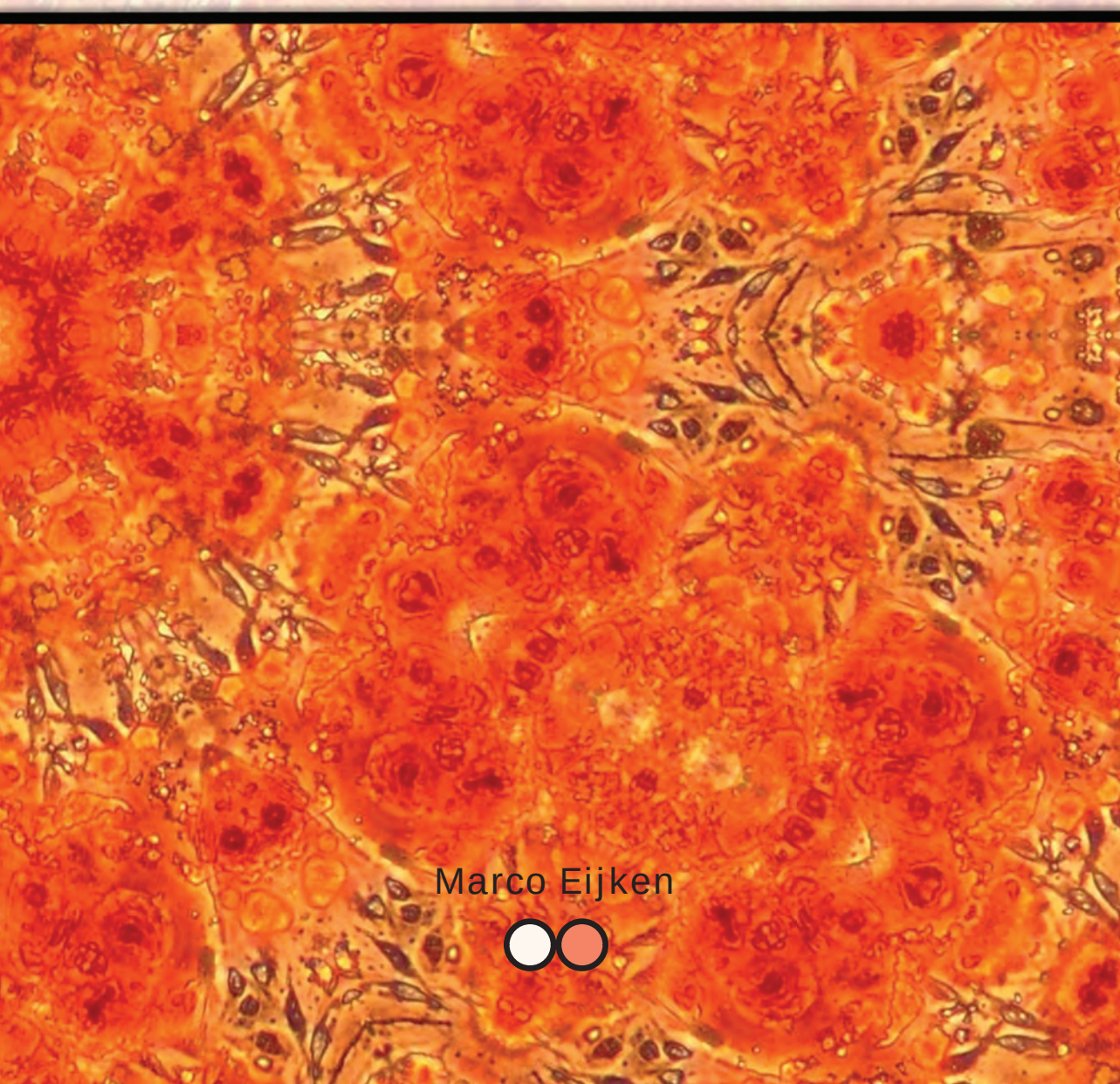
Human Osteoblast Differentiation and Bone Formation:

Growth Factors, Hormones and Regulatory Networks



Marco Eijken

Human Osteoblast Differentiation and Bone Formation: Growth Factors, Hormones and Regulatory Networks

Humane osteoblast differentiatie en botvorming: groeifactoren, hormonen en regulerende netwerken

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Chapter 1

General Introduction

1.1 Bone

Bone is a highly specialized form of connective tissue that provides internal support to the body in all higher vertebrates. In addition to its supportive function, it has several metabolic functions, it houses the brain, the spinal cord and bone marrow, and plays an essential role in maintaining blood calcium levels and supporting hematopoiesis [1]. Bones are built up from an organic extracellular matrix, which is strengthened by the deposition of mineral. Although bones are hard and mineralized, bone is a living tissue containing blood vessels and various cell types. A typical bone consists of a hard and compact outer shell, the cortex, which surrounds a hollow cavity that is filled with bone marrow. At specific locations in the bone cavity the bone is strengthened with a fine network of sponge-like trabecular bone. The combination of the relative heavy cortex together with the relative light trabecular bone gives the bone strength with minimal weight.

1.2 Bone cells

Bone is a dynamic tissue that is continuously maintained and renewed by four different kinds of bone cells; 1] osteoclasts, 2] osteoblasts, 3] osteocytes, and 4] lining cells (see figure 1.1). Osteoclasts are located at the bone surface where they degrade (resorb) small pieces of bone tissue. They are giant multinucleated cells and resorb bone via local acidification and secretion of various proteases. Acidification is necessary to dissolve the mineral in bone, whereas the proteases degrade the proteins of the extracellular matrix [2]. New bone is produced by osteoblasts that synthesize the organic matrix of bone by secretion of a wide variety of extracellular matrix proteins. In addition to matrix production, they also participate in the mineralization process and in the control of osteoclast function [3]. When an osteoblast is in its terminal differentiation stage and resides entrapped in its self produced bone matrix it is called an osteocyte. Osteocytes are the most abundant cells in bone and are believed to maintain the bone by sensing mechanical strains and bone damage [4-6]. They have a typical morphology with long thin cytoplasmic processes, which form a fine network of connections with other osteocytes and with the osteoblasts located at the surface of the bone (see osteocyte structures in figure 1.1). Another bone cell that is derived from osteoblasts is the lining cell; lining cells cover the bone surfaces and thereby separate the bone surface from the bone marrow. However, the exact function of bone lining cells remains unclear [3, 7].

1.3 Bone remodeling

To carry out its function, bone is continuously resorbed by osteoclasts and rebuild by osteoblasts at millions of microscopic sites. This process is called bone remodeling, which main function is to renew bone tissue to retain bone strength during adulthood [8, 9]. During bone remodeling, bone is first resorbed by osteoclasts and subsequently, osteoblasts arrive and rebuild the resorbed bone by synthesizing new bone matrix (figure 1.1).

Albeit that mechanical strength is of great importance for the skeleton, there is a certain hierarchy among factors that regulate bone mass. For example, calcium homeostasis overrides other functions of the skeleton. During hypocalcaemia due to

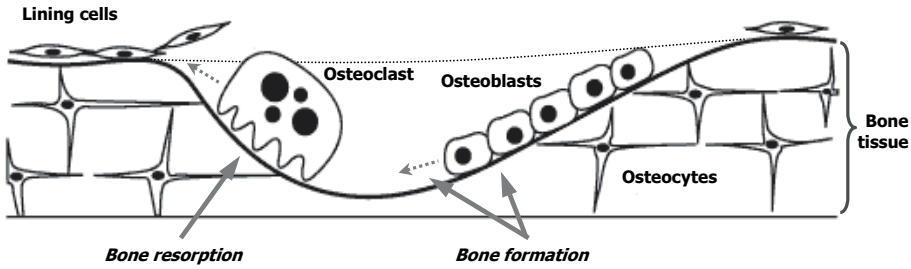

Figure 1.1. Bone remodeling. Four different bone cells reside in bone; 1] osteoclasts that resorb bone, 2] osteoblasts that produce new bone 3] osteocytes that sense mechanical strains, and 4] lining cells covering the bone tissue. During bone remodeling bone mass is controlled by the balance between osteoclastic bone resorption and osteoblastic bone formation. First osteoclasts resorb bone by acidification and secretion of a wide variety of proteases and, in turn, osteoblasts replace the resorbed bone by producing new bone matrix.

poor calcium diet, calcium deficiency, renal disease, or other pathology, osteoclast activity is increased to release calcium from the bone to restore calcium homeostasis. Other factors that initiate bone remodeling are mechanical loading of bone and bone damage [8]. During mechanical loading osteocytes respond to the mechanical stimuli, which results in bone remodeling at specific locations in the bone to obtain maximal bone strength [5, 10]. In case of bone damage, osteocytes go into apoptosis at the site of damage, which eventually initiates bone remodeling and subsequent bone repair [6].

Bone resorption and bone formation are coupled to each other, this means, if one goes up or down the other usually follows. This coupling between formation and resorption is controlled by several mechanisms, which are not yet completely understood. First, osteoblasts are able to stimulate osteoclast differentiation by production of osteoclast stimulating factors such as RANKL (receptor activator of NF-kB

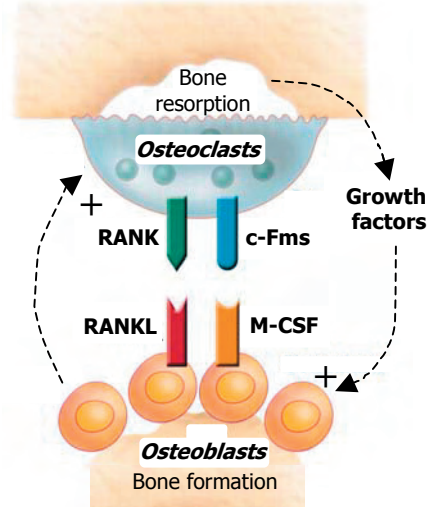

Figure 1.2. Coupling between bone formation and bone resorption. Osteoblast and osteoclast differentiation and function are coupled. Osteoblasts activate osteoclast differentiation by expressing RANKL and M-CSF, which bind to RANK and c-Fms receptors on the surface of osteoclasts. During bone resorption osteoclasts release various growth factors from the bone matrix, which in turn stimulate osteoblast differentiation. Picture adapted from Weitzmann *et al*. [14].

ligand) and M-CSF (macrophage colony-stimulating factor) [11]. RANKL is a key cytokine for osteoclast formation. Its discovery was followed by the identification of its soluble decoy receptor OPG (osteoprotegerin), which binds RANKL with high affinity [2, 12]. Besides that osteoclast activity is coupled to osteoblast activity, the activity of osteoblasts is also influenced by the activity of osteoclasts. During osteoclastic bone resorption multiple factors are released from the extracellular matrix, including IGF-I (insulin-growth factor I) and TGFβ (transforming growth factor β) [8, 13], which control the differentiation and activity of osteoblasts after their release (figure 1.2).

Although bone resorption and formation are coupled these processes are not always in balance. During growth the balance favors bone formation to reach peak bone mass. After adults reach their peak bone mass there is evidence of a reduction of bone formation, which eventually results in bone loss and structural bone damage during ageing [15, 16]. In case of hormonal imbalance the coupling between osteoblasts and osteoclasts can also be out of equilibrium (discussed in paragraph 1.9).

1.4 Bone quality & bone mineralization

The quality of bone is determined by its material composition and its structure. During loading, bone must be stiff and able to resist deformation, but on the other hand bone must be flexible to absorb energy by deformation [4]. The most abundant extracellular matrix (ECM) protein in bone is collagen type I which gives the bone its flexibility [17], whereas the mineral in bone gives the bone its mechanical strength. As a consequence variations in the amount of mineral and collagen affect the properties and quality of bone (*e.g.* stiffness and flexibility). When bone is too stiff (too much calcium) it will result in micro cracks and/or eventually complete fracture, when bone is too flexible (too little calcium) it is not sufficient for supportive function. An optimal mineral density is therefore essential. Mammalian bone is about 60 % (mass percentage) mineralized, whereas other bone-like structures responsible for other functions have a different mineral content [18]. For example, auditory ossicles contain 90 % mineral for efficient sound transmission, whereas deer antlers contain 40 % mineral needed to absorb the impact of head butting during fight [4]. Albeit that mineral is a crucial substance in bone, little is known about the optimal mineral density of bone and how bone becomes mineralized.

Osteoblasts as the bone forming cells are primarily responsible for the mineralization process in bone. First, osteoblasts produce a non-mineralized ECM (osteoid), which predominantly consists of collagen type I. Besides that collagen fibers provide structural strength and flexibility to the bone, collagen fibers also determine the structural organization for other ECM proteins and act as a template for mineral deposition [19]. Osteoblasts also produce a wide variety of so-called noncollagenous ECM proteins. This group of ECM proteins includes enzymes, growth factors, calcium binding proteins and several other proteins important for the structure of the ECM. Osteocalcin and osteopontin are well-known noncollagenous proteins localized in bone and are frequently used as markers of osteoblast differentiation. Moreover, in the clinic osteocalcin serum

levels are used as marker for bone turnover. But the exact roles of osteopontin and osteocalcin in the bone matrix are still unclear. The knowledge so far indicates that these proteins act as negative regulators of the mineralization process [20-25].

After osteoblasts have produced a complete and maturated ECM, hydroxyapatite mineral ($Ca_{10}[PO_4]_6[OH]_2$) is deposited in the ECM. Bone mineralization is a complicated process controlled by many factors including calcium and phosphate concentrations, enzyme activity, and the composition of the ECM. Initiation of mineralization requires the precipitation and attachment of hydroxyapatite crystals to the ECM [17]. One possible mechanism of hydroxyapatite crystal formation is via extracellular matrix vesicles (MVs). It has been demonstrated that osteoblasts produce MVs by polarized budding and pinching-off vesicles from specific regions of their outer plasma membranes. After MVs are released into the ECM they initiate formation of the first mineral crystals [17, 26, 27]. The osteoblast differentiation marker alkaline phosphatase is suggested to play a role in the mineralization process, where it hydrolyses organic phosphate substrates to release free inorganic phosphate [28]. This enzyme is highly expressed in MVs together with several phosphate and calcium transporters [29]. These transporters increase local calcium and phosphate concentrations and thereby initiate hydroxyapatite crystal formation [30, 31]. Although there is evidence for these MVs it might well be that mineralization in bone is initiated via other additional mechanisms as well [32, 33].

Mineralization of the ECM is not unique for bone tissue as demonstrated by ectopic calcification in pathological conditions [34]. In case of vascular calcification regions of the vascular wall are calcified [35]. There are important similarities and differences between bone mineralization and vascular mineralization. This led to increased interest to investigate how and why bone-like mineral deposits are formed in the arteries. Vascular smooth muscle cells are believed to initiate this calcification process in various types of vessel wall calcification. For unclear reasons these cells undergo an osteoblast-like differentiation process expressing osteoblastic factors including alkaline phosphatase and runt-related transcription factor 2 (RUNX2), causing mineralization of the ECM in the vessel wall [34, 36-39].

1.5 Mesenchymal stem cells

The cavity in bone is filled with bone marrow that retains both mesenchymal and hematopoietic stem cells. Both types of stem cells are able to self-renew and differentiate into various cell types, including osteoblasts and osteoclasts. Hematopoietic stem cells are the most abundant in the bone marrow and give rise to different kinds of blood cells. They can differentiate into myeloid (erythrocytes and megakaryocytes), lymphoid (T and B cells) and granulocyte/monocyte progenitors. Osteoclasts are also from hematopoietic origin and derived from the same lineage as monocytes.

Osteoblasts on the other hand are derived from mesenchymal origin. In addition to osteoblasts, mesenchymal stem cells (MSCs) give rise to a wide variety of other cell types such as chondrocytes, fibroblasts, adipocytes, and myoblasts [3, 40, 41] (figure 1.3). MSCs reside mainly in the bone marrow but is has been shown that they can be found in almost

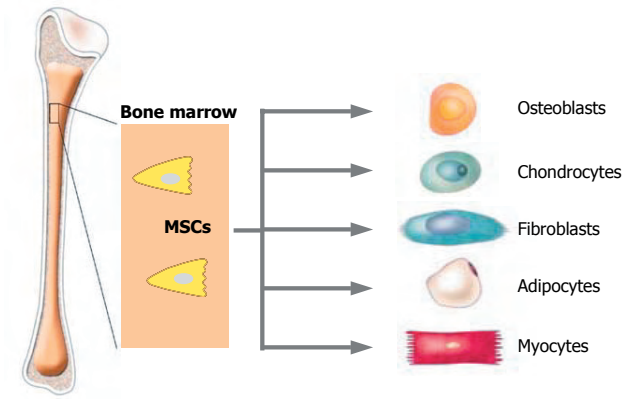

Figure 1.3. Mesenchymal stem cell differentiation. Bone marrow retains mesenchymal stem cells (MSCs), which give rise to a variety of cell types such as osteoblasts, chondrocytes, fibroblasts, adipocytes, and myoblasts. Picture adapted from Yin *et al*. [40].

all other tissues to maintain tissue homeostasis [41, 42]. In humans, fat tissue is nowadays also used as a source for MSC isolation. The use of MSCs in general has generated a great deal of interest because of their multipotency and relative easy accessibility. They have great potential as regenerative medicine and in tissue engineering [41, 43]. Some interesting examples of therapeutic use of mesenchymal stem cells have been reported, including cardiovascular repair, treatment of lung fibrosis, spinal cord injury and bone and cartilage repair [44]. However, albeit that MSCs can be studied and committed to different cell lineages *in vitro* [41], the molecular control of these processes are poorly understood. More knowledge is required about MSC commitment and differentiation in order to use MSCs in a robust and controlled manner in therapeutic applications.

