecular basis of BMP osteogenic properties have been extensively studied *in vitro*, studying the osteoblastic differentiation process and *in vivo* using transgenic and knockout mice along with animals and humans with naturally occurring mutations in the corresponding genes (D. Chen et al., 2004). Disruption of BMP7, leads to multiple skeletal defects, lack of eye and glomerular development, and subsequent renal failure and neonatal death (Jena et al., 1997). A wide number of preclinical studies have been demonstrating that these small molecules are capable of inducing ectopic bone formation upon intramuscular implantation and efficient bone healing/regeneration, when delivered in the appropriate concentration and on the appropriate scaffold into a bone defect site (Boden, 2005; Evans, 2011; Lattanzi et al., 2005). The use of recombinant human BMP2 and BMP7 has been approved both in Europe and the United States for selected clinical applications, as an alternative to autogenous bone grafts in the axial and appendicular skeleton. However, despite significant evidence of their potential benefit to bone repair there is, to date, a dearth of convincing clinical trials (Gautschi et al., 2007). On this regard, the main limitation of using recombinant proteins for inducing bone formation in clinical applications is the need for delivery systems that provide a sustained, biologically appropriate concentration of the osteogenic factor at the site of the defect (Lattanzi et al., 2008; Parrilla et al., 2010).

2.2.1 BMP receptors and intracellular signal transduction

BMP actions are mediated through the interaction with different sets of transmembrane serine/threonine kinase receptor complexes, grouped in two types, BMPRI and BMPRII (Massague, 1998; Zwijsen et al., 2003). BMP family members bind to their receptors with different affinities and in different combinations, determining the specificity of the downstream intracellular signals (F. Liu et al., 1995). BMPRI receptors include BMPR-IA (ALK-3), BMPR-IB (ALK-6), and ActR-IA (ALK-2) (Wan & Cao, 2005). BMPRII, activin type

IIA receptor (ActRIIA), and activin type IIB receptors (ActRIIB) are type II receptors, which bind exclusively BMP ligands, including BMP2, BMP4 and BMP7 (Wan & Cao, 2005). Generally, the type-I receptors are high-affinity binding receptors, whereas the type II receptors bind with lower affinity exclusively ligands belonging to the BMP family (X. Wu et al., 2007). BMPR-IB is the only receptor expressed within all types of cartilage and is required for correct chondrogenesis and osteogenic differentiation. ALK2 is expressed in isolated chick osteoblasts and chondrocytes, and overexpression of constitutively active ALK2 enhances chondrocyte maturation, suggesting that it is essential for normal endochondral ossification (X. Wu et al., 2007). Both type I and type II receptors are expressed on the cell surface; upon BMP2 binding, the type-II receptor transphosphorylates the type-I receptor. On its turn, the activated type I receptor phosphorylates of selected members of the Smad family of signal transduction proteins, namely Smad1, 5, and 8 (Kawabata et al., 1998; Nohe et al., 2002). The consequent activation of the Smad signaling cascade implies the formation of heterodimeric complexes with the common partner Smad (Co-Smad, Smad4), which translocate to the nucleus and recruit distinct transcription factors to regulate transcription, as illustrated in the following paragraphs (Wan and Cao, 2005; X.Wu et al., 2007). The cascade is sintetically depicted in Figure 1. As an alternative mechanism, BMP2 can also bind to preformed heteromeric receptor complexes and activate a Smad-independent transduction cascade, which results in the induction of alkaline phosphatase activity via p38-mitogen-activated protein kinase (MAPK; Kozawa et al., 2002; Guicheux et al., 2003; Nohe et al., 2002). BMP3 (osteogenin) appears to antagonize the osteogenic effects of BMP2 in stromal cells, likely acting via an activin-mediated pathway (Bahamonde et al., 2001).

2.2.2 BMP dowstream targets in bone biology

BMP2 is usually considered a paradigmatic model for studying bone formation mechanisms, since the original demonstration of its efficacy in inducing ectopic bone formation in muscles (E. A. Wang et al., 1990). *In vitro*, a wide number of studies demonstrated that BMP2 is able to induce the osteogenic differentiation of mesenchymal cells and transdifferentiation of myoblast into osteoblasts (reviewed by Ryoo et al., 2006). The BMP2 signaling involved in the osteoblast differentiation program, proceed downstream of the Smad cascade with the recruitment of osteo-specific transcription factors. The best characterized as the master osteogenic transcription factor induced by Bmp2 is the Runt-related transcription factor 2 (Runx2), because mice lacking Runx2 show complete arrest in osteoblast maturation and consequent absence of bone (Komori et al., 1997). Runx2 induces the expression of bone marker genes (including alkaline phosphatase, ALP, osteocalcin, osteopontin, bone sialoprotein and bone-specific collagens) through binding the osteoblast-specific cis-acting element-2 (OSE2), which is found in the promoter region of osteoblast-specific genes (Ziros et al., 2002). Runx2 is believed to function during the initial steps of osteogenic differentiation, from cell commitment to the chondro-osteogenic switch in endochondral ossification (Ryoo et al., 2006). The zinc finger-containing transcription factor Osterix (Osx) represents another piece in the mosaic work of Bmp2 action. Osx is expressed in developing bones and seems to act in the terminal differentiation of osteoblasts. As for Runx2, Osx-deficient mice display total absence of bone (Nakashima et al., 2002). Recent studies indicate that Runx2 and Osx mRNAs are not directly up-regulated by Bmp2, suggesting the existence of crucial intermediators, possibly represented by selected members of the Distal-less homeobox family (Dlx; further described in paragraph

2.10). Dlx5 is a homeodomain-containing transcription factor that is expressed in later stages during osteoblast differentiation and induces the expression of osteocalcin and the formation of a mineralized matrix (Ryoo et al., 2006). Dlx5 is believed to play an evolutionarily conserved role in skeletogenesis, as Dlx5-deficient mice display severe craniofacial abnormalities and delayed cranial ossification (Acampora et al., 1999). Dlx5 transcription is induced by Bmp2 signaling and could represent an upstream regulator of both Runx2 and Osx (Ryoo et al., 2006).

2.2.3 BMP antagnosists, inhibitors and further interactions

BMP signaling is modulated at different levels: extracellular BMP antagonists compete for receptor binding on the cell surface; inhibitory Smads and interacting molecules can interfere with the correct function of the intracellular Smad cascade; furthermore, alternative signals from co-existing transduction pathways converge on the same downstream targets to cooperate or antagonize BMP function on gene expression and protein processing.
BMP antagonists have been recently classified into three subfamilies based on the size of a cysteine-rich domain, known as cystine-knot, that characterizes many TGFβ superfamily members: the "Differential screening-selected gene aberrative in neuroblastoma" (DAN) family; the twisted gastrulation; and chordin and noggin. The DAN family is further subdivided in into four subgroups based on a conserved arrangement of additional cysteine residues outside of the cystine-knots: group 1 includes PRDC (Protein Related to Dan and Cerberus) and gremlin; group 2, coco and Cer1, homologue of Xenopus Cerberus; group 3 comprises the ; finally, group 4, sclerostin and USAG-1. All these molecules are involved in embryo development at various levels, representing also crucial node of intersection between Wnt- and BMP-signaling (Yanagita, 2005).
With regard to bone formation, the best studied antagonists of BMP signaling are noggin and chordin, which are structurally and fuctionally related, and regulate BMPs availability in the extracellular compartment (Rosen, 2006).