1.6 Osteoblast differentiation

The life of an osteoblast consists of multiple differentiation steps (osteoblastogenesis, see figure 1.4). First commitment of mesenchymal stem cells to the osteoblast lineage will arise. These committed osteoblasts are called osteoprogenitor cells. They proliferate

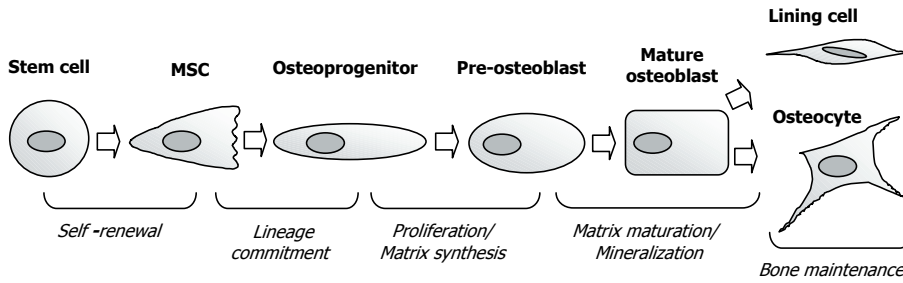

Figure 1.4. Osteoblast differentiation. Osteoblasts undergo a complex differentiation program. Each differentiation stage is controlled by different kinds of morphogens, hormones, growth factors, cytokines, and ECM proteins.

and will differentiate into pre-osteoblasts. Pre-osteoblasts in turn start to produce the ECM and differentiate into mature osteoblasts. Subsequently, these mature osteoblasts continue ECM synthesis and initiate mineralization of the ECM. Only a fraction of the mature osteoblasts will be incorporated into newly formed bone matrix and remains settled as an osteocyte. The remaining mature osteoblasts will die or become bone-lining cells [3].

It is believed that each of the differentiation steps during osteoblastogenesis is regulated by different kinds of morphogens, hormones, growth factors, cytokines and ECM proteins. These external signals initiate several signaling cascades and transcription factors that mediate and control osteoblastogenesis.

Steroid hormones including estrogens, androgens, progesterone, glucocorticoids and 1,25α(OH)$_2$D$_3$ (vitamin D$_3$) are well-known for their stimulatory effect on osteoblast differentiation and activity [3, 45, 46]. The most significant impact on osteoblastogenesis *in vitro* is accomplished by glucocorticoids. Glucocorticoid treatment is essential for the induction of osteoblastogenesis and initiation of matrix mineralization in rat and human osteoblast models [47-52]. The role of glucocorticoids in osteoblast differentiation is discussed in more detail in paragraph 1.8.

Members of the TGFβ superfamily such as TGFβ and bone morphogenic proteins (BMPs) are other well-known regulators of bone formation. Both TGFβ and BMPs promote bone development by stimulating osteoprogenitor cells [53, 54]. However, TGFβ also seems to act negatively on later phases of osteoblast differentiation by inhibiting mineralization [53, 55, 56].

The Wnt signaling pathway is only relatively recently known to be involved in bone metabolism [57]. Wnt signaling plays a widespread role in skeletogenesis from embryonic skeletal patterning, through fetal skeletal development, and bone remodeling in adults. Multiple skeletal defects have been described in mice deficient for various genes involved in the Wnt signaling pathway [57]. In humans, loss-of-function mutations in the Wnt co-receptor LDL receptor-related protein 5 (LRP5) are associated with the osteoporosis-pseudoglioma syndrome, which is characterized by low bone mass and skeletal fragility [58]. In contrast, gain-of-function mutations in LRP5 are associated with a high bone mass phenotype [59, 60]. Despite all these data, the functions of Wnt signaling in bone biology remain unclear. The Wnt-induced skeletal defects are at least accomplished by regulating osteoblast differentiation and activity. Several reports show that Wnt signaling contributes to osteoblast differentiation [61-63], whereas other reports show the ability of Wnt signaling to inhibit osteoblast differentiation [64-66]. These conflicting data are likely to reflect the diverse function of Wnt signaling at different stages of osteoblastogenesis. More data in different models will be required to understand the complex role of Wnt signaling in osteoblast differentiation.

Several transcription factors are known that control bone development and osteoblast differentiation. Two specific osteoblast transcription factors are RUNX2 [67-69] and SP7 (SP7 transcription factor, also known as osterix) [70]. RUNX2 and SP7 are crucial for skeletogenesis and osteoblast differentiation. They target osteoblast-related genes such as osteocalcin, bone sialoprotein, osteopontin and collagen type Iα1 [71]. In addition, several other non-osteoblast specific transcription factors have been identified that

control osteoblast differentiation, including TWIST1 (twist homolog 1), ZBTB16 (zinc finger and BTB domain containing 16), DLX5 (distal-less homeobox 5) and MSX2 (msh homeobox homolog 2) [3, 8, 72-74].

1.7 Activin signaling

Activins are members of the TFGβ superfamily, that besides activins, includes inhibins, BMPs, TGFβs, growth and differentiation factors (GDFs), Nodel, Myostatin, anti-Müllerian hormone (AHM) and several other growth and differentiation factors [75, 76]. Activins and inhibins were initially purified from gonadal fluids and characterized on basis of their ability to modulate follicle stimulating hormone (FSH) secretion from pituitary gonadotropes [76, 77]. Nowadays, however, it is known that activins can affect the function of several other cell types and tissues, like the adrenal gland, liver, neurons, pancreas and bone [78-82]. They control processes such as proliferation, differentiation, immune responses, wound repair, tumor development and various endocrine activities [76, 83].

The inhibin subunits βA, βB, and α (INHBA, INHBB and INHA) are produced as precursors, which after cleavage dimerize to form inhibin or activin proteins. Like in all other members of the TGFβ superfamily covalent disulfide bond links the two subunits. Homo- or heterodimerization of only β-subunits results in the formation of activin A (βAβA), activin AB (βAβB), or activin B (βBβB) [76]. Heterodimerization of an α-subunit with a β-subunit results in the formation of inhibin A (αβA) or B (αβB) (see figure 1.5). Additional β-subunits have been identified in humans (INHBC and INHBE) but their biological roles remain unclear.

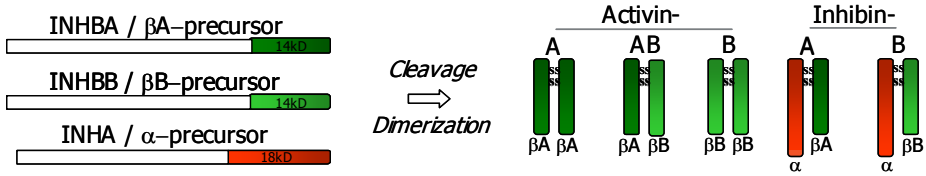

Figure 1.5. Structure of activins and inhibins. Homo- and heterodimerization of only β-subunits results in the formation of activins. Heterodimerization of a α-subunit together with a β-subunit results in formation of inhibins.

TGFβ family members elicit their cellular response via formation of heteromeric complexes of specific type I and II serine/threonine kinase receptors which are located at the cell surface of target cells. Upon ligand binding and type I and II receptor heterodimerization the type I receptors activate Smad proteins through phosphorylation. Phosphorylated Smad complexes in their turn accumulate in the nucleus where they bind to DNA and regulate gene transcription of target genes [75, 84].

Five type II and seven type I receptors have so far been identified. Activins use the type II receptors ACVR2A or ACVR2B (also known as ActRII or ActRIIB) and the type I receptor ACVRIB (also known as ALK4) which results in phosphorylation of Smad2 and 3. Interestingly, TGFβs have their own type I and II receptors but share Smad2 and 3 with activins for signaling to the nucleus. BMPs on the other hand signal via distinct Smads

(Smad 1,5 and 8) but bind to the same type II receptors as activin. This illustrates the complexity of the system and indicates that so far only little is known about the signaling of these family members and ligand specificity.

The activity of activins is inhibited by the extracellular action of inhibins and follistatin [85]. Inhibins need the presence of a type III TGFβ receptor, betaglycan, to inhibit activin signaling via competitive binding to the activin type II receptor for activin [86, 87]. Follistatin is a soluble extracellular protein that functions as an activin binding protein. It binds activins with high affinity preventing activins to interact with their cell-surface receptors and thereby inhibits activin signaling [88]. The binding affinity of follistatin is ten time higher for activin A than for activin B. Follistatin is found in the circulation but is also expressed and secreted by several activin target cells [76].

Several studies have now demonstrated that activins are involved in bone metabolism. In the extracellular matrix of bovine bone large quantities of activin are found [89] and activin A seems to promote bone formation and fracture healing in rodents [89-91]. At the cellular level it has been shown that activin A enhances osteoclast-like cell formation as well as osteoblastogenesis in murine bone marrow cultures [92, 93]. However, other reports demonstrated an inhibitory effect of activin on osteoblast differentiation in rat and murine osteoblasts [94, 95].

1.8 Glucocorticoid signaling in bone

Glucocorticoids are named after their role in maintaining glucose homeostasis. Glucocorticoids regulate numerous processes in the human body such as mediation of the stress response [96], regulation of lipid and glucose metabolism [97], immunosuppressive and anti-inflammatory actions [98], and cellular differentiation [48, 99, 100]. The active glucocorticoid in humans is cortisol, which is produced in the adrenal gland [101]. After cortisol is released in the circulation about 80 to 90 % binds with high affinity to cortisol-binding globulin (CBG) [102], 6 to 15 % binds with low affinity to albumin, whereas 4-5 % is unbound. The albumin-bound and free levels are considered as biological active [103]. The primary role of CBG is to regulate the bioavailability and metabolic clearance of cortisol [102]. However, CBG might also contribute to the localization of cortisol. Localization of CBG in certain tissues may serve to locally enhance or inhibit free cortisol action. To enhance cortisol action at the tissue level it has been shown that cortisol can be released by local cleavage of CBG or by cellular uptake of the CBG-cortisol complex [102].

At the cellular level glucocorticoids mediate signaling via binding to the glucocorticoid receptor (GR) [104]. The GR belongs to the superfamily of nuclear steroid receptors which also includes the thyroid-hormone receptor, estrogen receptor, androgen receptor and vitamin D receptor [105]. GR is expressed in almost all cell-types including osteoblasts [106], where it regulates gene expression by binding to glucocorticoid responsive elements (GREs) in the regulatory regions of glucocorticoid target genes [104] such as osteocalcin, collagen Iα1 and TGFβ1 [107-109].

In vitro, glucocorticoids (GCs) such as cortisol are crucial initiators of human osteoblast differentiation [47-52]. Despite clear stimulation of bone formation by GCs

in vitro, GCs are still regarded as negative regulators of bone formation. This is mainly based on clinical use of GCs, which frequently results in bone loss and increased risk of fractures (GC-induced osteoporosis) [110] (see further below in section 1.9). The exact mechanism of GCs in regulating bone formation is unclear. It might depend on the duration and concentration of GC treatment and on the differentiation stage of both osteoblasts and osteoclasts [111-114].

In glucocorticoid target tissues glucocorticoid signaling is regulated at the pre-receptor level by 11β-hydroxysteroid dehydrogenases (11β-HSDs) (figure 1.6). Two distinct 11β-HSDs have been described in humans. 11β -HSD type 2 acts as an inactivating enzyme converting cortisol into biologically inactive cortisone. This enzyme is found primarily in mineralocorticoid target tissues in which it protects the nonselective mineralocorticoid receptor against activation by glucocorticoids [115-117]. 11β-HSD type 1 on the other hand is found in almost all glucocorticoid target tissues and acts as an activating enzyme converting cortisone into biologically active cortisol [118, 119]. The principle source of circulating cortisone is the kidney which expresses high levels of 11β-HSD type 2 [120].

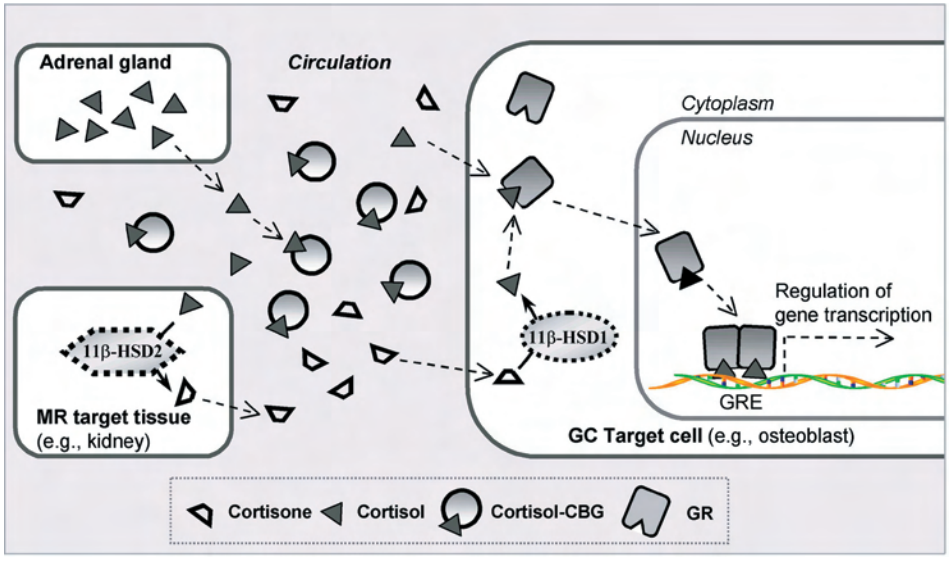

Figure 1.6. Simplified scheme of glucocorticoid signaling. Cortisol is produced by the adrenal gland. After release in the circulation, the majority of cortisol is bound by cortisol binding globulin (CBG). Free cortisol can enter target cells where it binds to the glucocorticoid receptor (GR). After binding the GR is translocated into the nucleus where it binds to glucocorticoid responsive elements (GRE) to regulate gene transcription. Cortisol is regulated at the pre-receptor level by 11β-HSDs. 11β-HSD type 2 acts as an inactivating enzyme converting cortisol into biologically inactive cortisone to protect the nonselective mineralocorticoid receptor (MR) from activation by glucocorticoids. 11β-HSD1 acts as an activating enzyme converting inactive cortisone into cortisol.

In bone cortisol action is also controlled by action of 11β-HSDs. 11β -HSD type 1 is the most prominent isozyme in bone, whereas 11β -HSD type 2 is barely detectable [121, 122]. This indicates that in bone tissue cortisone is converted to active cortisol resulting in enhanced glucocorticoid signaling in bone.

1.9 Osteoporosis

Osteoporosis is the most common bone disease and can be characterized by low bone mass, micro architectural deterioration and decreased bone quality, which results in bone fragility and increased risk for fractures. Osteoporosis is an important public health issue since 50 % of women and 20 % of men older then 50 years will have a fragility fracture in their remaining lifetime [123]. In elderly people fractures have a great impact on the quality of life. Hip fractures are the most devastating; they require the patient to be admitted to hospital and cause serious disability and excess mortality. The costs and other economic implications of treatment and rehabilitation of osteoporosis related fractures are high. The combined annual cost of all osteoporotic fractures in Europe only are 25 billion euros [124]. Osteoporosis is an ageing related disease and since elderly people represent worldwide the fastest growing age group osteoporosis will impose a major economic burden on healthcare worldwide.

Estrogen deficiency is a well-known cause of osteoporosis. Estrogens play a fundamental role in skeletal growth and homeostasis. Postmenopausal women who suffer from estrogen deficiency have frequently imbalanced bone remodeling. The absence of estrogens results in increased osteoclast activity resulting in significant bone loss and increased risk for fractures [14].