2.2.3.1 Noggin

Noggin (Nog) is a glycosylated chemokine protein, which is able to form a neutralizing complex that prevents BMPs from binding to BMPRs (Groppe et al., 2002; Krause et al., 2011). Nog action intervene as early as during gastrulation, where it antagonizes BMP-2, -4 and -7 and generates a dorsal-ventral BMP gradient which is crucial for the germ layer formation. Nog effects are pleiotropic during embryo development, although they are mainly explicated toward the formation of ectoderm and mesoderm derivatives (Krause et al. ,2011). In fact, Nog expression is essential for proper skeletal development, as excess BMP activity in Nog-null mice results in excess cartilage and failure to initiate joint formation, along with additional severe developmental abnormalities leading to premature embryo lethality (Brunet et al., 1998; McMahon et al., 1998; Tylzanowski et al., 2006). Conversely, ectopic expression of Nog in developing embryos results in suppression of lateral somite differentiation and complete inhibition of chondrogenesis in limbs (Capdevila & Johnson, 1998). Transgenic mice overexpressing noggin in mature osteoblasts show a dramatic decrease in bone mineral density and bone formation rate due to impaired osteoblast recruitment and function (X.B. Wu et al.., 2003). Most known Nog functions are based on its antagonism against BMP4 signalling, during both endochondral bone formation and teeth formation and eruption (Groppe et al., 2002; Tucker et al., 1998). Nog expression is regulated through a feedback system, as diverse BMPs (namely BMP2, -4, -5, -6 and -7) induce Nog

expression in osteoblasts, while Indian hedgehog (Ihh) induce Nog expression in chondroytes (Krause et al., 2011). Finally, Nog expression is down-regulated by fibroblast growth factor-2 and -9 (FGF-2 and FGF-9) in the mesenchyme during cranial suture fusion in mice. Hence, it is speculated that the biological effects of gain-of-function mutations of the FGF receptor genes, typically associated to abnormal skeletal penotypes with cranial and limb malformations (see paragraph 2.6.2), are partially due to inappropriate inhibition of noggin expression (Warren et al., 2003).

2.2.3.2 Chordin

Similarly to noggin, chordin (CHRD) contains the cysteine-knot structure in its biochemical structure and is able to bind BMPs and sequester them in latent non-functional complexes. The formation of the Chrd-BMP complex completely prevents binding of Bmp2 to both BMPR1A and BMPR2 receptors, leading to dorsalization of early vertebrate embryo (Scott et al., 1999). Chrd binds predominantly to BMP2 and BMP4, although it has been demonstrated that BMP1 overexpression counteract the dorsalizing effects of chordin, suggesting that also BMP1 should be among the major chordin antagonists in early mammalian embryogenesis and in pre- and postnatal skeletogenesis (Scott et al., 1999). Furthermore, in mouse, Chrd binds also to Bmp7 with similar affinity (Zhang et al., 2007). Chordin is supposed to play a role during the very early embryo patterning, as it is expressed in the anterior primitive streak, in the node and subsequent axial meso-endoderm. Similarly to Nog, Chrd deficiency is early lethal in mice and is associated to a ventralized gastrulation phenotype. Stillborn animals have normal early development and neural induction but display later defects in inner and outer ear development and abnormalities in pharyngeal and cardiovascular organization (Bachiller et al., 2000). The expression and function of Chrd and Nog in the midgastrula strictly overlap. It seems that they can compensate for each other during early mouse development, as suggested by studying the phenotypic effects of combined Chrd/Nog mutations in mice. When both gene products are removed, antero-posterior, dorso-ventral, and left-right patterning are all affected in mice, with severe disruption of mesoderm development (Bachiller et al., 2000).

2.2.3.3 Sclerostin and USAG-1

Sclerostin (Sost), recently classified among BMP antagonists, was originally identified as the gene responsible for the autosomal recessive progressive sclerosing bone dysplasia, known as sclerosteosis (MIM#269500; Brunkow et al., 2001). In scleosteosis, loss of SOST prolongs the active bone-forming phase of osteoblasts, resulting in the increased bone mass. Sost is abundantly expressed in long bones and cartilages and binds to BMP-6 and -7 with highest affinity (Kusu et al., 2003; Winkler et al., 2003). Sost is expressed exclusively by osteocytes, and inhibits the differentiation and mineralization of murine preosteoblastic cells (van Bezooijen et al., 2004).

Transgenic mice overexpressing Sost exhibited low bone mass and decreased bone strength due to reduced osteoblast activity and bone formation (Winkler et al., 2003). The mechanism of action of Sost should be based on the inhibiton of BMP-induced Smad phosphorylation and alkaline phoaphatase (ALP) activity, though there are still controversies regarding this issue (Yanagita, 2005). It also acts as an inhibitor of the Wnt-signalling pathway, by binding and blocking the Wnt receptor LRP-5 (see paragraph 2.3.2).

Also another rare skeletal disease characterized by high bone mass, osteopetrosis or van Buchem's disease, already associated to LRP5 gene mutations, has been linked to inactivating mutations in the SOST gene (see Table 1 and 2). This finding highlighted the role of sclerostin in the homoeostasis of bone mass, and provided the rationale to target sclerostin with monoclonal antibodies to enhance bone formation. In a rat model of post-menopausal osteoporosis due to ovariectomy, treatment with a sclerostin antibody increased bone mass at all skeletal sites and completely prevented bone loss associated with oestrogen deficiency.
Another cysteine-knot secretory protein recently classified among the BMP antagonists is the uterine sensitization-associated gene-1 (USAG-1), originally found in rat endometrium upon sensitization, hence its name, and found expressed in the human kidney (Yanagita, 2005). USAG-1 share 38% identity to SOST aminoacid sequence, so that, also based on their functional overlap, the two moelcules could be grouped in a distinct family of BMP antagonists. Recombinant USAG-1 protein binds and inhibits BMP-2, -4, -6, and -7 with high affinity, reducing ALP activity in mesenchymal cells and pre-osteoblasts.