Another well-known cause of osteoporosis is caused by long-term glucocorticoid treatment. Glucocorticoids (*e.g.*, prednisone) are one of the most frequently used drugs and are prescribed to control acute and chronic inflammation, auto immune disease, for treatment of leukemia and lymphomas and after organ transplantation. However, therapeutic use of glucocorticoids has negative side effects on bone remodeling and frequently results in glucocorticoid-induced osteoporosis (GIO). GIO occurs in two phases: first a rapid early phase in which bone mineral density is reduced as a result of excessive bone resorption. Secondly a slower progressive phase exists in which bone mineral density declines because of impaired bone formation [125, 126]. Although the adverse effects of therapeutic glucocorticoid use on bone are known for a very long time little is known about the mechanism behind GIO. It is believed that GIO is at least in part induced by increased osteoblast and osteocyte apoptosis and stimulation of osteoclast formation [127-129]. This negative effect is in marked contrast to the essential role of glucocorticoids for osteoblastogenesis as mentioned above. It should be considered, however, that the negative effect on bone is caused by high pharmacological levels of glucocorticoids for longer periods of time and does not reflect the normal physiological role of glucocorticoids in bone.

1.10 Treatment of osteoporosis

The majority of bone diseases are caused by increased bone resorption, rendering its inhibition a primary therapeutic objective [130]. Inhibition of bone resorption can be accomplished by inhibiting osteoclast differentiation and/or osteoclast activity. Examples of drugs that can inhibit bone resorption are: estrogens and selective estrogen

receptor modulators (SERMs), calcitonin and bisphosphonates. Estrogens and SERMs inhibit osteoclast differentiation, whereas calcitonin and bisphosphonates inhibit the activity of osteoclasts. Bisphosphonates are, to date, the most effective drugs that inhibit bone resorption. Bisphosphonates are chemically stable analogues of inorganic pyrophosphate. They concentrate in bone and are absorbed by osteoclasts, where they inhibit osteoclast activity and survival. Consequently, this results in reduced bone resorption, lower bone turnover, and a positive bone balance. Since bisphosphonates inhibit osteoclast activity regardless of cause, they are used to treat several forms of bone loss including postmenopausal and glucocorticoid-induced osteoporosis, and cancer-induced bone loss [130]. Currently, several novel drugs are in development that inhibit bone resorption. A promising one is based on inhibition of RANKL, a key cytokine in stimulating osteoclast differentiation [131, 132].

In contrast to the drugs that target bone resorption only a few therapies are known that promote bone formation *in vivo*. Parathyroid hormone (PTH) is an example of a bone-stimulating hormone. The anabolic activity of PTH on bone has been demonstrated in several clinical studies, which showed that intermittent PTH treatment enhances bone mass and reduces the risk of fractures [130, 133-136]. In contrast, continuous PTH treatment decreases bone mass by an indirect stimulation of bone resorption [136]. A relatively novel drug that seems to affect both bone resorption and bone formation is strontium ranelate. Although its mechanism of action is poorly understood it seems to inhibit bone resorption and stimulate bone formation *in vitro* and *in vivo*, resulting in enhanced bone quality and reduced risk for fractures [137].

1.11 Aim of this thesis

In the treatment of bone diseases only little attention has been paid to the development of compounds that promote bone formation. Inhibitors of bone resorption only prevent additional bone loss in the patient but cannot restore the initial loss in bone mass. Additional therapies that promote bone formation are therefore highly desirable and will be a valuable adjunct therapy in patients receiving inhibitors of bone resorption.

To develop novel drugs that enhance bone formation, it is crucial to understand the biology of osteoblasts. In this thesis the control of human osteoblast differentiation and function (mineralization of the ECM) was studied in detail. This was achieved by culturing osteoblasts that under the proper stimuli produce a mineralized extracellular matrix. Importantly, we only used cells from human origin, which in the end will make the translation from *in vitro* finding to *in vivo* significance easier, especially since several discrepancies have been described in the control of osteoblasts derived from humans and osteoblasts derived from other species.

In general, the following approaches were used to gain more insight into the biology of osteoblasts; 1] the impact of osteoblast regulatory factors on osteoblast differentiation and bone formation was studied, 2] the endogenous control of these regulatory factors during osteoblast differentiation was investigated, 3] genome-wide gene expression arrays were used, to map the genetic control of osteoblast differentiation. Together

these approaches should generate more knowledge about bone biology, which might guide us to the development of novel therapies that stimulate bone formation. The studies presented in this thesis primarily focused on the role of glucocorticoid, activin and Wnt signaling pathways in osteoblasts using the 3 approached described above.

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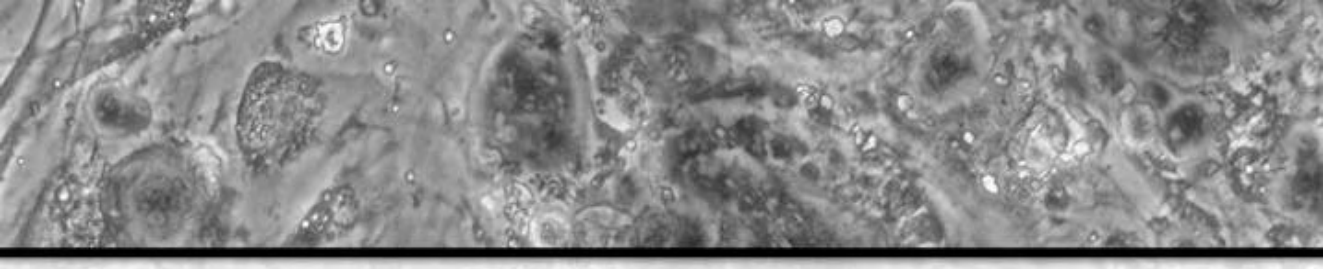

Chapter 2

The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization

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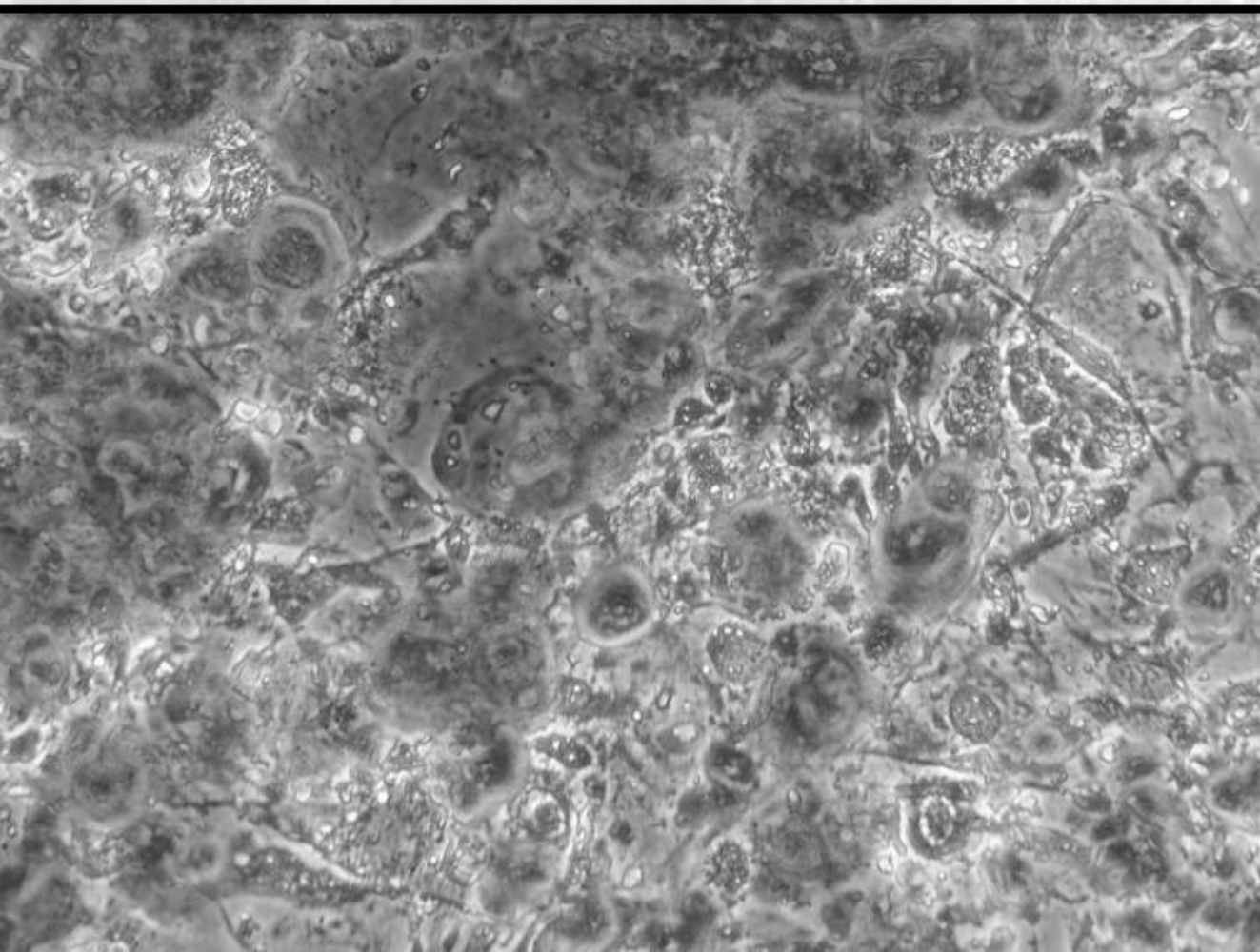

Abstract

Glucocorticoids (GCs) exert profound effects on bone and are essential for human osteoblast differentiation. However, GCs are still interpreted as negative regulators of bone formation, mainly caused by the detrimental effects on bone after clinical use of GCs. In this paper we emphasize the importance of GCs for proper human osteoblast differentiation and matrix mineralization. We show that human osteoblast differentiation needs to be triggered by GCs in a specific time-window during the early stages of development. Exposure to GCs in the beginning of osteoblast development induces a dose dependent increase in alkaline phosphatase activity and matrix mineralization. GC-induced differentiation stimulated expression of genes involved in bone formation and suppressed genes that negatively regulate bone formation and mineralization. Furthermore, we highlight the importance of local cortisol activation in osteoblasts by expression of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1)

2.1. Introduction

Osteoblast differentiation is a complex process regulated by many endocrine, paracrine and autocrine factors. In human and rat bone marrow stromal cells GCs are crucial for the induction of osteoblast differentiation and formation of a mineralized extracellular matrix [1-6]. Although the exact role of GCs in regulating bone formation is unclear, it might be dependent on the duration and concentration of GC treatment and on the differentiation stage of both osteoblasts and osteoclasts [7-10].

At a molecular level GC signaling is mediated via the GC receptor (GRα). GRα is expressed in almost all cell-types including osteoblasts [11] where it regulates gene expression by binding to GC responsive elements in the regulatory regions of several target genes, including osteocalcin, collagen Iα1 and transforming growth factor-β1 [12-14].

Despite clear stimulation of bone formation by GCs *in vitro*, GCs are still interpreted as negative regulators of bone formation. This idea is mainly caused by the clinical use of GCs. GC therapy frequently results in bone loss and increased risk for fractures [15-17]. This negative effect on bone is caused by high levels of GCs for longer periods and might not reflect the normal *in vivo* role of GCs in bone. Furthermore, most studies on osteoblast differentiation are performed using mouse osteoblasts that lack the need for GC treatment for the induction of differentiation [18, 19], which is in contrast to the human situation.

Therefore we emphasize in this paper the importance of GCs for proper human osteoblast differentiation and matrix mineralization. Moreover, we want to highlight the significance of pre-receptor regulation of GCs in osteoblasts by expression of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1). 11β-HSD1 is found in almost all GC target tissues including osteoblasts [20, 21] and primarily displays reductase activity converting relative high levels of free cortisone into the biologically active cortisol. As a consequence 11β-HSD1 activity in osteoblasts provides an efficient mechanism for the local activation of GCs in bone and is an important autocrine determinant of osteoblast proliferation, differentiation and function [22-24].

2.2. Results and discussion

The essential role of glucocorticoids for proper osteoblast function

To demonstrate the importance of GCs for human osteoblast differentiation in more detail we used the pre-osteoblast model SV-HFO [25], which is a well-characterized osteoblast cell-line showing a controlled GC-induced differentiation process in a 3-week period. During this differentiation process an extracellular matrix (ECM) is formed and the process of mineralization is initiated around day 14 (Fig. 2.1 A). Moreover, GC-induced differentiation leads to increased sensitivity to mechanical loading [26]. GC-induced differentiation was dose dependently increased by DEX as demonstrated by increased alkaline phosphatase (ALP) activity (Fig. 2.1 B) and mineralization. Matrix mineralization was induced at a minimal dose of 10 nM (data not shown). When we used mesenchymal stem cells derived from bone marrow GC treatment was as important for the induction of osteogenesis and proper matrix formation/mineralization (data not

shown), which is also demonstrated in several other studies [18, 17].

Studies using human osteosarcoma cells and mouse osteoblasts show that GC treatment inhibits osteoblast cell number and proliferation [29-32]. In contrast, other experiments show increased cell number and proliferation after GC treatment [33, 1, 5], which is supported by our observation of increased proliferation and cell number after GC-induced differentiation in human osteoblasts. Furthermore the number of apoptotic cells was not changed. This is shown in Fig. 2.1 C where we show increased cell number measured by the cultures DNA content, higher proliferation measured by thymidine incorporation and rate of apoptosis measured by annexin V staining.

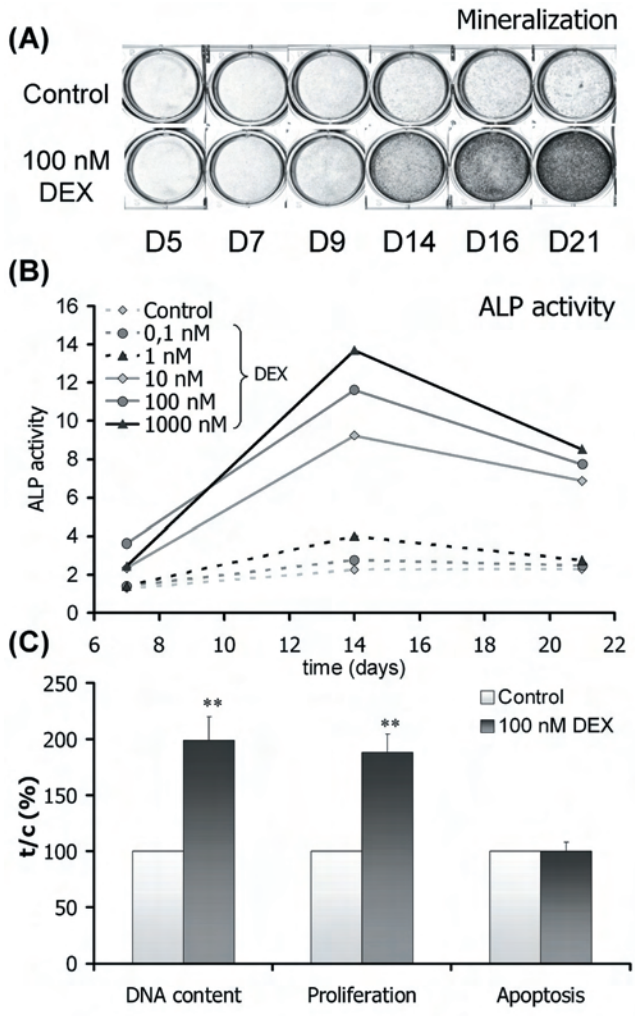

Fig. 2.1. (A) DEX-induced matrix mineralization by SV-HFO osteoblasts. Cells were cultured in the presence and absence of 100 nM DEX. Cultures were stained for calcium deposition using Alizarin Red S. **(B)** Dose dependent increase of ALP activity (nmol/µgDNA/min) by DEX at days 7, 14 and 21 of SV-HFO culture, data shown represent a typical SV-HFO culture. **(C)** Effects of DEX on cultures DNA content (day 14), proliferation (day 6) and apoptosis (day 14).Values are means ± SEM **p < 0.01 compared to control.

Osteoblasts need to be directed by GCs at the early stages of development

Data clearly show that osteoblasts need to be directed by GCs to differentiate and mineralize. Moreover, osteoblasts need to be triggered by GCs in a specific time-window during the early stages of development. This is demonstrated in Fig. 2.2 where we treated osteoblasts with DEX in different time periods followed by quantification of mineral deposition at day 21. Continuous DEX treatment resulted in strong mineralized cultures as shown in Fig. 2.1 A and in the upper line in Fig. 2.2. Interestingly, shortening the treatment until days 16 and 12 also resulted in strongly mineralized cultures. When the treatment was further shortened (to day 9 or 7) matrix mineralization was decreased but still occurred. In contrast, cultures that were only treated with DEX starting at day 12 or later failed to initiate mineralization. In summary, osteoblasts are directed by GCs at the beginning of development to differentiate, which eventually leads to matrix mineralization. Once the cells have entered the osteoblast differentiation pathway the mineralization process seems to be independent of GCs.