2.2.3.4 LIM-mineralization proteins

Among the BMP-interacting molecules, it is worth to mention the recently discovered Lim mineralization protein (LMP), an intracellular LIM-domain protein acting as a potent positive regulator of the osteoblast differentiation program (Bernardini et al., 2010). LMP was originally identified and cloned from rat calvarial osteoblasts stimulated by glucocorticoids, and subsequently emerged as a novel attractive osteogenic molecule able to induce the activation of the BMP signaling pathway (Boden et al., 1998; Boden et al., 1998b; Viggeswarapu et al., 2001). In humans, three different splice variants are transcribed from the LMP-coding gene (PDZ and LIM doamin-7, PDLIM7): LMP1 is the longest transcript, encodes the full-length protein isoform comprising conserved PDZ and LIM domains plus a non-conserved region, and has demonstrated osteogenic properties; LMP2, misses over 100 nucleotides within the non-conserved region and does not induce bone formation; finally the LMP3 isoform is the result of a more complex post-transcriptional processing that produces a smaller peptide missing all LIM domains while retaining a shorter non-conserved sequence (H.S. Kim et al., 2003; Y. Liu et al., 2002; Viggeswarapu et al., 2001). Despite the truncation of nearly two third of the full-length isoform, LMP3 retains efficient osteogenic properties, demonstrated *in vitro* and in different animal models (Lattanzi et al., 2008; Parrilla et al., 2010; Pola et al., 2004). *In vitro*, both LMP1 and LMP3 induce osteogenic differentiation of mesenchymal progenitors, fibroblasts and pre-osteoblasts, through the transcriptional activation of BMP-family members (mainly BMP2, BMP4 and BMP7) and TGFβ1 protein (Bernardini et al., 2010 ; Minamide et al., 2003). LMP1 osteogenic properties are also based on interactions with the Smad ubiquitin regulatory factor 1 (Smurf1), thus preventing Smads ubiquitination and potentiating BMP signaling, along with the insulin-growth factor biniding protein-6 (IGFBP6), a potent indcutor of osteoblast differnetiation (Sangadala et al., 2006; Strohbach et al., 2008). In addition, it suppresses osteoclast activity acting through the mitogen-activated protein kinase (MAPK)-signaling, which implies more pleiotropic effects to be exerted by the peptide (H. Liu et al., 2010). The effectiveness of LMPs in inducing bone formation *in vivo* has been demonstrated, using different strategy to deliver the gene/peptide at the site where the bone healing was required (H.S. Kim et al., 2003; Lattanzi et al., 2008; Parrilla et al, 2010; Pola et al., 2004; Sangadala et al., 2009; Strohbach et al., 2008b; X. Wang et al., 2011; Yoon et al., 2004). So far, the expression of

human LMPs has been detected in the iliac crest bone, in teeth and in calvarial tissues and cells (Bernardini et al., 2011; Bunger et al., 2003; Fang et al., 2010; X. Wang et al., 2008).

2.2.4 Skeletal phenotypes associated to BMPs

At least four molecules that are directly involved in the BMP-TGFβ pathway are mutated in human syndromes comprising skeletal malformations in their phenotypes, due to the actions carried out during throughout development in mesoderm induction, tooth development, limb formation, bone induction, and fracture repair. Table 1 summarizes the mendelian disorders allelic to BMP-related gene mutations categorized in the "online mendelian inheritance in man" (OMIM) database (www.omim.org). Main differences in human phenotypes associated to different genes are evident, reflecting the diversified gene functions during skeletal development and patterning. In particular, while heterozygous mutations in the BMP4 gene are associated to minor facial malformations, different point mutations in the human homolog of the murine Nog (NOG) can cause five different phenotypes, invariably carachterized by limb malformations, and other skeletal anomalies due to impaired endochondral ossification. This could possibly reflect more pleiotropic roles for NOG (see Table 1).

Gene[a]	Diseases	MIM[b]
BMP4	Syndromic microphtalmia	607932
	Orofacial cleft	600625
BMPRIB	brachydactyly, type A2	112600
	Acromesomelic chrondrodysplasia with genital anomalies	609441
NOG	brachydactyly, type B2	611377
	multiple synostosis syndrome	186500
	stapes ankylosis with broad thumb/toe	184460
	proximal symphalangism	185800
	tarsal-carpal coalition syndrome	186570
SOST	Sclerosteosis	269500

a. gene symbol is provided; b. Mendelian Inheritance in Man (MIM) ID code.

Table 1. Mendelian diseases associated to BMP family mutations

2.3 WNT signaling

Since the original discoveries of the role of Wnt signalling in bone formation and skeletogenesis (Hartmann & Tabin, 2001; Lako et al., 1998), there has been a stream of research aimed at elucidating the complex roles that Wnt proteins, endogenous Wnt inhibitors and interacting molecules play in the regulation of bone mass, revelaing that they play a vital role in adult tissue maintenance (Milat & Ng, 2009).

2.3.1 Wnt proteins

The "wingless-type mouse mammary tumor virus (MMTV) integration site" family (WNT) consists of structurally related genes which encode secreted signaling glycoproteins, which

are extremely conserved in evolution. They are implicated in oncogenesis and in several developmental processes, including regulation of cell fate, early axis specification, organ development and patterning during embryogenesis (Hartmann & Tabin, 2000, 2001). Hence, aberrations in Wnt signalling lead to complex developmental diseases.

WNT family members are defined by sequence homology to the Drosophila wingless (wg) and the murine int-1 proto-oncogene, hence the family name. The first Wnt gene was cloned 30 years ago (Nusse & Varmus, 1982); to date, nineteen Wnt genes have been identified in the mouse and human genomes (http://www.stanford.edu/rnusse/wntwindow.htlm; Milat & Ng, 2009). Wnt proteins are traditionally categorized into two classes, canonical and non-canonical, based on their *in vivo* and *in vitro* activities that are exerted through distinct molecular signal transduction mechanisms. Canonical Wnts (including Wnt1, Wnt3A, Wnt8, Wnt10b) activate a cascade that results in the translocation of β-catenin (cadherin-associated protein, beta 1, CTNNB1) to the nucleus, where it associates to the lymphoid-enhancer binding factor/T-cell specific transcription factors (TCF/LEF) that finally induces the expression of target genes (Logan et al., 2004; Milat & Ng, 2009). The canonical pathway, the best studied, initiate when a canonical Wnt-ligand binds to one of the Frizzled (Fz) receptors. This event inactivates the glycogen synthase kinase 3 (GSK3), through the developmental transducer phosphoprotein Dishevelled (Dvl), and therefore prevents phosphorylation and consecutive proteosomal-degradation of β-catenin (Westendorf et al., 2004; Behrens et al., 1996).

Non-canonical Wnts (including Wnt4, Wnt5a and Wnt11) activate transcription through β-catenin-independent signaling pathways, involving alternative intracellular second messengers. At least three alternative non-canonical Wnt pathways could be described. One is based on the intracellular release of Ca2+ that activates calcium-sensitive enzymes, which on their turn activate specific transcription factors (A.E. Chen et al., 2005). This Wnt-cGMP/Ca2+-protein kinase C dependent pathway plays important roles during dorso-ventral patterning of the embryo, regulating cell migration, as well as heart development, and might play a role during tumor suppression (Piters et al., 2008). The planar cell polarity (PCP) signaling (Mlodzik, 2002) represent another non-canonical Wnt-pathway, required for embryonic morphogenesis, activated through Fz receptors binding and results in the coactivation of Rho and Rac, two small GTPases that are able to regulate cytoskeletal architecture (Piters et al., 2008). Finally, the Wnt-protein kinase A (PKA) pathway is based on the increase cAMP levels, which activates PKA and the transcription factor CREB and has been implicated in myogenesis in mice (Kuhl et al., 2000; Semenov et al., 2007).

Recent evidences suggested that the distinct signaling pathways are alternatively activated through the binding of distinct sets of receptors, regardless of the original classification of canonical versus non-canonical Wnts (van Amerongen et al., 2008).

2.3.2 Wnt receptors

The mechanism by which Wnt binding to its receptors triggers the downstream signalling has been extensively studied, although a conclusive and comprehensive view has not been achieved so far (Fuerer et al., 2008). The best known Wnt receptors belong to the Frizzled (Fzd) family, which includes ten G protein-coupled transmembrane receptors (FZD1-to-10; Wodarz & Nusse, 1998). Thereafter, various members of the density lipoprotein receptor (LRP) family (LRP5 and LRP6) have been shown to be essential co-receptor for Wnt

signalling (Tamai et al., 2000; Wehrli et al., 2000). LRP phosphorylation by activated Wnt is critical and requires the cooperative roles of FZD, the cytoplasmic scaffolding proteins dishevelled (Dsh/Dvl) and axin, and GSK3, within the canonical pathway (Bilic et al.., 2007; Davidson et al., 2005).

2.3.3 Wnt targets

A large number of Wnt target genes have been identified to date, in over twenty studies based on genome-wide approaches (e.g. microarray-based gene expression profiling) in different cell lines and tissues (http://www.stanford.edu/~rnusse/pathways/targets.html). Distinct *in vitro* studies provided the evidence of "feedback targets" of Wnt, that are Wnt signalling components whose expression can be regulated by the signaling itself, indicating that feedback control is a key feature of Wnt signaling regulation. In particular, over 150 genes have been identified as direct transcriptional targets of the Wnt canonical pathways, as they contain Tcf/Lef binding sites, including basic regulators of cell proliferation/tumorigenesis (c-myc, cyclin D, c-jun, among others), growth factors (FGF9, FGF20, VEGF, BMP4), transcription factors (RunX2), and a wide number of genes implicated in cell adhesion and differentiation (see the gene-lists available at the web site above indicated).

2.3.4 Wnt in bone biology

An extensive review of the scientifc literature reveals that Wnt proteins play a leading role in bone development and homeostasis. In this respect, the canonical Wnt pathway has been most extensively studied for its role in skeletogenesis,

There is *in vitro* evidence that Wnt proteins are produced by calvaria, primary osteoblasts (Wnt1, Wnt4, Wnt14) and osteosarcoma cell lines. Wnt genes (e.g. Wnt7b) are up-regulated during osteogenic differentiation of bone marrow stromal cells (Kato et al., 2002; Gregory et al, 2005). Some Wnt proteins (Wnt10b, Wnt1,Wnt2 and Wnt3a) have an effect on bone physiology, as they regulate bone marker gene expression (osteocalcin, RunX2, Osterix, alkaline phosphatase), stimulate osteoblastogenesis and inhibit adipogenesis, mainly through the canonical pathway (Takada et al, 2009). Mice lacking either Wnt10b or Wnt5a display impaired bone structure organization and reduced bone mass due to hypoplasia (Bennet et al., 2005; Takada et al., 2007). Wnt5a seems to act through CaMKII rather than TCF/LEF, thus suggesting that both the canonical and non-canonical Wnt signalling pathways play a role in osteoblastogenesis (Milat & Ng, 2009). Mice lacking Lrp5 display a low bone mass secondary to reduced osteoblast proliferation (Kato et al., 2002). Similarly in humans, a loss-of-function mutation in LRP5 occurs in the osteoporosis-pseudoglioma syndrome (OPPG, MIM#259770; Table 2), an autosomal recessive syndrome characterized low bone mass, ocular defects, and predisposition to fractures (Gong et al., 2001). Conversely, the gain-of-function mutation in Lrp5 may not have an effect on bone density when expressed in mature osteoblasts and can be associated to increased bone mass, due to inhibition of Wnt sigaling (Yadav et al., 2008).

Moreover, LRP5 inhibits tryptophan hydroxylase (Tph1) expression, a rate-limiting enzyme in the gut-derived serotonin biosynthetic pathway, impairing serotonin synthesis. Serotonin on its turn regulates the bone mass (Warden et al., 2005), in fact, gut-specific deletion of LRP5 results in low bone mass, similarly to the phenotype observed in LRP5-null mice (Yadav et al., 2008). Reasonably, this could provide the first possible explanation

of the complex gut–bone interactions in the regulation of bone mass (Milat & Ng, 2009). Conversely, osteoblast-specific deletions of LRP5 do not cause osteoblast defects. LRP5 may be involved in postatal regulation of osteoblast differentiation and it is possible that LRP6, rather than LRP5, is the critical co-receptor for Wnt signalling in bone. As a proof of evidence, LRP6 loss of function bone phenotype is much more severe than that associated to LRP5 loss. The LRP6−/− genotype is lethal in mice, while heterozygous mice display reduced bone mass (Pinson et al., 2000). In humans, a missense mutation in LRP6, with consequent impairment of Wnt signalling, has been associated to an autosomal dominant early coronary artery disease, to metabolic risk factors and to osteoporosis (Mani et al.., 2007).

β-catenin (CTNNB1) mutations appeared to affect bone resorption by regulating, in differentiated osteoblasts, the expression of osteoprotegerin (OPG), which controls osteoclast differentiation (Glass et al., 2005; see paragraph 2.8). Also, conditional deletion of Ctnnb1 in mouse embryo limb and head mesenchyme resulted in blockage of osteoblastic differentiation of mesenchymal precursors (Day et al., 2005; Hill et al.,2005). Ctnnb1 is indeed crucial in determining the correct osteoblastic fate of mesenchymal progenitors in the developing embryo (Hill et al., 2005).

Wnt signaling is also involved in the transcriptional modulation of the molecular events leading to cartilage differentiation. Ectopic canonical Wnt signaling leads to enhanced ossification and suppression of chondrocyte formation during skeletogenesis (Day et al., 2005). On the other hand, during both intramembranous and endochondral ossification, b-catenin inactivation induces ectopic chondrocyte formation in place of osteoblast differentiation. Moreover, Wnt signaling is essential for skeletal lineage differentiation, preventing transdifferentiation of osteoblasts into chondrocytes, and control stem cell self renewal, proliferation and fate lineage specification, by regulating the balance between FGF and BMP signaling (Hill et al., 2005).

2.3.5 Wnt-signaling inhibitors

Other ligands can bind the Fzd–LRP5/6 receptor complex, thus antagonizing Wnt signal transduction pathway. Dickkopfs (Dkk) proteins, compete for the LRP5/6 receptor and prevent canonical signalling. Dkk are involved in epithelial-mesenchymal transition during mesodermal tissue development and play an important role in bone biology. DKKs role in vertebrate development is due to their local inhibitory funtion on Wnt-regulated processes, such as antero-posterior axial patterning, limb development, somitogenesis and eye formation (Pinzone et al, 2009).

Dkk1 binds to LRP6 with high affinity and is expressed by osteocytes and osteosarcoma cells. When overexpressed, DKK1 induce osteopenia, while Dkk1 loss induces increased bone formation in mice (Milat & Ng 2009). In humans, an increase of DKK1 expression in leukocytes was associated to the presence of bone lesions in myeloma patients (Milat & Ng, 2009). The role in bone homestasis of other Wnt inhibitors, such as the secreted frizzled related protein-1 (sFRP1) and the Wnt inhibitory factor 1, (WIF-1) has been explored and demonstrated so far exclusively in animal and cellular models (Milat & Ng, 2009).