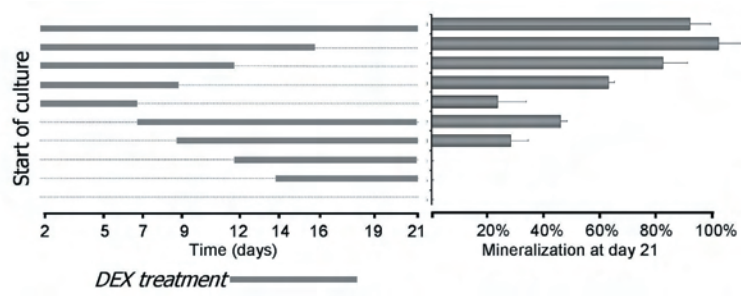

Fig. 2.2. Time dependent induction of mineralization by DEX. SV-HFO cultures were treated with DEX for different time periods indicated by the horizontal bold gray lines. Mineralization was measured at day 21.

GC-induced differentiation results in differential expression of genes involved in osteoblast differentiation and bone formation

Besides clear phenotypic characteristics like matrix mineralization, GC-induced differentiation regulates a wide variety of genes that control proper osteoblast differentiation and matrix mineralization. Here we show several examples of genes that are regulated after GC-induced differentiation. In these experiments we analyzed gene expression (mRNA) in the first, second and third week of culture in both differentiated (DEX-treated) and non-differentiated cultures (non-treated control). GC-induced differentiation resulted in up-regulation of osteoblast markers like Runx2, collagen Iα1 and ALP (Fig. 2.3 A–C). In contrast, the non-collagenuous ECM protein osteopontin (OPN) was decreased by GC-induced differentiation together with the collagenase MMP1 (matrix metalloproteinase-1), which showed a strong suppression by DEX (40-fold reduction) (Fig. 2.3 E and F). MMPs are extracellular proteins involved in ECM degradation and tissue turnover [34, 35]. MMPs in turn are modulated by tissue inhibitors of metalloproteinase (TIMPs). Interestingly, GC-induced differentiation resulted in a strong (30-fold) induction of TIMP4 (Fig. 2.3 D). TIMPs are the major endogenous inhibitors of the MMPs and four TIMPs have been identified, although their specific inhibitory activities

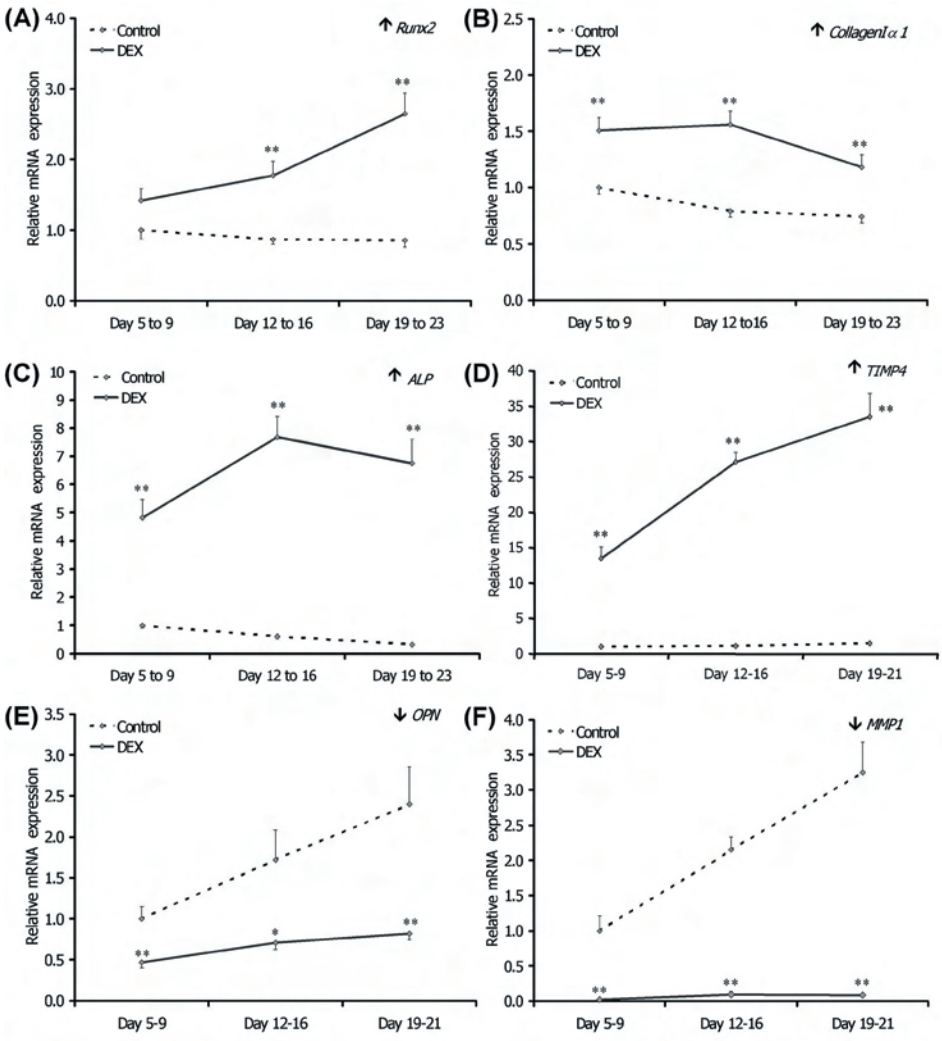

Fig. 2.3. Q-PCR data (mRNA expression) from non-differentiating (control) and differentiating (DEX treated) SV-HFO cultures at weeks 1–3. **(A)** Runx2/Cbfa1, **(B)** collagen Iα1, **(C)** alkaline phosphatase, **(D)** TIMP4, **(E)** osteopontin and **(F)** MMP1. Values are means ± SEM *p < 0.05; **p < 0.01 compared to control.

towards the MMPs are not specified [35, 36, 37]. Together, this indicates that during osteoblast differentiation and bone formation when matrix synthesis is needed, matrix degradation is suppressed by decreasing MMP1 expression and increasing TIMP4 expression.

A more complex regulation of gene expression by DEX is shown for osteocalcin (OCN). OCN is the most abundant noncollagenuous protein in bone matrix, which gene contains a Vitamin D responsive element (VDRE) [38] resulting in increased levels of OCN mRNA and protein after 1α,25-dihydroxyvitamin D_3 (vitD) treatment (10 nM, Fig. 2.4). At basal unstimulated conditions DEX did not influence OCN expression, however, vitD-induced OCN expression was significantly reduced by DEX (Fig. 2.4) (day 14 of culture).

Both OCN and OPN are markers of osteoblast differentiation and OCN is used as a

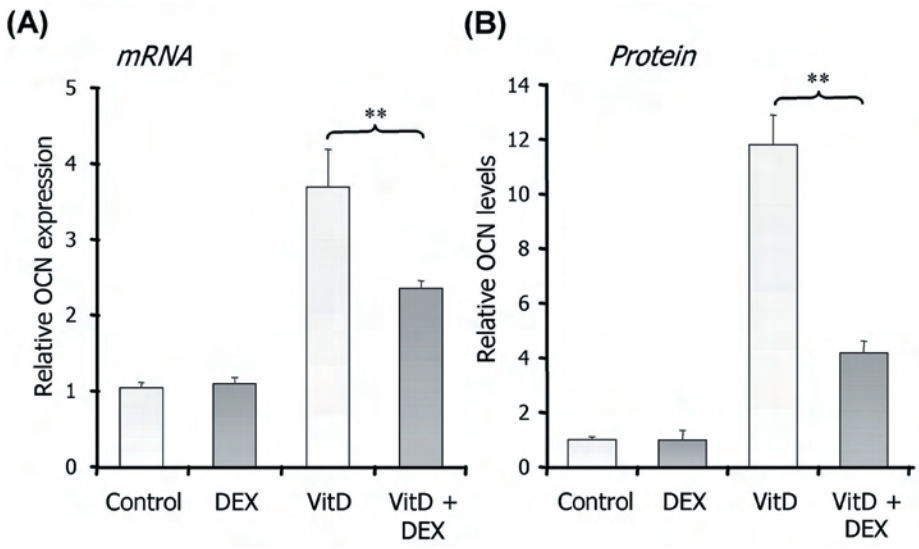

Fig. 2.4. DEX inhibition of vitD-induced osteocalcin (OCN) expression. **(A)** OCN mRNA expression after DEX treatment in basal and vitD stimulated cultures (day 14). **(B)** OCN protein levels in the cultures supernatant after DEX treatment in basal and vitD stimulated cultures (day 14). Values are means ± SEM **p < 0.01 compared to vitD stimulated cultures.

serum marker for bone turnover. Nevertheless, these genes are down regulated by GC-induced differentiation. At first sight this seems to contradict a positive role of GCs in osteoblast differentiation. However, despite their use as markers of bone formation, the exact role of OPN and OCN in the bone matrix is not fully understood. In contrast, they seem to act as negative regulators of the mineralization process. OPN- and OCN-deficient mice show increased amounts of mineral in their bones [39-41] and *in vitro* mineralization studies show that they directly inhibit hydroxyapatite crystal formation [42-44].

In summary, during GC-induced osteoblast differentiation genes are either up-regulated or down-regulated to stimulate osteoblast differentiation and proper matrix development. The genes discussed here are only a small set of the genes that account for osteoblast differentiation and matrix mineralization. In the literature also other genes are described that are regulated by DEX treatment in osteoblasts like, TGFβ1, bone sialoprotein, osterix and others [45, 46]. GC-induced osteoblast differentiation probably results in differential expression of a large set of genes influencing pathways and processes involved in osteoblast differentiation and bone formation.

Osteoblasts produce cortisol via 11β-HSD1 expression

The importance of GCs for proper osteoblast differentiation and bone forming function is emphasized by osteoblastic expression of 11β-HSD1. Expression of 11β-HSD1 in osteoblasts provides an efficient mechanism for the local activation of GCs in bone. In a previous study we showed that 11β-HSD1 is regulated in a differentiation dependent manner in osteoblasts [24]. This differentiation dependent regulation is summarized in Fig. 2.5 A, which shows that non-differentiated osteoblasts have low ALP activity but have strong increased levels of 11β-HSD1 to compensate for the lack of cortisol. Their differentiating

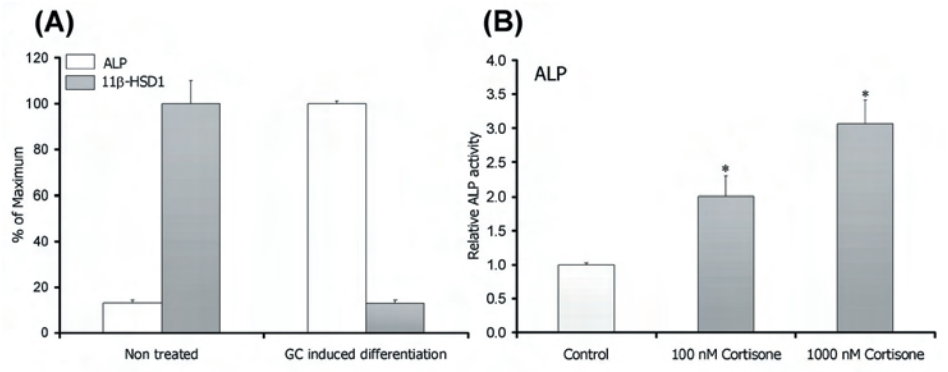

Fig. 2.5. Significance of 11β-HSD1 expression in human osteoblasts. **(A)** ALP activity (nmol/μgDNA/min) and 11β-HSD1 activity (cortisone to cortisol conversion per μg DNA) in non-differentiating and differentiating osteoblasts at day 21 of culture. **(B)** SV-HFO cultures continuously incubated with 100 and 1000nM cortisone after which ALP activity was measured at day 30 of culture. Values are means ± SEM **p < 0.01 compared to control.

counterparts have high ALP activity but low 11β-HSD1. Fig. 2.5 B summarizes our previous results demonstrating the significance of 11β-HSD1 for osteoblast differentiation. High 11β-HSD1 activity induced ALP activity by converting cortisone into biologically active levels of cortisol. This process eventually leads to matrix mineralization [24]. Pre-receptor hormone regulation is not unique for GCs. Local levels of androgens and estrogens are also regulated by enzymes present in target tissues [47]. Our previous work also showed that human osteoblasts express all the enzymes involved in the production of estradiol, *e.g.*, aromatase, sulfatase and 17β-HSD isoenzyme [48].

Conclusion

The major aim of this study was to underscore the significance of GCs for proper human osteoblast differentiation. GCs are essential for human osteoblast differentiation, which is in marked contrast to the widely studied murine osteoblast differentiation. The human GC-induced differentiation process is summarized in Fig. 2.6 A. In the beginning of osteoblast development precursor cells need to be directed by GCs to differentiate into bone forming osteoblasts. During this process several genes are regulated which either stimulate or inhibit bone formation (Fig. 2.6 B). The importance of GCs on osteoblast differentiation is emphasized by the increasing conversion of inactive cortisone into active cortisol via 11β-HSD1 when cells are refrained from GCs. This process might then act as a feed-back mechanism by the cells for delayed differentiation.

The detrimental effects on bone after clinical use of GCs is in this respect unclear but might be caused by various potential mechanisms. It might be duration (*i.e.* long term) or concentration dependent. However, in our studies we examined a range of concentrations and treated the cells continuously. GC effect might be dependent on osteoblast differentiation stage and act inhibitory at very late stages of osteoblast differentiation and osteocytes [49, 50]. Alternatively, the predominant negative effect of GCs could be mediated by the osteoclasts instead of the osteoblasts. Alternatively, *in vivo* interaction of GCs with other hormones/factors might be important. Moreover several secondary effects (*e.g.*, at the intestine, kidney, parathyroid glands) after GC

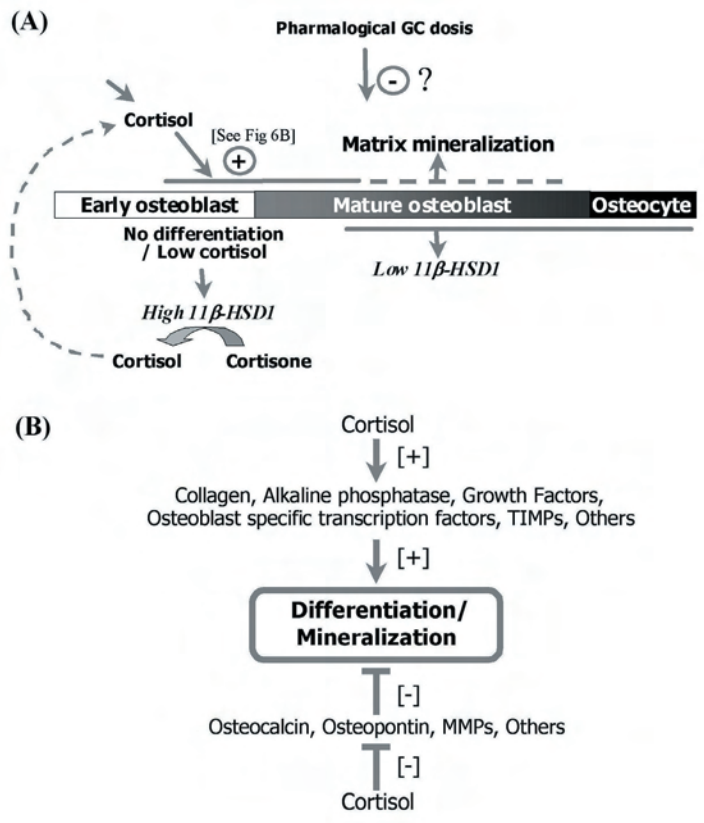

Fig. 2.6. (A) Schematic diagram depicting the role of GCs and 11β-HSD1 on osteoblast differentiation. **(B)** Regulation of gene expression by GCs on genes having either a positive or negative effect on bone formation.

treatment

could impair proper bone formation and might not reflect the normal *in vivo* role of GCs in bone. To address these issues and to pinpoint the mechanism of the negative effect of GCs on bone extensive additional research is needed. Nevertheless the current study in conjunction with other data in literature unequivocally demonstrates the significance of GCs for proper osteoblast differentiation.