It is worth to mention that close relationships between the BMP- and the WNT-signaling occur at this level; Dkk1 and Nog cooperate in mammalian head induction (del Barco Barrantes et al., 2003); the expression of DKK1 is regulated by BMP-4 in limb development (Grotewold et al., 2002). Furthermore, the mutlifunctional antagonist called Cerberus, a

potent inducer of head formation during vertebrate development, has distinct binding sites for Wnt proteins and BMPs (Piccolo et al., 1999). Finally, USAG-1 might also have dual activities, and play as a molecular link between Wnt and BMP signaling pathway (Yanagita et al., 2004).

2.3.6 Skeletal phenotypes associated to Wnt signalling members

Over 70 mendelian syndromes presenting with skeletal abnormalities in humans are associated to mutations in Wnt signaling-related genes. This large group include complex developmental disorders with multi-system implication due to severe imbalance of body patterning in embryo. Besides very rare and incompletely characterized syndromes, some of the most significant WNT-associated skeletal phenotypes are summarized in table 2. Overall, these diversified phenotypes are characterized alternatively by limb malformations and defective/eccessive ossification.

Refer to the OMIM database (www.omim.org) for complete clinical information about the listed phenotypes and for additional Wnt-related genetic disorders.

Gene[a]	Diseases	MIM[b]
LRP5	Hyperostosis, endosteal	144750
	Osteopetrosis, autosomal dominant 1	607634
	Osteoporosis-pseudoglioma syndrome	259770
	Osteosclerosis	144750
	van Buchem disease, type 2	607636
	Bone mineral density variability 1	601884
WNT5A	Robinow syndrome, autosomal dominant	180700
SOX9	Acampomelic campomelic dysplasia	114290
	Campomelic dysplasia	
	Campomelic dysplasia with autosomal sex reversal	
WNT10B	Split-hand/foot malformation 6	225300
WISP3	Arthropathy, progressive pseudorheumatoid, of childhood	208230
	Spondyloepiphyseal dysplasia tarda with progressive arthropathy	
PRKAR1A	Acrodysostosis with hormone resistance	101800
FRZB	Osteoarthritis susceptibility 1	165720
ROR2	Brachydactyly, type B1	113000
	Robinow syndrome	268310

a. gene symbol is provided; b. Mendelian Inheritance in Man (MIM) ID code.

Table 2. Main mendelian syndromes associated to mutations in genes related to the Wnt-signaling

2.4 Hedgehog homologs family

Drosophila hedgehog (Hh) and its vertebrate orthologs, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Ihh) are secreted proteins involved in the establishment cell fates at several points during development. The vast majority of information on the Hh system derives from the studies performed in Drosophila. Hh production occurs through a well documented process of synthesis and post-translational processing leading to cholesterol-modification. This biochemical feature allows the Hh proteins to permeate the plasma membrane and form diffusible multimeric complexes. These represent represent the biologically active forms required for long-range signaling across different tissues in the developing embryo (Ehlen et al., 2006). Further transduction depends on the presence of the tramsmembrane protein dispatched-1 (Disp1), which is essential for driving long-range Hh signaling in flies and mice (Burke et al., 1999). Once they reach the target site of action, Hh molecules bind to the surface receptor Patched-1 (Ptch1) and induce the release and functional activation of the transmembrane protein smoothened (Smo). Smo activation on its turn induce downstream signaling, including the de-repression of the transcription factors belonging to the glioma-associated family of zinc finger oncogenes (Gli-1, -2 and -3; vertebrate orthologs of the Drososphila Cubitus interruptus), resulting in the activation of target gene expression (Cohen, 2003). Gli proteins are DNA-binding transcription factors which localize in the cytoplasm and play important roles during embryogenesis (Lum & Beachy, 2004). This is also substantiated by the implication of human GLI genes in complex developmental disorders (see paragraph 2.4.2 and table 3).

The signal transduction events downstream of Smo are best understood in Drosophila, while the complete sequence of molecular events involved in Hh signaling in vertebrate skeletogenesis is still unclear. A growing number of studies during the last decade have been trying to clarify this issue (Ehlen, 2006). New evidences are taking root in suggesting that vertebrate Hh signaling is related to the function of the primary cilium, a specialized cell surface projection intensively involved in intercellular signaling, which is essential for the correct coordination of organogenesis. In fact, some molecules involved in the Hh signalling cascade localize to the primary cilium (Corbit et al., 2005; Haycraft et al., 2005; Singla and Reiter, 2006). In addition, mutations in components of the intraflagellar transport machynery are able to indirectly disrupt Hh signaling and lead to a wide range of human disorders, comprising severe impairment of organ patterning in their phenotypes (van Reeuwijk et al., 2011).

2.4.1 Hh in bone biology

Hedgehog signaling coordinates a variety of embryo patterning processes through a series of inductive interactions (Ehlen et al., 2006). In particular, in vertebrates Shh and Ihh are essential regulators of skeletogenesis as they provide positional information and initiate or maintain cellular differentiation programs, regulating the formation of cartilage and bone. They basically regulate endochondral ossification, acting either as paracrine modulators on adjacent cells or over long distances, leading to the axial and appendicular skeleton patterning (Ehlen et al., 2006). Indian hedgehog (Ihh) can be regarded as the osteogenic HH, being a signaling molecule expressed predominantly in pre-hypertrophic chondrocytes where it plays a major role in endochondral bone formation. Ihh functions in the growth plate are mediated through the parathyroid hormone-related peptide (PTHrP) (Vortkanp et al., 1996). In the absence of Ihh, PTHrP mRNA is undetectable in fetal bones (Kronenberg, 2006).

The parathyroid hormone-related peptide (PTHrP) also named PTH-like hormone (PTHLH), is a key regulator of endochondral bone formation synthesized by perichondrial cells and chondrocytes in growing bones. This hormone was originally discovered as a circulating peptide increased in the hypercalcemia occurring during malignancies (Suva et al., 1987). As a circulating hormone the PTHrP has pleiotropic functions, while during endochondral bone formation it acts in a juxtacrine/paracrine manner, binding to the same receptor as paratohomone (PTH/PTHrP Rec or PTHR1); the signal is transduced in the intracellular compartment via the recruitment of multiple G proteins and subsequent activation of adenylate cyclise, which generates cAMP. cAMP-dependent PKA then promote a downstream cascade leading to the suppression of target genes, including RunX2. PKA also phosphorylates and activate SOX9. The PTHR1 is expressed by chondrocytes as they stop proliferating. PTHrP then induces proliferation and delays chondrocyte hypertrophy (Kronenberg, 2006). Mice missing either the PTHrP or its receptor genes undergo a truncated endochondral sequence leading to shorter or absent column of proliferating chondrocytes in fetal bones and usually die shortly after birth, due to severe impairment of rib cage development (Amizuka et al., 1994; Chung et al., 1998). Conversely mice overexpressing either genes show bones with increased number of proliferating chondrocytes giving rise to delayed endochondral sequence (Weir et al., 1996).