2.3. Materials and methods

Cell culture

SV-HFO cells were cultured in αMEM (GIBCO, Paisley, UK) supplemented with 20mM HEPES, pH 7.5 (Sigma, St. Louis, MO); streptomycin/penicillin; 1.8mM $CaCl_2$ (Sigma); and heat-inactivated FCS (GIBCO) at 37°C and 5 % CO_2 in a humidified atmosphere. Thawed cells were precultured for 1 week in the presence of 10 % FCS. In this preculture, cells were seeded in a density of 5×10^3 vital cells/cm^2 and were subcultured every week. During this preculture, SV-HFO cells remained in an undifferentiated stage. After preculturing,

cells were seeded in a density of 10×10^3 vital cells/cm^2 in the presence of 2 % charcoal-treated FCS supplemented with 10 mM β-glycerophosphate (Sigma). Medium freshly supplemented with hormones was replaced every 2–3 days. Dexamethasone was purchased from Sigma and $1\alpha,25\text{-}(OH)_2D_3$ was generously provided by Dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark). For analysis, medium was collected and stored at −20°C and cells were scraped from the culture dish in PBS containing 0.1 % Triton X-100 and stored at −80°C. Cell lysates were sonicated on ice in a sonifier cell disrupter for 2×15 sec. before analysis.

DNA content

For DNA measurements, 100 µl SV-HFO cell lysates were treated with 200 µl heparin (8 IU/ml in PBS) and 100 µl ribonuclease A (50 µg/ml in PBS) for 30 min at 37ºC. This was followed by adding 100 µl ethidium bromide solution (25 µg/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA) using an extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma) was used.

Alkaline phosphatase (ALP) activity

ALP activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20mM in 1M diethanolamine buffer supplemented with 1mM $MgCl_2$ at pH 9.8) in the SV-HFO cell lysates for 10 min at 37ºC. The reaction was stopped by adding 0.06M NaOH. Absorption was measured at 405 nm. Results were adjusted for DNA content of the cell lysates.

Mineralization

For quantification of the mineral content cell lysates were incubated overnight in 0.24M HCl at 4°C. Calcium content was colorimetrically determined with a calcium assay kit (Sigma) according to the manufacturer's description. Results were adjusted for DNA content of the cell lysates. For Alizarin Red staining cell cultures were fixed for 60 min with 70 % ethanol on ice. After fixation, cells were washed twice with PBS and stained for 10 min with Alizarin Red solution (saturated Alizarin Red in demineralized water adjusted to pH 4.2 using 0.5 % ammonium hydroxide).

Apoptosis assay

Apoptosis was measured through the binding of annexin V and uptake of propidium iodide (PI) by flow cytometry using a Apoptest-FITC kit (Nexins research, Kattendijke, The Netherlands). For analysis 10,000 osteoblasts were counted using a FACScalibur (Becton Dickinson). Percentage total apoptotic cells was calculated by counting vital (unlabeled), apoptotic (annex in V stained), necrotic (PI stained) and late apoptotic cells (double stained).

Proliferation assay

Proliferation was examined by [^{3}H]-thymidine uptake measurements. SV-HFO cells were seeded into 96-well plates, in differentiating (100 nM DEX) and non-differentiating

conditions. After culture for 6 days [^{3}H]-thymidine (Amersham, UK) was added to a concentration of 1 µCi/ml and incubated for 7 h. Cells were then harvested onto glass fibre mats (TomtecHarvester96; Hamden, CT) and radioactivity measured with a Wallac MicroBeta scintillation counter.

Osteocalcin production

Osteocalcin was determined in the cultures medium. Osteocalcin was assayed by radioimmunoassay by incubating overnight with ^{125}Iodide labeled human osteocalcin and anti-human osteocalcin, kindly provided by Dr. C.M. Gundberg, Department of Orthopaedics and Rehabilitation, School of Medicine, New Haven, Connecticut, USA. Results were adjusted for DNA content of the cell lysates.

11β-HSD1 activity

Cortisone to cortisol conversion in SV-HFO cells was measured as described previously [24].

Quantification of mRNA expression

Total RNA was isolated using RNA-Bee solution (Tel-Test, Friendwood, TX) according to the manufacturer's protocol. To remove calcium (derived from extracellular matrix), RNA was precipitated by overnight incubation with 4M LiCl and 50mM EDTA at −20°C. After precipitation and centrifugation for 30 min at 14,000 rpm and 4°C, the RNA pellet was washed four times with 70 % ethanol and dissolved in H_2O. The total amount of RNA was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). One microgram total RNA was reverse transcribed into cDNA using a cDNA synthesis kit and according to the protocol of the manufacturer (MBI Fermentas, St. Leon-Rot, Germany), using 0.5 µg oligo(dT)18 and 0.2 µg random hexamer primers. Quantitative real-time PCR was carried out using an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in 25µl volumes using a qPCR core kit (Eurogentec, Seraing, Belgium). Reaction mixes contained 20 ng cDNA, 5mM $MgCl_2$, 200 /M dNTPs, and 0.025 U/µl Hot GoldStar enzyme. Primer and probe sets were designed, using the Primer Express software (version 1.5; Applied Biosystems), amplicons overlapped at least one exon boundary. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistics

Data presented are the results of at least two independent experiments performed in triplicate. Values are the means ± SEM Significance was calculated using the Student's *t*-test.

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Chapter 3

11ß-Hydroxysteroid dehydrogenase expression and glucocorticoid synthesis are directed by a molecular switch during osteoblast differentiation

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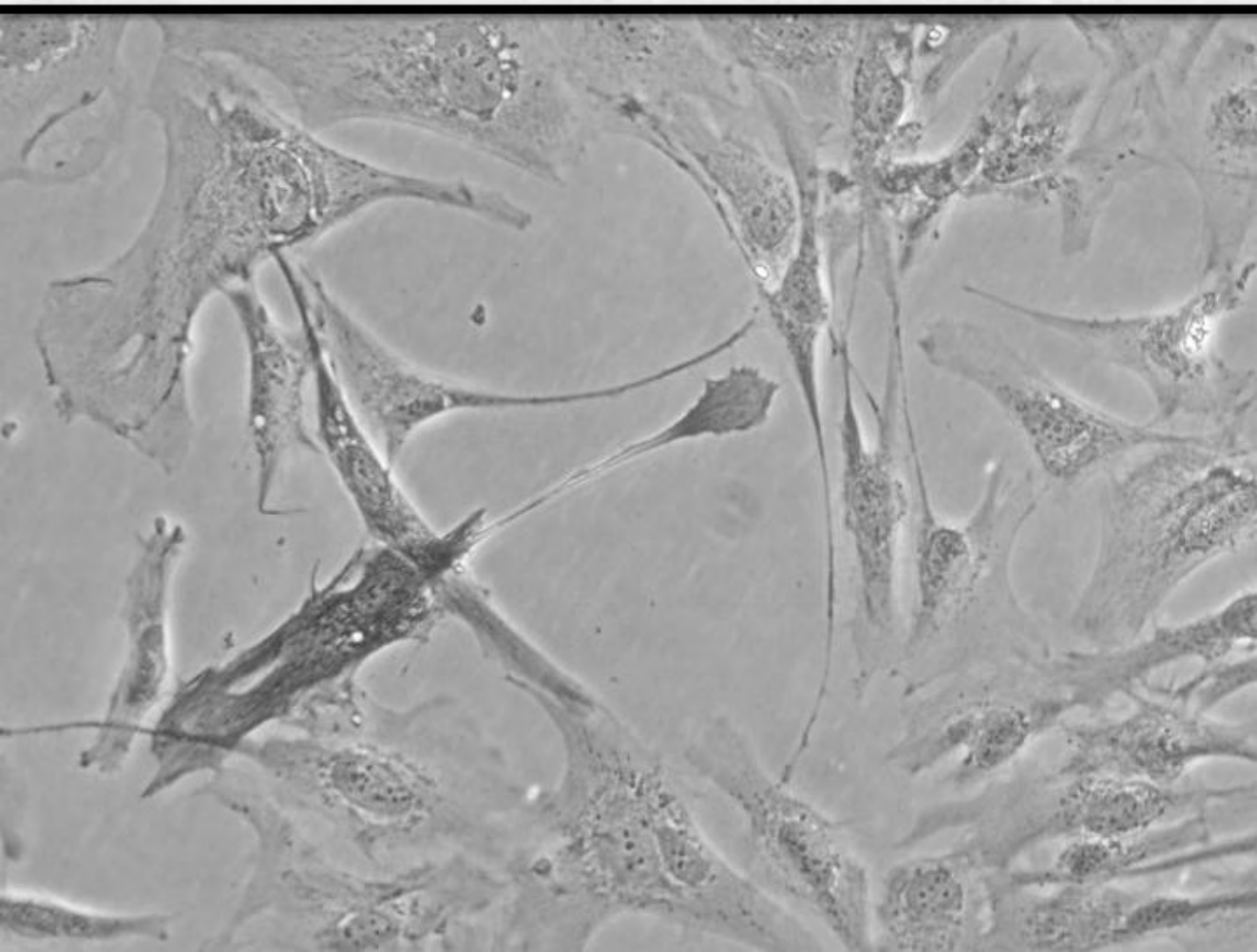

Abstract

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays an important role in the pre-receptor regulation of corticosteroids by locally converting cortisone into active cortisol. To investigate the impact of this mechanism on osteoblast development, we have characterized 11β-HSD1 activity and regulation in a differentiating human osteoblast cell line (SV-HFO). Continuous treatment with the synthetic glucocorticoid dexamethasone induces differentiation of SV-HFO cells during 21 days of culture. Using this cell system, we showed an inverse relationship between 11β-HSD1 activity and osteoblast differentiation. 11β-HSD1 mRNA expression and activity were low and constant in differentiating osteoblasts. However, in the absence of differentiation (no dexamethasone), 11β-HSD1 mRNA and activity increased strongly from day 12 of culture onward, with a peak around day 19. Promoter reporter studies provided evidence that specific regions of the 11β-HSD1 gene are involved in this differentiation controlled regulation of the enzyme. Functional implication of these changes in 11β-HSD1 is shown by the induction of osteoblast differentiation in the presence of cortisone. The current study demonstrates the presence of an intrinsic differentiation-driven molecular switch that controls expression and activity of 11β-HSD1 and thereby cortisol production by human osteoblasts. This efficient mechanism by which osteoblasts generate cortisol in an autocrine fashion to ensure proper differentiation will help to understand the complex effects of cortisol on bone metabolism.

3.1 Introduction

Glucocortoids exert diverse effects on bone metabolism. In human and rat bone marrow stromal cells, glucocorticoids are crucial for the induction of osteoblast differentiation and formation of a mineralized extracellular matrix [1-3]. Paradoxically, glucocorticoids in pharmacological doses cause osteoporosis, mainly by suppressing bone formation but also through effects on bone resorption [4-6]. Overall, the action of glucocorticoids on bone is complex, poorly understood, and is dependent on the duration and concentration of glucocorticoid treatment and the differentiation stage of both osteoblasts and osteoclasts [7-10].

At a molecular level, glucocorticoid signaling is mediated via the glucocorticoid receptor (GRα). GRα is expressed in almost all cell types including osteoblasts [11], in which it regulates gene expression by binding to glucocorticoid responsive elements in the regulatory regions of several target genes, including osteocalcin, collagen Iα1, and transforming growth factor-β1 [12-14]. Regulation of glucocorticoid responsiveness may occur at several levels, including changes in GRα expression [15] and at a postreceptor level through variation in GRα accessory proteins [16]. Glucocorticoid action has also been shown to be regulated at prereceptor level by isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD), which catalyze tissue-specific synthesis and metabolism of GRα ligands [17]. 11β-HSD type 1 (11β-HSD1) is found in almost all glucocorticoid target tissues and primarily displays reductase activity, converting cortisone into the biologically active cortisol [18, 19]. 11β-HSD2 converts cortisol into inactive cortisone and is found primarily in mineralocorticoid target tissues, in which it protects the nonselective mineralocorticoid receptor from activation by glucocorticoids [20, 21]. In bone, both isozymes of 11β-HSD (mRNA, protein, and enzyme activity) have been demonstrated, with 11β-HSD1 being the most prominent isozyme in human osteoblasts whereas 11β-HSD2 is barely detectable [22, 23]. Reductase activity of 11β-HSD1 in osteoblasts provides an efficient mechanism for the local activation of glucocorticoids in bone. As a consequence, the regulation of 11β-HSD1 activity in osteoblasts is likely to be an important autocrine determinant of osteoblast proliferation, differentiation, and function [24, 25].

Previous studies have highlighted the potential importance of osteoblastic 11β-HSD1 in predicting the detrimental effects of corticosteroids on bone [26, 27], but, as yet, the role of 11β-HSD1 in normal osteoblast development remains unclear. In studies presented here, we have used a nonneoplastic human osteoblast cell line to study the regulation of 11β-HSD1 and the specific impact on osteoblast differentiation and mineralization. Data indicate that regulation of 11β-HSD1 expression and activity are integral features of osteoblast differentiation, providing a potent mechanism for autocrine regulation of bone formation

3.2 Results

Glucocorticoids induce osteoblast differentiation and mineralization

During SV-HFO culture, osteoblast differentiation was monitored by measuring DNA levels, alkaline phosphatase (ALP) activity, and mineralization. In basal untreated cultures, ALP activity remained very low throughout the entire 23-days culture period. In contrast, cultures that were treated continuously with dexamethasone (DEX) showed a dose-dependent increase in ALP activity, which peaked around day 14 (see Figs. 3.1 A and 3.4 A). In these DEX-treated cultures, mineralization was initiated around day 14 of culture and increased up to day 19, after which it seemed to level off (Fig. 3.1 B). In the absence of DEX, cultures showed no evidence of osteoblast differentiation and mineralization (Fig. 3.1 A and B). Both DEX-treated and non-DEX-treated cultures showed a continuous increase in DNA content during culture, although differentiating cultures

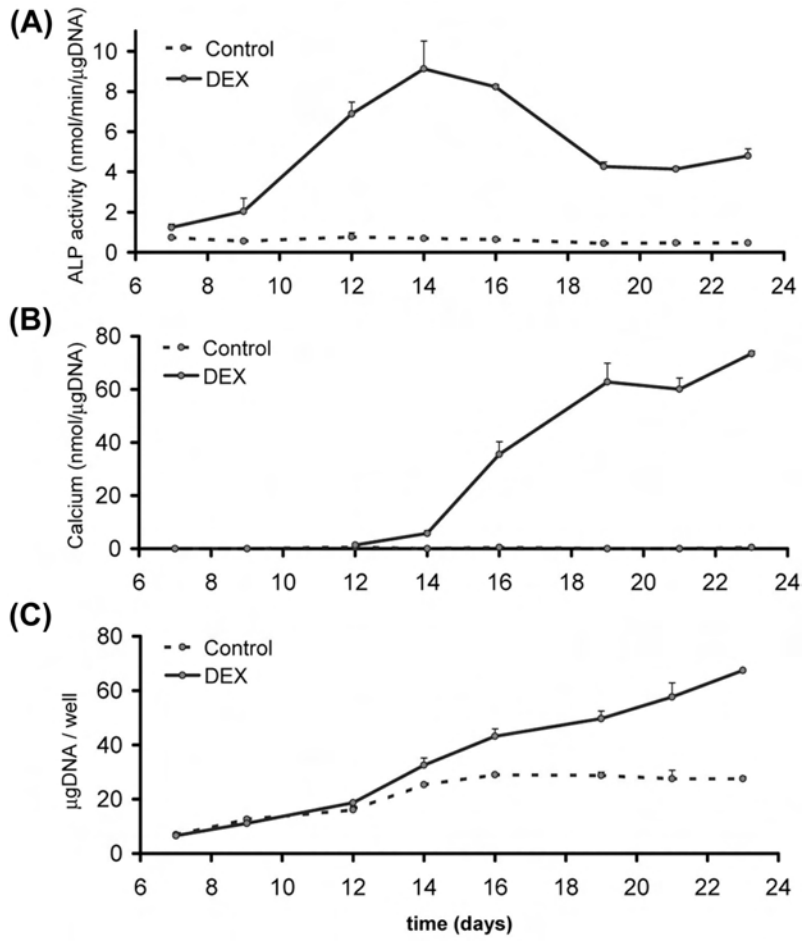

Fig. 3.1. ALP Activity (**A**), Mineralization (**B**), and DNA content (**C**) during SV-HFO culture in DEX-treated (1 µM) (Differentiating) and nontreated (nondifferentiating) cells. Values shown are the results of one typical SV-HFO culture.

showed a stronger increase in DNA content (Fig. 3.1 C). DEX-induced differentiation and mineralization was completely blocked by the GR antagonist mifepristone (2 μM) (data not shown). Throughout the remainder of the study, DEX-treated (1 μM) and non-DEX-treated cultures were referred as differentiating and nondifferentiating osteoblasts, respectively.