2.4.2 Skeletal phenotypes associated to HH family members

Due to their essential role in embryo development, mutations of the HH family genes imply severe alteration of vertebrate body patterning and affect the cell proliferative homeostasis leading to tumorigenesis. There are at least three defined phenotypes that are directly ascribable to HH gene mutations in humans. Noticeably, being essential for the endochondral ossification throughout the developing skeleton, human IHH mutations in homozygosis lead to malformations involving both appendicular and axile skeletal structures (see table 3). Main mendalian disorders of the whole HH signaling are listed in table 3.

Gene[a]	Diseases	MIM[b]
IHH	Acrocapitofemoral dysplasia	607778
	Brachydactyly, type A1	112500
SHH	Single median maxillary central incisor	147250
PTHLH	Brachydactyly, type E2	613382
GLI3	Greig cephalopolysyndactyly syndrome	175700
	Pallister-Hall syndrome	146510
	Polydactyly, postaxial, types A1 and B	174200
	Polydactyly, preaxial, type IV	174700

a. gene symbol is provided; b. Mendelian Inheritance in Man (MIM) ID code.

Table 3. Selected mendealian malformation syndromes of HH signaling molecules

2.5 The FGF/FGFR signaling

Fibroblast growth factors (FGFs) and corresponding receptors (FGFRs) are known to play important roles during bone development. FGF signaling is essential for maintaining bone

homeostasis and during fracture healing. The FGF family currently comprises over 20 structurally related members that bind to tyrosine kinase transmembrane receptors. Upon binding FGFR on its extracellular ligand-binding domain, FGF causes the dimerization of receptor monomers, leading to autophosphorylation of tyrosine residues on the intracellular signal transduction domain (Su et al., 2008). Alternative downstream signal transduction pathways have been described that basically imply the activation of the mitogen-activated protein kinase (MAPK) signaling. (Eswarakumar et al., 2005).

2.5.1 FGFs/FGFRs in bone biology

FGF sigaling is crucial in both endochondral and intramembranous ossification. Different FGF ligands are expressed in the mesenchyme of the limb bud at the stage of condensation and later in chondrocytes and osteoblasts in the growth plate of developing long bones (Colvin et al., 1999; Lazarus et al., 2007; Yu et al., 2003). During the cyclic proliferation/hypertrophy stages of endochondral bone formation, more then ten different FGF molecules are expressed in a perfectly spatio-temporal coordinated fahion (Lazarus et al., 2007). Particularly, converging evidences indicate FGF2 as the earliest marker gene to be expressed in prechondrocyte condensation stages, while FGF1 and FGF3 appear later in differentiated chondrocytes (Lazarus et al., 2007; Yu et al., 2003).

Lessons from genetically modified mouse models and *in vitro* studies indicated that excessive Fgf2 inhibits chondrogenesis, resulting in decreased bone elongation, hypertrophic diffentiation and abnormal chondrocyte proliferation (Montero et al., 2000; Sobue et al., 2005). Conversely, mice lacking Fgf2 display reduced bone formation and abnormal bone structure (Montero et al., 2000). Overall, the correct dosage of Fgf2 is essential for the bone growth and homeostasis (Su et al., 2008). Similarly, other FGF ligands have a documented role in limb outgrowth and patterning, from the stage of condensation till osteoblastic differentiation, indicating some kind of redundancy among FGF signaling (de Lapeyriere et al., 1993; Fiore et al. , 1997; Finch et al., 1995; Guo et al., 1996; Haub et al., 1991; Hebert et al., 1994).

With regard to intramembranous ossification, all Fgfs, except Fgf-3 and -4, are expressed in coronal suture in the mouse embryo and in other mesenchymal sutures during craniofacial development (Su et al., 2008). The role of FGF/FGFR signaling in promoting intramembranous ossification is indeed strongly supported by the association of FGFR1-3 genes in human craniosynostosis syndromes (see table 4). Increased FGF signaling cause increased proliferation rates in suture-derived calvarial cells leading to premature suture closure (i.e. synostosis; H.J. Kim et al., 1998). FGF signaling also regulates calvarial cell differentiation. Therefore, FGF signaling exerts a dual effect on osteoblast biology, inducing proliferation of immature osteoblasts and apoptosis in differentiated osteoblasts (Mansukhani et al., 2000). The pro-differentiation effects should be the result of Runx2-induced expression of osteocalcin, enhanced by Fgf2 (H.J. Kim et al., 2003). FGF signaling actually cross-talks with the other osteogenic pathways, including Msx2, Twist, Bmp and other TGF-β superfamily members, during calvarial suture morphogenesis (Opperman, 2000; Rice at al, 2005).

2.5.2 Skeletal phenotypes associated to FGF signaling; The FGFR syndromes

Missense mutations in either FGFs or FGFRs human genes cause a variety of congenital skeletal disorders, including syndromic craniosynostosis (CRS) and hypo/achondroplasia,

among others (extensively reviewed by Su et al., 2008). In particular, both CRS syndromes and chondrodysplasias, are associated to gain-of-function mutations in either of the FGFR-1, -2 and -3 genes, which imply the constitutive activation of the kinase receptor activity regardless of ligand binding on the extracellular domain. A list of the best characterized malformation syndromes associated to FGFR gene mutations (the autosomal dominant "FGFR syndromes") is provided in table 4, as paradigmatic examples of the effects of FGF signaling impairment in human diseases. The functional relevance of the most common FGFR gene mutations of syndromic craniosynostosis has been further examined using animal models and *in vitro* studies (Su et al., 2008).

Gene[a]	Diseases	MIM[b]
FGFR2	Antley-Bixler syndrome	207410
	Apert syndrome	101200
	Beare-Stevenson cutis gyrata syndrome	123790
	Crouzon syndrome	123500
	Gastric cancer, somatic	137215
	Jackson-Weiss syndrome	123150
	LADD syndrome	149730
	Pfeiffer syndrome	101600
	Saethre-Chotzen syndrome	101400
	Scaphocephaly with maxillary retrusion and mental retardation	609579
FGFR3	Achondroplasia	100800
	CATSHL syndrome	610474
	Crouzon syndrome with acanthosis nigricans	612247
	Hypochondroplasia	146000
	LADD syndrome	149730
	Muenke syndrome	602849
	Thanatophoric dysplasia, type I	187600
	Thanatophoric dysplasia, type II	187601
FGFR1	Jackson-Weiss syndrome	123150
	Osteoglophonic dysplasia	166250
	Pfeiffer syndrome	101600
	Trigonocephaly	190440

Table 4. The FGFRs syndromes: skeletal malformations associated to FGFRs: a. gene symbol is provided; b. Mendelian Inheritance in Man (MIM) ID code.

2.6 TWIST

The Twist homolog 1 (Drosophila), TWIST1, belongs to the basic helix-loop-helix (bHLH) class of transcriptional regulators that dimerize to form a bipartite DNA binding groove, which recognize a consensus DNA element and alter the chromatin structure (Pan et al., 2009). Twist1 represents a critical modulator of mesenchymal cell fate during skeletal development, inducing differentiation toward both the chondrogenic and the osteogenic lineages, while inhibiting myogenesis (Miraoui & Marie, 2010). In Drosophila, twist homozygous mutations are associated to a lethal phenotype, due to disruption of gastrulation and failure in mesodermal-derived organ development and leading to complete eversion of head; the embryo was twisted in the egg, hence the name given to the gene

(Simpson, 1983). Heterozygous mutations in the Twist1 gene usually determine loss-of-function due to haploinsufficiency (Zackai and Stolle, 1998). In fact, mice heterozygous for a Twist null mutation exhibited cranial and limb defects (Bourgeois et al.. 1998; El Ghouzzi et al.. 1997). Such findings, allowed to confirm TWIST1 as the candidate gene for the Saethre-Chotzen syndrome (MIM#101400, autosomal dominant), characterized by craniofacial and limb malformations and indicated a key role of TWIST in n the mesodermal development of the head and limbs (El Ghouzzi et al., 1997; Gripp et al., 2000; see following sections). Thereafter, Twist role in calvarial bone/suture patterning and development has been intensively investigated (Miraoui & Marie, 2010). In this context. Twist1 appears to act as an upstream transcriptional regulator of FGFRs (Shishido et al., 1993).

2.7 The RANK/RANKL/OPG system in bone biology

Bone resorption, required during endochondral bone formation and bone remodeling, is driven by functional activation of osteoclasts. It is now clear that the complete maturation of osteoclasts occurs only in presence of osteoblasts (Grano et al., 1990; Teti et al, 1991). The molecular basis of this fundamental interaction resides in the interplay between the receptor activator of nuclear factor kappa B (RANK), its ligand (RANKL) and the osteoprotegerin (OPG).

RANK is a homotrimeric transmembrane receptor, belonging to the tumor necrosis factor (TNF) receptor superfamily, expressed on the cell surface of osteoclast precursors and mature osteoclasts. RANKL, also called OPG-ligand or osteoclast differentiation factor (ODF), also belong to the TNF superfamily and is expressed in osteoblasts (Boyce and Xing, 2008). The interaction between RANK and RANKL induces a downstream signalling, that involves the nuclear factor kB (NFKB), with subsequent transcriptional activation of target genes leading to recruitment, differentiation, activation and survival of osteoclasts.

OPG, also known as tumor necrosis factor receptor superfamily member 11 (TNFRSF11) or osteoclastogenesis inhibitory factor (OCIF) is a soluble receptor of RANK, exrpressed in osteoblasts, stromal cells and other non-skeletal cells. OPG acts as a decoy receptor for RANKL, as it prevents RANK/RANKL interaction and inhibits osteoclastogenesis and bone resorption (Khosla, 2001). The RANKL/OPG ratio represents a major determinant in bone mass regulation, as inferred from the OPG-deficient mouse, which displays an osteoporotic phenotype with increased osteoclasts (Bucay et al., 1998). Conversely, OPG overexpression leads to osteopetrosis (Simonet et al., 1997).

OPG expression is regulated by the canonical Wnt pathway, which regulates osteoclasts by increasing the OPG/RANKL ratio (Suzuki et al., 2008). The OPG/RANKL ratio in osteoblasts is also regulated by bone remodeling hormones. Both parathormone (PTH) and vitamin D decrease the ratio by the transcriptional up-regualtion of RANKL. Conversely, estrogens increase OPG production in osteoblasts, hence increase bone formation and reduce resorption (Zallone, 2006). More recently a role in the control of bone remodeling has been emerging for leptins, serotonin and insulin, probably acting through the RANKL/OPG system (Elefteriou, 2005; Huang et al, 2009).

2.7.1 Skeletal phenotypes associated to OPG mutations

Besides the documented involvement of the RANK/RANKL/OPG system in the pathogenesis of acquired multifactorial bone remodeling disorders, such as osteoporosis and osteoarthritis, OPG is also emerged as a genetic disease-associated gene.

Homozygous or compound heterozygous mutations of TNFRSF11 have been found in juvenile Paget disease of bone (MIM#239000), characterized by systemic hyperhostosis, with typically increased skull bone thickness, hyperphosphatemia and progressive skeletal deformities.

2.8 Notch signaling

Evolutionarily conserved Notch signaling plays an important role in developmental processes and adult tissue homeostasis by regulating cell fate determination, proliferation, differentiation and apoptosis in a spatio-temporal coordinated manner. The Notch receptor and its ligands are transmembrane proteins whose signaling requires cell to cell contact between neighboring cells (Engin & Lee, 2010). Four Notch receptors (Notch1-4) are known in mammals, while Notch ligands fall into two classes: Delta and Jagged. Subsequent cleavage steps occur upon Notch receptor/ligand interaction to activate the Notch intracellular domain (Notch ICD) that is then released from the membrane, and translocated to the nucleus (Hayes et al, 2003). In the nucleus, Notch ICD binds specific transcription factors and recruits transcriptional co-activators to induce the expression of a basic helix-loop-helix (bHLH) family of genes (Engin & Lee, 2010).

Notch signaling is involved in skeletal patterning and somitogenesis, as related molecules are expressed in the presomitic mesoderm (PSM) of mouse embryos. Notch1 null mouse embryos exhibits significantly delayed and disorganized somitogenesis (Conlon et al., 1995; Turnpenny et al., 2007).

Converging evidences suggest that Notch pathway is active in the early stages of osteoblast differentiation, also by acting on Runx2-dependent osteogenic gene expression (McLarren et al., 2000; Tezuka et al., 2002). Nonetheless, this issue is currently debated. Notch could also regulate osteoclastogenesis, through the up-regulation of RANKL and OPG genes, suggesting that the functional cross-talk between osteoblasts and osteoclasts might be also mediated by Notch signaling (Engin & Lee, 2010). Finally, Notch signaling is believed to act also in chondrogenic differentiation, although its exact role and its temporal effects during chondro/osteoblastogenesis are still unclear.

Getting into the clinical field, mutations in Notch pathway genes are the etiology of two genetic disorders with severe skeletal impairment: Spondylocostal dysostosis type 1 (SCDO1, MIM#602768) and Alagille syndrome (AGS, MIM#118450), both characterized by vertebral column defects (Bulman et al., 2000; L. Li et al., 1997). This clinical evidence, confirm the best characterized function of NOTCH signaling as a regulator of somitogenesis.

2.9 Homeobox genes

The homeobox genes belonging to the muscle segment homeobox gene (Msx) family, Msx-1 and Msx-2, are implicated in the regulation of craniofacial skeletal morphogenesis (AÏoub et al., 2007; Berdal et al., 2009; Orestes-Cardoso et al., 2002). Msx genes are expressed in the cranial neural crest (CNC) cells where they regulate migration and proliferation and specify tissue lineage differentiation fates. They preferentially support skeletal tissue development and they contribute extensively to the formation of the craniofacial skeleton (Bendall & Abate-Shen, 2000). Msx1 and Msx2 genes are expressed in osteoclasts and drive the membranous ossification. In particular, Msx1 has been functionally associated with mandible development, while Msx2 as been implicated in tooth eruption and elongation (AÏoub et al., 2007; Orestes-Cardoso et al., 2002).