GRα mRNA expression in SV-HFO cells

In both differentiating and non-differentiating cultures, GRα mRNA could be easily detected and mRNA expression was constant during the entire culture period (day 5 to 23). However, at all days of culture differentiating osteoblasts showed lower GRα mRNA levels (0.7 ± 0.1) compared with their nondifferentiating counterparts (Fig. 3.2).

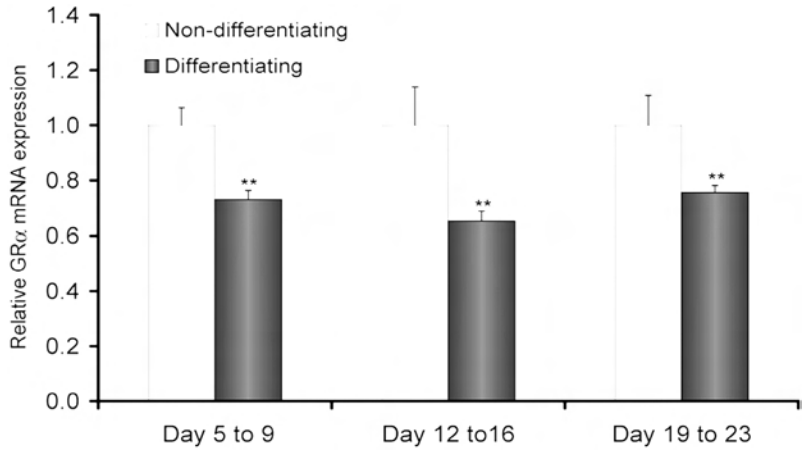

Fig. 3.2. GRα mRNA expression in differentiating and nondifferentiating SV-HFO cells. Results are presented as expression relative to control at week 1, 2, and 3 of culture. Values are means ± SEM (n ≥ 6). **, P < 0.01 compared with nondifferentiating cultures.

11β-HSD1 mRNA expression in differentiating and nondifferentiating osteoblasts

At the start of culture (day 5 to 9), 11β-HSD1 mRNA was expressed at similar levels in both differentiating and nondifferentiating SV-HFO cultures. However, after day 12, 11β-HSD1 mRNA expression increased strongly in nondifferentiating osteoblasts. This resulted in an up-regulation of about 2-fold at day 12, increasing to a 15-fold higher expression at day 19 (Fig. 3.3 A). By contrast, 11β-HSD1 mRNA expression in differentiating cultures remained constant during the entire culture period. Induction of differentiation using 10 nM DEX resulted in similar levels of 11β-HSD1 mRNA when compared with treatment with 1 μM DEX (data not shown). To demonstrate that increased 11β-HSD1 mRNA expression was not due to differentiation into adipocytes, we tested SV-HFO cultures for Oil-Red-O staining as well as mRNA expression of the adipocyte marker adipocyte lipid-binding protein (aP2). Cultures were negative for Oil-Red-O staining and, after 40 cycles of RT-PCR, we could not detect any aP2 expression in both differentiating and nondifferentiating cultures (data not shown).

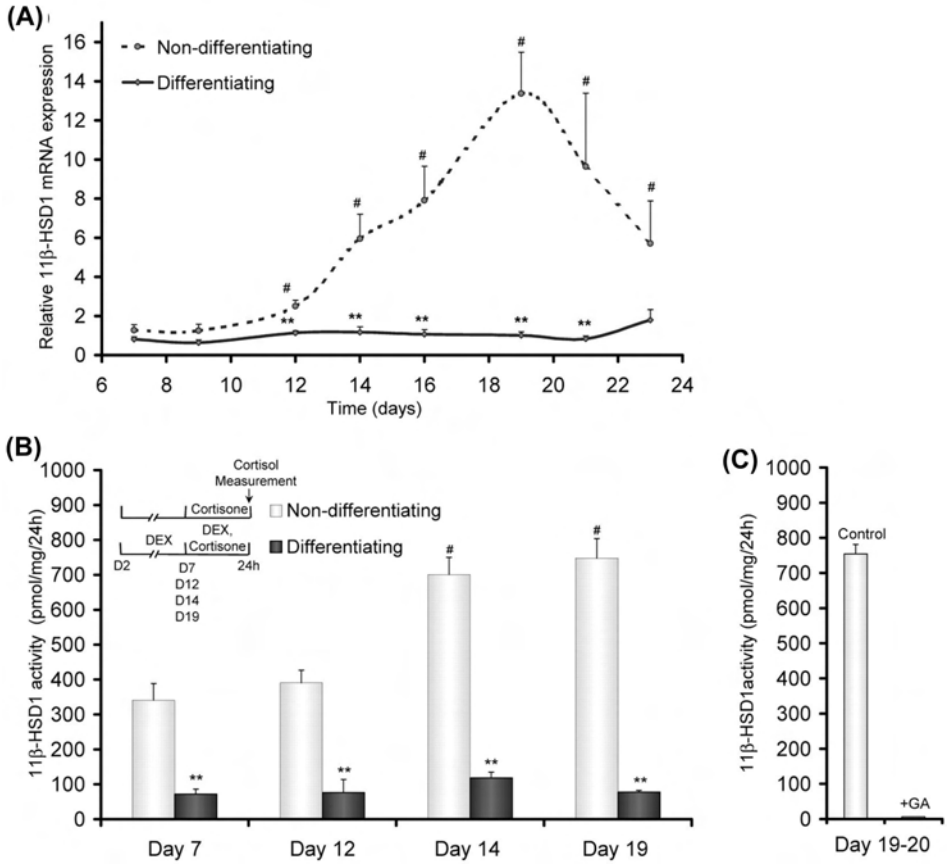

Fig. 3.3. Relative 11ß-HSD1 mRNA expression (**A**) and 11ß-HSD1 activity (**B**) in differentiating and nondifferentiating SV-HFO cultures for incubation protocols, see insets and §3.4 Materials and methods. Values are means ± SEM (n = 4). (**C**) Inhibition of 11ß-HSD1 activity by GA in SV-HFO cells. After 19 days of culture, 11ß-HSD1 activity was measured in absence and presence of 2.5 μM GA. Values are means ± SEM (n = 4). **, P < 0.01 compared with nondifferentiating osteoblasts. #, P < 0.05 compared with levels at day 7 of nondifferentiating osteoblasts.

11β-HSD1 activity in differentiating and nondifferentiating osteoblasts

Next we examined 11β-HSD1 activity in relation to osteoblast differentiation and mineralization. To demonstrate 11β-HSD1 reductase activity in SV-HFO cells, we analyzed cortisone to cortisol conversion after addition of 1 μM cortisone to the culture medium. In differentiating cultures, 11β-HSD1 activity was constant (86 ± 11 pmol/μg·24 h; mean ± SEM), whereas nondifferentiating cultures showed significantly higher 11β-HSD1 activity: 341 ± 48 and 391 ± 36 pmol/μg·24 h at day 7 and 12, which increased to 701 ± 49 and 748 ± 56 pmol/μg·24 h at day 14 and 19, respectively (Fig. 3.3 B). This activity profile shows similarity to the 11β-HSD1 mRNA expression profile in differentiating and nondifferentiating cultures. 11β-HSD1 activity was completely blocked by addition of the 11β-HSD inhibitor 18β-glycyrrhetinic acid (GA) (Fig. 3.3 C). Finally, the presence or absence of β-glycerophosphate had no effect on 11β-HSD1 activity in either differentiating and nondifferentiating cultures (data not shown).

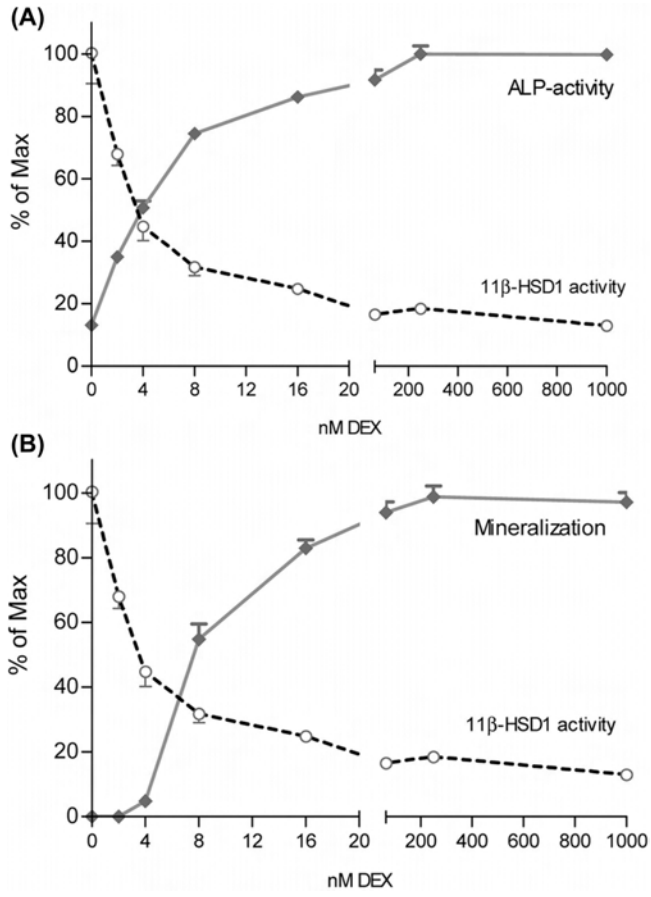

Fig. 3.4. Inverse relation between DEX-induced differentiation and 11ß-HSD1 activity. SV-HFO cells were cultured for 19 days in the presence of 0, 2, 4, 8, 16, 63, and 250 nM and 1 µM DEX. Subsequently, ALP activity, 11β-HSD1 activity (**A**), and mineralization (**B**) were measured. Values are means ± SEM (n = 4).

Inverse relationship between osteoblast differentiation and 11β-HSD1 activity

The data presented thus far suggested an inverse relationship between osteoblast differentiation and 11β-HSD1 expression and activity. To investigate this in more detail, SV-HFO cells were cultured for 19 days with various concentrations of DEX. DEX treatment caused a dose-dependent increase in ALP activity and mineralization, which coincided with a dose-dependent decrease in 11β-HSD1 activity (Fig. 3.4 A and B), substantiating the inverse relation between 11β-HSD1 activity and differentiation. This inverse relationship between ALP and 11β-HSD1 activity was tightly coupled as indicated by the DEX EC_{50} values of 3 ± 0.5 and 5 ± 0.5 nM, respectively. Mineralization was induced by DEX with an EC_{50} value of 9 ± 1 nM DEX.

Effects of short-term DEX treatment on 11β-HSD1 expression and activity

In the study so far, osteoblast differentiation was induced by continuous DEX treatment, resulting in a low and constant 11β-HSD1 mRNA expression and activity. These results are

in contrast to those of other studies using a variety of cell types in which DEX treatment stimulated 11β-HSD1 activity. However, these results were obtained after short-term DEX incubation. Therefore, we studied the effect of short-term DEX incubation on the regulation of 11β-HSD1 mRNA expression and activity in nondifferentiating SV-HFO osteoblasts.

At day 7 of culture, 11β-HSD1 mRNA expression decreased after short-term DEX treatment (1 μM, 24 h). However, at later time points during SV-HFO culture (day 14 and 19), 11β-HSD1 mRNA expression was not affected by short-term DEX treatment (Fig. 3.5 A). To study short-term DEX effects on 11β-HSD1 activity, cells were exposed for 48 h to DEX (1 μM); within this 48 h, the final 24 h was used to measure 11β-HSD1 activity. This resulted in decreased 11β-HSD1 activities at day 7 matching the observed data at the mRNA level. At later time points, 11β-HSD1 activity was not decreased by short-term DEX treatment. At day 12 and 14, even a significant increase was observed (Fig. 3.5 B). These data show that short-term DEX-treatment does not

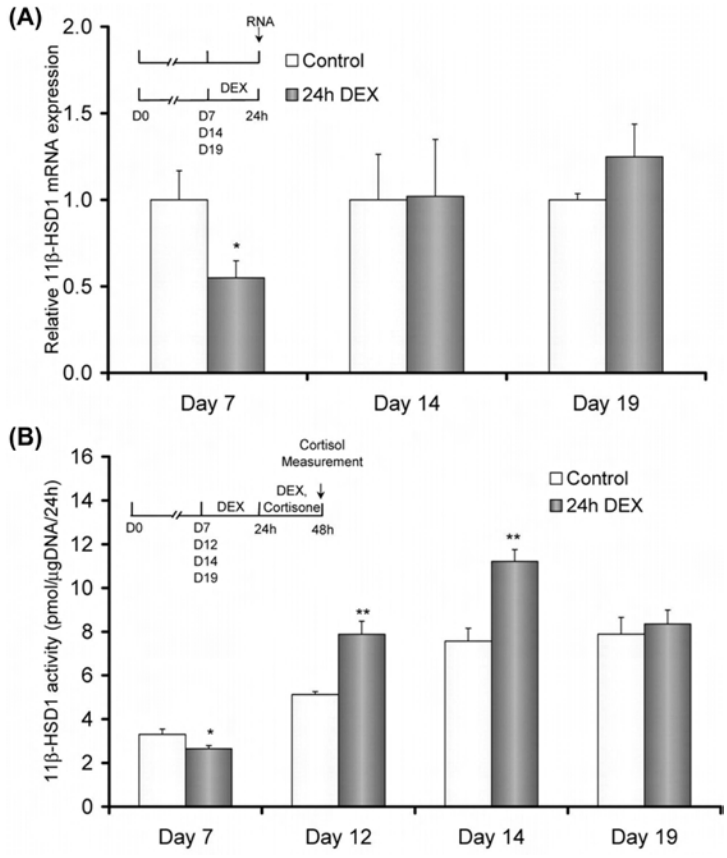

Fig. 3.5. Short-term effects of DEX on 11β-HSD1 mRNA expression and activity during SV-HFO culture. (**A**) Effects of 24 h DEX treatment (1 μM) on 11β-HSD1 mRNA expression. Results are presented as expression relative to control at day 7, 14, and 19 of culture. For incubation protocols see inset. Values are means ± SEM (n ≥ 4). (**B**) 11β-HSD1 activity after 24–48 h of DEX treatment (1 μM DEX) at day 7, 12, 14, and 19 of culture. For incubation protocols see insets. Values are means ± SEM (n = 4). *, P < 0.05; **, P < 0.01 compared with control.

decrease 11β-HSD1 expression and activity at later stages of culture in contrast to DEX-induced differentiation.

11β-HSD1 promoter activity in differentiating and nondifferentiating cultures

To gain more insight into the background of the differentiation-driven regulation of 11β-HSD1 expression, we used a luciferase reporter gene under control of the 11β-HSD1 promoter (HSD11B1). We used several lengths of the HSD11B1 promoter containing the –261 to +77, –301 to +77, –804 to +77, –1382 to +77, and –2506 to +77 region of the promoter. During SV-HFO culture, these reporter constructs were transiently transfected at day 7, 12, 14, or 19 of culture. At day 7 of culture, all the constructs showed similar promoter activity in differentiated and nondifferentiated cultures. Activity of the short –261 to +77 region of the HSD11B1 promoter was identical at all time points in differentiating and nondifferentiating cultures (Fig. 3.6 A). However, reporter constructs containing more upstream regions of the HSD11B1 promoter were sensitive to induction in the absence of differentiation from day 12 onward (Fig. 3.6 B, C, D, and E). The reporter construct containing the –1382 to +77 region of the HSD11B1 promoter was the most sensitive to induction in the absence of differentiation (Fig. 3.6 D). Further increasing the length of the promoter up to –2506 resulted in a lower induction (Fig. 3.6 E). These results show that the HSD11B1 promoter is induced from day 12 onward in the absence of osteoblast differentiation. Moreover, important differentiation-dependent regulatory elements are located in region –1382 to –261 of the HSD11B1 promoter. To show that promoter activity is not silenced shortly after DEX exposure, we analyzed promoter activity after 24 and 48 h of DEX exposure (1 μM) at day 7, 12, 14, and 19. We studied both the –804 to +77 (data not shown) and –1382 to +77 (Fig. 3.6 F) region of the HSD11B1 promoter in relation to short-term DEX treatment. These two promoters showed a clear suppression after DEX-induced differentiation (Fig. 3.6 C and D); however, short-term DEX treatment did not chance promoter activity (Fig. 3.6 F). These results support the mRNA and activity data obtained after short-term DEX treatment.

CAAT/Enhancer binding protein α (C/EBPα) and C/EBPβ mRNA expression in differentiating and nondifferentiating osteoblasts

Two important candidates for HSD11B1 promoter regulation are C/EBPα and C/EBPβ. The presence of both C/EBPα and C/EBPβ mRNA was demonstrated in SV-HFO cells. However, no difference in mRNA expression between differentiating and nondifferentiating osteoblasts was found (data not shown).

Functional consequences of osteoblastic 11β-HSD1 activity

To demonstrate a functional role of 11β-HSD1 in osteoblast differentiation, we cultured SV-HFO cells in the continuous presence of 100 or 1000 nM cortisone. To prevent total removal of osteoblast-produced cortisol at every medium replacement, medium was only partially replaced. Treatment with cortisone resulted in a dose-dependent increase in ALP activity up to 3.2-fold at day 30 (Fig. 3.7 A). Despite this clear induction of ALP activity, cortisone did not induce mineralization. To further study the effect on

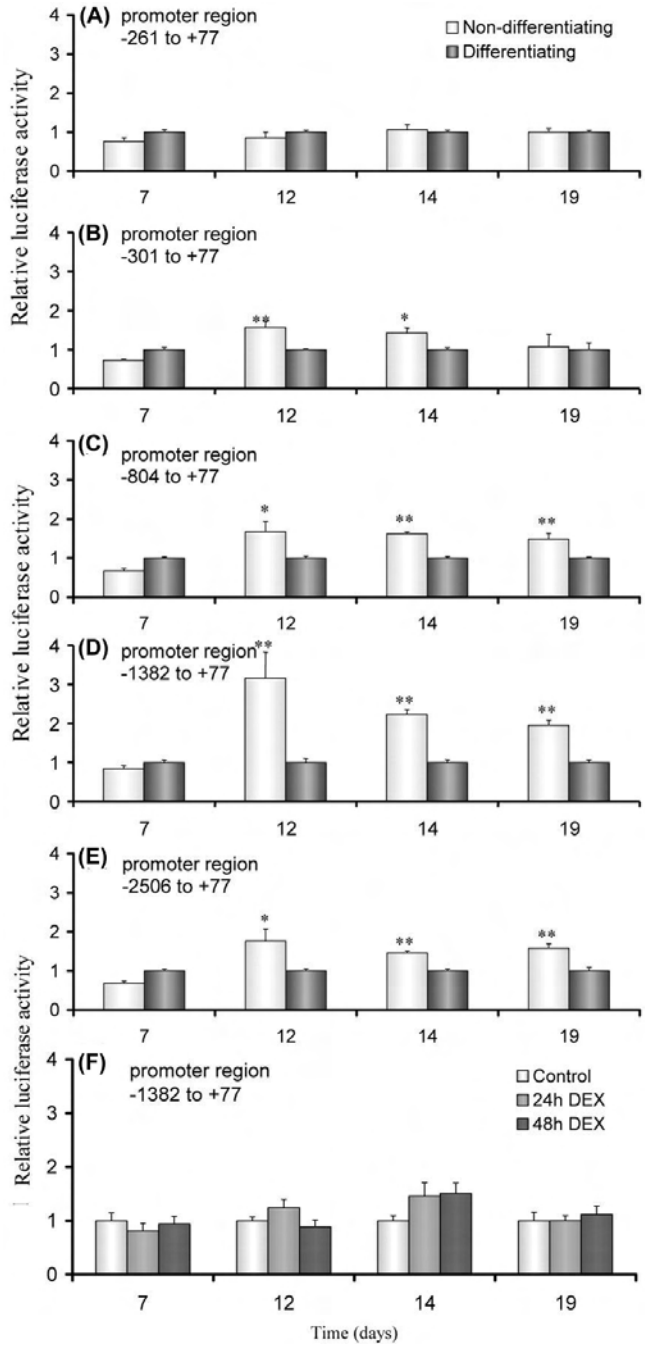

Fig. 3.6. HSD11B1 promoter activity in differentiating and nondifferentiating SV-HFO cultures. Cultures were transiently transfected with luciferase reporter constructs containing the –261 to +77 (**A**), –301 to +77 (**B**), –804 to +77 (**C**), –1382 to +77 (**D**) and –2506 to +77 (**E**) region of the HSD11B1 promoter at day 7, 12, 14, and 19 of culture. Values are means ± SEM (n ≥ 5). *, $P < 0.05$; **, $P < 0.01$ compared with differentiating cultures. (**F**) Promoter activity of the –1382 to +77 region after short-term DEX treatment (1 μM, 24 and 48 h) at day 7, 12, 14, and 19 of culture.

mineralization, we cotreated the cells with 1α,25-dihydroxyvitamin D3 (1α,25-$[OH]_2D_3$) as a facilitator of mineralization. Treatment with 10 nM 1α,25-$(OH)_2D_3$ alone increased ALP activity, whereas it was not sufficient to induce mineralization. However, cotreatment of 1000 nM cortisone with 1α,25-$(OH)_2D_3$ resulted in a strong induction of mineralization and ALP activity after 30 days of culture (Fig. 3.7 C and D). This effect on mineralization and on ALP activity was completely blocked by the addition of the GR antagonist mifepristone (2 μM) (Fig. 3.7 C and D). To prove a specific role for 11β-HSD in cortisone-induced ALP activity, cells were incubated with the 11β-HSD inhibitor GA. Cortisone-induced ALP activity was completely blocked by the addition of 2.5 μM GA. Addition of GA had no effect on either basal ALP activity or cortisol-induced ALP activity (Fig. 3.7 B).

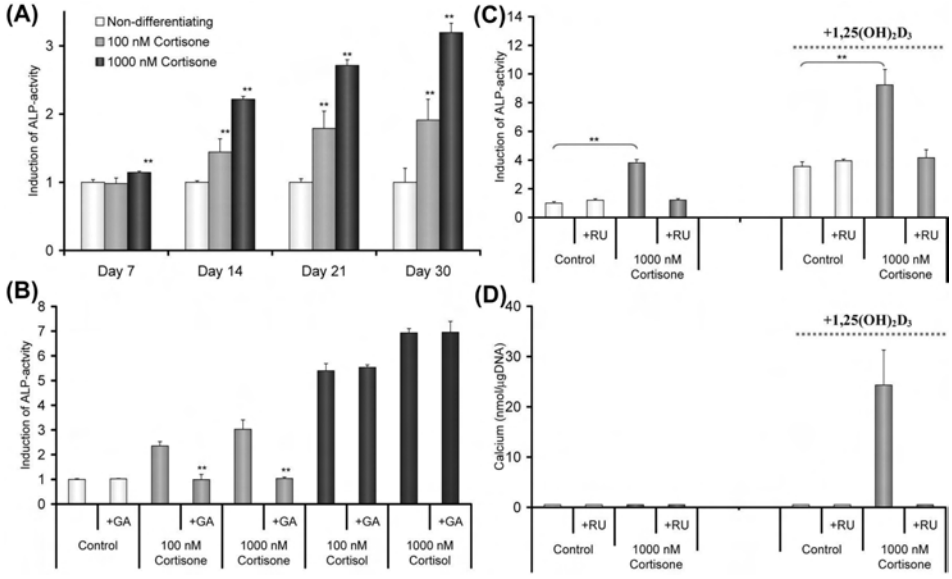

Fig. 3.7. Functional consequences of osteoblastic 11β-HSD1 expression. **(A)** Induction of ALP activity after cortisone incubation. Cells were cultured in the continuous presence of 100 or 1000 nM cortisone. Results are presented as ALP activity compared with non treated controls at day 7, 14, 21, and 30 of culture. Values are means ± SEM (n = 6). **, P < 0.01 compared with nondifferentiating osteoblasts. **(B)** Inhibition of cortisone induced ALP activity by GA. SV-HFO cells were cultured for 21 days in the presence of cortisone (100 and 1000 nM), cortisol (100 and 1000 nM), and vehicle with or without 2.5 μM GA. Values are means ± SEM (n = 3). **, P < 0.01 compared with control without GA. **(C** and **D)** Cortisone induced ALP activity and mineralization in the presence or absence of 1α,25-$(OH)_2D_3$ (10 nM) and the GR antagonist mifepristone (RU) (2 μM) after 30 days of culture.

3.3 Discussion

The current study demonstrates the presence of an intrinsic differentiation-driven molecular switch that controls the expression and activity of 11β-HSD1 and thereby cortisol production in human osteoblasts. In the absence of exogenously added glucocorticoids and osteoblast differentiation, 11β-HSD1 expression and activity are strongly induced. By contrast, cells induced to differentiate and mineralize in the

presence of glucocorticoids show low levels 11β-HSD1 activity. In other words, 11β-HSD1 acts to generate cortisol in an autocrine fashion to facilitate proper differentiation and mineralization. Conversely, when sufficiently high levels of active glucocorticoids (cortisol, dexamethasone, prednisolone) are available to induce osteoblast differentiation, endogenous glucocorticoid activation via 11β-HSD1 remains minimal. An additional benefit of this latter phenomenon is that unwanted and detrimental increases in local glucocorticoid concentrations is prevented. This mutual regulation provides an elegant and efficient mechanism by which osteoblast differentiation and local glucocorticoid levels are regulated in a balanced manner.

Under the influence of glucocorticoids, SV-HFO cultures proceed through a tightly controlled process of differentiation resulting in an extracellular protein matrix that fully mineralizes. Without glucocorticoid treatment, SV-HFO cultures do not differentiate and show no mineralization, demonstrating the importance of glucocorticoids for human osteoblast differentiation and mineralization. As a consequence, the regulation of 11β-HSD1 activity in osteoblasts is likely to be an important autocrine determinant of osteoblast proliferation, differentiation, and function [24, 25] .

The actions of glucocorticoids are mediated via GRα, which is expressed in almost all cell types, including osteoblasts [11]. In SV-HFO cells, GRα mRNA was clearly expressed and, in addition to increased 11β-HSD1 activity, nondifferentiated osteoblasts expressed GRα at a higher level than differentiating osteoblasts. Together, these observations indicate that absence of glucocorticoid-induced differentiation results in a cellular sensitization to the glucocorticoid endocrine system.

In agreement with previous studies using primary cultures of human osteoblasts [22, 23], 11β-HSD1-mediated cortisol generation appears to be the predominant form of glucocorticoid metabolism in SV-HFO cells. Regulation of 11β-HSD1 expression and activity by glucocorticoids have been reported for hepatocytes, adipocytes, and fibroblast and primary osteoblasts, all of which showed enhanced 11β-HSD1 activity after glucocorticoid exposure [26, 28-30], resulting in a "feed-forward" mechanism after glucocorticoid exposure. *In vivo*, Jamieson *et al*. [31] demonstrated that glucocorticoids regulate 11β-HSD1 mRNA in a complex tissue- and time-specific manner, suggesting a reduction of 11β-HSD1 by DEX. Glucocorticoid-induced differentiation (continuous DEX treatment) of SV-HFO osteoblasts suppressed 11β-HSD1 mRNA expression and activity, whereas short-term treatment with DEX did not suppress 11β-HSD1 activities, particularly at later stages of culture. Moreover an increase in 11β-HSD1 activity was measured after short-term DEX treatment. This demonstrates that, in osteoblasts, glucocorticoids can regulate 11β-HSD1 in two different ways. Firstly, short-term glucocorticoid exposure directly increases 11β-HSD1 activity, resulting in a feed-forward of glucocorticoid action. Secondly, we show that, in osteoblasts, continuous exposure to glucocorticoid indirectly regulates 11β-HSD1 expression by inducing a differentiation-driven switch that represses 11β-HSD1 activity. In the first week of culture, short-term DEX treatment resulted in the suppression of 11β-HSD1 activity and mRNA expression. In this first week of culture, SV-HFO cells are very sensitive to the induction of differentiation by glucocorticoids. Therefore, 24 h of DEX exposure may be sufficient to initiate osteoblast differentiation and, as a result, suppression of 11β-HSD1 levels.

In the absence of glucocorticoid-induced differentiation, 11β-HSD1 mRNA expression was strongly induced in the second week of culture. Interestingly, at this same time point of culture, differentiating cultures showed peak ALP activity and initiated the process of mineralization. Why this induction of 11β-HSD1 expression is initiated after exactly 2 weeks of culture is unclear. Promoter reorganization or differential expression of transcription factors at this time point might mediate this differentiation-controlled switch in 11β-HSD1 expression. In rat osteoblasts, it has been demonstrated that, in more differentiated osteoblasts, osteocalcin expression is strongly induced after the osteocalcin gene is opened by remodeling of the chromatin structure [32, 33]. Luciferase reporter-plasmids under the control of various HSD11B1 promoter fragments provided evidence that specific regions of the HSD11B1 promoter are sensitive to activation in the absence of osteoblast differentiation. This indicates that regulation of the HSD11B1 promoter during this switch is at least partly regulated by differences in transcription factor binding to the HSD11B1 promoter in differentiating and nondifferentiating osteoblasts. The absence of promoter induction by the short –261 +77 promoter indicates that important differentiation dependent regulatory elements driving expression in nondifferentiating cells are located in region –1382 to -261. Two important candidates for 11β-HSD1 promoter regulation are C/EBP α and C/EBPβ. These proteins are known to regulate 11β-HSD1 promoter activity in liver cells, in which C/EBPα promotes and C/EBPβ represses 11β-HSD1 transcription [34]. Furthermore, these genes are known to be regulated by cortisol [35, 36]. Both C/EBPα and C/EBPβ are expressed in SV-HFO cells. However, mRNA expression of these genes was identical in nondifferentiating and nondifferentiating SV-HFO osteoblasts.

The current study shows that 11β-HSD1 activity is tightly related to osteoblast differentiation and has significant consequences for osteoblast differentiation and function. In the presence of sufficient amounts of cortisone, SV-HFO osteoblasts induce, in an autocrine/paracrine way, differentiation by generating biologically active levels of cortisol. Autocrine regulation of glucocorticoids action has also been shown in adipocytes and is a potentially important target for obesity therapy [37]. Freshly isolated adipose stromal cells mainly convert cortisol into cortisone. However, when adipocyte differentiation is initiated, 11β-HSD1 dehydrogenase activity is switched into reductase activity generating cortisol and thereby promoting adipocyte differentiation [38]. This shows that both osteoblast and adipocytes use 11β-HSD1 activity to regulate their differentiation in an autocrine manner. However, in osteoblasts there is an inverse relation between 11β-HSD1 activity and differentiation, whereas adipocytes increase their cortisol production during further differentiation.

In vivo, glucocorticoids are bound to cortisol binding globulin. Cortisol binding globulin binds cortisol with approximately 10-fold higher affinity than cortisone [39], resulting in higher free levels of the 11β-HSD1 substrate cortisone than the active glucocorticoid cortisol. Therefore, local glucocorticoid metabolism might indeed play a significant role in regulating osteoblast differentiation and function *in vivo*. This pre-receptor hormone regulation is not unique for glucocorticoids. Local levels of androgens and estrogens are also regulated by enzymes present in target tissues [40]. Our previous work has shown that human osteoblasts express all the enzymes involved in the production of estradiol, *e.g.*

aromatase, sulfatase, and 17β-HSD isoenzymes [41]. This means that human osteoblasts have the capacity to produce active estrogens and glucocorticoids locally. Moreover, production of these hormones is regulated in a differentiation dependent manner.

This study demonstrates the importance of autocrine 11β-HSD1 action for proper osteoblast differentiation and function. However, the 11β-HSD1 knockout (-/-) mouse does not show a clear change in bone phenotype [42]; the authors of this study highlighted important caveats why this 11β-HSD1-/- mouse might not be necessarily a good model to study the effects of local glucocorticoid metabolism on human bone. Firstly, the global loss of 11β-HSD1 leads to a profound alteration in the hypothalamic-pituitary-adrenal axis, which may completely alter the set-point of circulating glucocorticoid effects. Secondly, the background strain of the 11β-HSD1-/- mouse shows expression of both 11β-HSD1 and 11β-HSD2 in bone, which is in direct contrast to humans, which show only expression of 11β-HSD1. A bone-specific knockout of 11β-HSD1 will clearly provide some answers to the issues outlined above. Evidence that endogenous glucocorticoid metabolism has significant effects *in vivo* on bone is illustrated by the 11β-HSD2 bone specific transgenic mouse [43]. These animals are essentially the bone-specific, transgenic equivalents of the 11β-HSD1-/- mice and show changes in a variety of bone parameters when compared with wild-type controls.