The MSX homeoproteins are co-expressed with the distal-less homeobox gene (DLX) family in CNC cells and in various developing tissues. MSX and DLX molecules structurally and functionally interact *in vitro* by forming heterodimers via their homeodomains and reciprocally inhibit each other's activities (Bendall & Abate-Shen, 2000). These homeoprotein families are jointly implicated in the control of craniofacial, axial, and appendicular skeletal morphogenesis (Alappat et al., 2003; Kraus and Lufkin, 2006; Lallemand et al.., 2005). In osteoblast differentiation, Msx activity counteracts the osteogenic-inducing property of Dlx during osteoblast differentiation. In mouse, Msx2 was found indeed to repress the expression of osteocalcin (OC), while Dlx genes were recruited to initiate OC transcription via RunX2 , leading to the final mineralization stage of osteoblast differentiation (Hassan et al., 2004). Thus, functional antagonism through heterodimer formation may provide a mechanism for regulating the transcriptional actions of Msx and Dlx *in vivo*. They were also shown to regulate one another's expression , and to share target genes such as amelogenin (Alappat et al., 2003; Kraus and Lufkin, 2006). On the other hand, it has also been suggested that Msx and Dlx genes act independently in regulating the development of craniofacial skeleton (Levi et al., 2006).

2.9.1 Skeletal disorders associated to MSX2

The human homolog of Msx-2 (MSX2, MIM*123101), originally cloned in 1993 (X. Li et al., 1993), has been associated to three distinct phenotypes, inherited as autosomal dominant tracts and sharing distinctive clinical features, such as craniofacial deformities of variable degrees, no limb involvement, and neurological symptoms (mainly represented by headache and seizures) among others (see www.ncbi.nlm.nih.gov/omim for detailed descriptions). A proline-to-hystidine substitution in the homeodomain of MSX2 was found in the Boston-type Craniosynostosis syndrome (type 2 Craniosynostosis, MIM#604757), a skeletal developmental disorder characterized by skull malformations (from mild asymmetry to trilobular skull with craniosynostosis, the so-called cloverleaf skull) and neurological symptoms usually without mental retardation (Muller et al., 1993; Warman et al., 1993). Deletion of the entire gene or mutations affecting the DNA-binding affinity of MSX2 where found in kindreds affected by the parietal foramina type 1 syndrome (PFM1; MIM#168500), featured by symmetric calvarial defects in the parietal bone, variably associated with additional craniofacial malformations (including cleft lip/palate, scalp defects and cranium bifidum), headache and seizures. Finally, parietal foramina with cleidocranial dysplasia (PFMCCD, MIM#168550) has been recently classified as a distinct MSX2-related mendelian disorder, in which the PFM phenotype is associated with clavicular hypoplasia and mild craniofacial dysmorphisms . In this latter case, a frameshift mutation in the homeodomain leading to premature termination of MSX2 translation was found (Garcia-Minaur et al., 2003). The association of these three allelic genetic diseases, strengthened the idea of MSX2 involvement in the control of membranous ossification.

2.10 MicroRNAs

The fine regulation of gene function necessary for the correct orchestration of osteogenesis and skeletal development may occur at several levels. One mechanism possibly acting in this process is represented by the post-transcriptional modulation operated by micro ribonucleic acids (microRNA or miRNA). miRNAs are a growing group of small (~22

ribonucleotides), single-stranded, noncoding RNAs that generally attenuate gene function. They regulate the translation of specific messenger RNA (mRNA) by base pairing with target complementary sequences (Carthew & Sontheimer, 2009). miRNA were first discovered in the nematode *Caenorhabditis elegans* and then found in diverse species, showing high degrees of evolutionary conservation. MicroRNAs are involved in many developmental signaling pathways and in housekeeping regulation of organ physiology (Schramke & Allshire, 2003). Over 1400 human miRNAs are deposited in the widest miRNA database (Griffiths-Jones et al, 2004, 2006, 2008; Kozomara et al., 2011; http://www.mirbase.org/). With regard to the regulation of bone formation, a growing number of miRNAs are found to be expressed in the developing skeletal system of metazoan, where are reasonably involved in regulating skeletal tissue homeostasis and development (He et al, 2009). Besides the emerging role of miRNAs during embryo skeletogenesis, miRNA-dependent modulation of gene function can alter skeletal phenotypes across individuals and also within same individual over time. This interpretation derives from recent studies focusing on miRNA biology in the pathogenesis of osteoporosis. Different miRNAs are upregulated in sencescent mesenchymal cells, leading to reduced cell plasticity and multilineage potential. Being able to modify the cell differentiation fate, miRNAs should interfere with the main developmental signaling pathways. Particularly, recent evidences suggest that miRNAs might have a regulatory function in osteoblast differentiation (Z. Li et al, 2008). BMP signaling pathway can be modulated at various level by miRNA; relevant results that recently emerged on this issue are shortly cited as examples. Mir125b inhibits osteoblast differentiation by reducing the cell proliferation rate; miR26a has been shown to inhibit the osteogenic differentiation of adipose tissue-derived stromal cells through intereference on Smad1 translation ; on their turn, Smads participate in miRNA biogenesis (Mizuno et al, 2008); miR-2861 enhances BMP2-induced osteoblastogenesis by repressing the histone deacetylase 5 gene, which induce Runx2 degradation (H. Li et al, 2009). Another miRNA, namely miR-138, regulates the osteogenic differentiation of human mesenchymal stem cells *in vivo* (Eskildsen et al, 2011). In addition, during BMP2-induced osteogenic differentiation of mesenchymal cells, the expression of miRNAs that target osteogenic genes is decreased (Z. Li et al, 2008).

Hence, the pathogenic increase or decrease of miRNAs targeting osteogenic genes, along with the action of BMPs on miRNA biogenesis, may influence bone develoment, concurring in the pathogenesis of bone-defective or hyper-ossification disorders.

Interestingly, several miRNA-coding genes are located within HOX gene clusters on evolutionary conserved chromosomal loci, and share their expression pattern. How this genomic situation could imply a HOX-miRNA functional interaction is still unclear, although Hox genes figure among miRNA targets (Yekta et al, 2008).

Finally, distinct studies have demonstrated that sequence variations occurring in miRNA genes can be associated to human diseases or disease-predisposition (Calin et al, 2005; Duan et al, 2007; Jazdzewsky et al, 2008). A homozygous mutation in miR-2861 results in reduced osteoblast activity leading to osteoporosis (H. Li et al, 2009). The evolution of craniofacial variation among species, the development of human craniofacial disease, and the physiological changes leading to osteopenia/osteoporosis that increases with aging, could result from evolutionary variation in miRNA expression patterns and/or structural modifications in miRNA binding sites in mRNAs, thus depicting a brand new era in the genetics of human diseases (He et al, 2009).

3. Conclusion

Overall, the molecular scenario that has been systematically described in this chapter could allow understanding the complexity of bone tissue homeostasis. Far from being considered a "resting" differentiated tissue, bone appears to be extremely plastic, as the control of its homeostasis is driven by thousand of genes interacting in complex developmental networks, along with post-translational and epigenetic mechanisms that can modify the genome performance.

The number of actors playing in this scene is much wider than that described here, thus the screenplay of osteogenesis is still too complex to allow depicting an exhaustive overview. This complexity implies an extremely high number of human diseases affecting bone development, formation and integrity, that possibly can grow over as the genetic body of knowledge further develops.

4. References

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