In conclusion, the current study demonstrates a tight control of 11β-HSD1 activity during osteoblast differentiation, thereby supplying adequate amounts of cortisol to support osteoblast differentiation and preventing excessive and detrimental amounts of cortisol in mature osteoblasts. Furthermore, the data presented indicate the presence of a molecular switch that regulates HSD11B1 promoter activity dependent on the state of cellular differentiation.

3.4 Materials and methods

Cell culture

SV-HFO [44] cells were cultured in MEM (GIBCO, Paisley, UK) supplemented with 20 mM HEPES, pH 7.5 (Sigma, St. Louis, MO); streptomycin/penicillin; 1.8 mM $CaCl_2$ (Sigma); and heat-inactivated FCS (GIBCO) at 37°C and 5 % CO_2 in a humidified atmosphere. DEX, cortisone, and mifepristone (RU 38486) were purchased from Sigma; $1\alpha,25(OH)_2D_3$ was generously provided by Dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark). Thawed cells were precultured for at least 1 week in the presence of 10 % FCS. In this preculture, cells were seeded in a density of 5 x 10^3 vital cells per centimeter squared and were subcultured every week. During this preculture, SV-HFO cells remained in an undifferentiated stage and cells were only used between passages 8 and 13. After preculturing, cells were seeded in a density of 10 x 10^3 vital cells per centimeter squared in the presence of 2 % charcoal-treated FCS supplemented with 10 mM β-glycerophosphate (Sigma) (added as a phosphate donor to facilitate the mineralization process) and were grown without subculturing. Medium freshly supplemented with hormones or other additives was replaced every 2-3 days. In experiments in which medium was partially replaced, replacements were as follows: day 2 and 5, 60 %; day 7 and 9, 75 %;

day 12, 14, 19, 21, 26 and 28, 86 %; day 16 and 23, 100 % fresh medium was added. For analysis, medium was stored at –20°C and cells were scraped form the culture dish in PBS containing 0.1 % Triton X-100 and stored at –80°C. Before analysis, cell lysates were sonicated on ice in a sonifier cell disrupter for 2 x 15 sec.

DNA content

For DNA measurements, 100 µl SV-HFO cell lysates were treated with 200 µl heparin (8 IU/ml in PBS) and 100 µl ribonuclease A (50 µg/ml in PBS) for 30 min at 37°C. This was followed by adding 100 µl ethidium bromide solution (25 µg/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (PerkinElmer, Wellesley, MA) using an extinction filter of 340 nm of and emission filter of 590 nm. For standards, calf thymus DNA (Sigma) was used.

ALP activity

ALP activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamine buffer supplemented with 1 mM $MgCl_2$ at pH 9.8) in the SV-HFO cell lysates for 10 min at 37°C. The reaction was stopped by adding 0.06 M NaOH. Absorption was measured at 405 nm. Results were adjusted for DNA content of the corresponding cell-lysates.

Mineralization

SV-HFO cell lysates were incubated overnight in 0.24 M HCl at 4°C. Calcium content was colorimetrically determined with a calcium assay kit (Sigma) according to the manufacturer's description. Results were adjusted for DNA content of the corresponding cell lysates.

Oil-Red O staining

Cell cultures were fixed for 10 min with 10 % formalin in PBS. After fixation, cells were washed with PBS and stained for at least 20 min with Oil-Red-O solution (2:3 vol/vol H_2O : n-propanol containing 0.5 % Oil-Red-O). After staining, cells were washed twice with demineralized water and examined under the microscope for staining.

11β-HSD1 activity during SV-HFO culture

Cells were cultured in the absence or presence of DEX and at various days during culture 1 µM cortisone was added to the culture medium. After 24 h, culture medium was collected and cortisol concentrations were analyzed by a chemoluminescence-based immunoassay on the Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA).

Human HSD11B1 promoter-reporter constructs

A yeast artificial chromosome clone of chromosome 1 (kindly provided by Prof. J. Adamski, GSF-National Research Center for Environment and Health, Institute of Experimental Genetics, Neuherberg, Germany) was used to sequence the promoter for the 11β-HSD1 gene (HSD11B1) (GenBank accession no. AL031316). The resulting DNA was then used to produce smaller DNA fragments corresponding to different lengths of the HSD11B1

promoter including a 77-bp region of 5' untranslated region (exon 1) downstream of the ATG site at 2506 bp. The promoter fragments were generated by PCR amplification using a single reverse primer (+77 to +65 bp), 5'-TCA*CCCGGG*TCCTCGTTTGCAG-3' in conjunction with various forward primers specific for different regions of the human HSD11B1 promoter/exon 1: promoter region –261 to +77, forward primer (–261 to –249 bp), 5'-TCA*GGTACC*GTCTCCTCTTGCT-3'; promoter region –301 to +77, forward primer (–301 to –289 bp), 5'-TCA*GGTACC*GAATCCAGTCCTG-3'; promoter region –804 to +77, forward primer (–804 to –791 bp) 5'-GCTCA*GGTACC*TTTACAAGACCCAG-3'; promoter region -1382 to +77, forward primer (-1382 to -1370 bp), 5'-TCA*GGTACC*GGCCTTT GTTGAC-3'; promoter region –2506 to +77, forward primer (–2506 to –2494 bp), 5'-TCA*GGTACC*GAGAACCAGCCAT-3'. Each primer contained restriction sites shown in italics to facilitate insertion into the pGEM-T Vector (Promega, Madison, WI): reverse primer, SmaI (*CCCGGG*); and forward primers, KpnI (*GGTACC*). After ligation, the promoter constructs were transformed in DH5α competent cells, selected (Luria broth + ampicillin), and DNA was extracted by midi-prep (Wizard plus SV Minipreps Purification System by Promega). The resulting HSD11B1 promoter constructs were then digested with SmaI and KpnI, separated on 1 % agarose gels, reextracted, and recloned into SmaI- and KpnI-digested luciferase pGL3-enhancer vector (Promega). Ampicillin-selected clones were used to produce DNA, which was then sequenced to confirm the identity of each HSD11B1 promoter-reporter fragment.

Luciferase activity

Four hours after medium replacement, SV-HFO cultures were transiently transfected with 200 ng pGL3 luciferase reporter plasmid, using FuGENE6 transfection reagent according to the manufacturer's protocol (Roche, Basel, Switzerland). One day after transfection, SV-HFO cultures were lysed in 100–200 µl 1x lysis buffer (Promega) for 20 min with gentle shaking. During the final stages of differentiation when the culture was mineralized, a cell scraper was needed to release the cells and initiate cell lysis. Luciferase activity was measured using 25 µl cell lysate and the Steady-Glo Luciferase Assay System (Promega). Luciferase values were corrected for luciferase activity of empty pGL3-enhancer vector.

Quantification of mRNA expression

Total RNA was isolated using RNA-Bee solution (Tel-Test, Friendwood, TX) according to the manufacturer's protocol. To remove calcium (derived from extracellular matrix), RNA was precipitated by overnight incubation with 4 M LiCl and 50 mM EDTA at –20°C. After precipitation and centrifugation for 30 min at 14,000 rpm and 4°C, the RNA pellet was washed four times with 70 % ethanol and dissolved in H_2O. The total amount of RNA was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). One microgram total RNA was reverse transcribed into cDNA using a cDNA synthesis kit according to the protocol of the manufacturer (MBI Fermentas, St. Leon-Rot, Germany), using 0.5 µg oligo(dT)18 and 0.2 µg random hexamer primers.

Quantitative real-time PCR was carried out using an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in 25 µl volumes

Table 3.1 Primer and Probe Concentrations and Sequences.

	Forward primer (nM, 5'-3')	**Reverse primer** (nM, 5'-3')	**Probe** (nM, 5'-Fam – 3' Tamra)
11β-HSD1	100 AGGAAAGCTCATGGGAGGACTAG	100 ATGGTGAATATCATCATGAAAAAGA	50 CATGCTCATTCTCAACCACATCACCAACA
aP2	800 TACTGGGCCAGGAATTTGAC	800 AATGCGAACTTCAGTCCAGG	
C/EBPα	400 TGGACAAGAACAGCAACGAG	400 TTGTCACTGGTCAGCTCCAG	75 CACCTTCTGCTGCGTCTCCACGTT
C/EBPβ	800 GACAAGCACACAGCGACGAGTA	800 GTGCTGCCGTCTCCAGGT	100 ATCTTGGCCTTGTCGCGGCTCTT
GAPDH	300 ATGGGGAAGGTGAAGGTCG	300 TAAAAGCAGCCCTGGTGACC	150 CGCCCAATACGACCAAATCCGTTGAC
GR	100 TGTTTTGCTCCTGATCTGA	100 TCGGGGAATTCAATACTCA	50 TGACTCTACCCTGCATGTACGAC

using a qPCR core kit (Eurogentec, Seraing, Belgium). Reaction mixes contained 20 ng cDNA, 5 mM $MgCl_2$, 200 µM dNTPs, and 0.025 U/µl Hot GoldStar enzyme. Primer and probe sets were designed, using the Primer Express software (version 1.5; Applied Biosystems). Primer and probe concentrations and sequences were as described as in Table 1. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The amount of human glyceraldehyde-3-phosphate dehydrogenase mRNA was used as internal control to normalize for possible differences in RNA extraction and degradation as well as efficiency of the cDNA synthesis. The expression of this gene was not significantly affected by hormonal treatment and during osteoblast differentiation. Data were presented as relative mRNA levels calculated by the equation: $2^{-\Delta Ct}$ (ΔCt = Ct of target gene minus Ct of glyceraldehyde-3-phosphate dehydrogenase). For semiquantitative RT-PCR, 80 ng of cDNA was amplified in a 25 µl reaction mix containing 200 µM dNTPs, 2.5 mM $MgCl_2$, 400 nM of each primer, and 1.25 U Amplitaq Gold (Applied Biosystems).

Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 56°C for 30 sec, and 72°C for 30 sec and a final extension step of 5 min at 72°C. Primer sequences for aP2 are described in Table 1.

Statistics

Data presented are the results of at least two independent experiments performed in triplicate. Values are the means ± SEM. Significance was calculated using the Student's *t* test.

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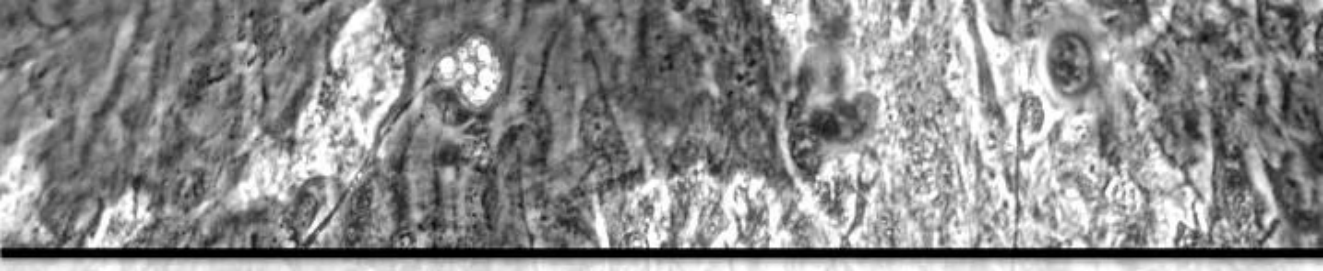

Chapter 4

Identification of regulatory genes, gene networks and cellular processes in glucocorticoid-induced human osteoblast differentiation and mineralization

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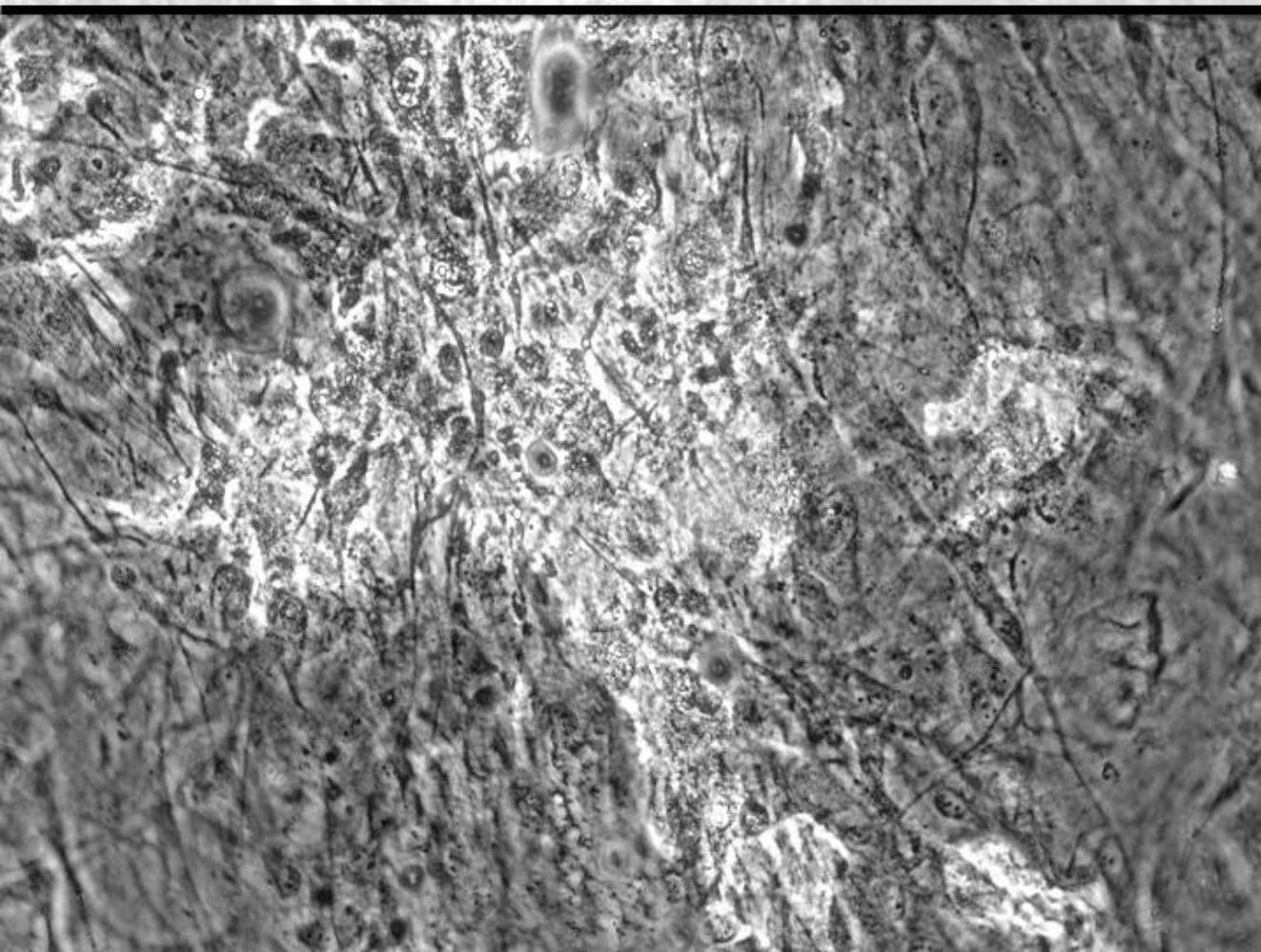

Abstract

Glucocorticoids (GCs) are important inducers of human osteoblast differentiation and extracellular matrix (ECM) mineralization. Yet the molecular consequences of GC-induced matrix mineralization are largely unknown. In the present study gene profiles of mineralizing human osteoblast cultures (GC-treated) were compared with nonmineralizing cultures. This identified 489 differentially expressed genes that consisted of both osteoblast-related and so far -unrelated genes. The expression of the osteoblast transcription factor ZBTB16 was specifically increased in mineralizing cultures and several other genes, including the cannabinoid receptor 1, paralleled its expression. Activation of this receptor caused increased mineralization. The differentially expressed genes could be categorized in several biological processes and gene networks. Gene ontology enrichment analyses identified a cluster of ECM genes differentially expressed between mineralizing and nonmineralizing cultures emphasizing the significance of ECM composition for proper mineralization. Time course studies identified several novel direct GC target genes and genes that were under control of the osteoblast differentiation program. To extend our observation beyond skeletal development, a vascular smooth muscle cell (VSMC)-based model for arteriosclerosis was used. This showed that GC-treatment also enhanced matrix mineralization in VSMC cultures. Moreover, we showed that a large subset of genes was similarly regulated in VSMCs and osteoblasts, demonstrating similarities between bone and vascular mineralization. In conclusion, differential expression profiling of mineralizing and nonmineralizing cultures proved to be a powerful tool to unravel the molecular mechanisms underlying GC-induced matrix mineralization in bone and arteriosclerosis. Several novel direct GC targets and targets to control bone formation were identified.

4.1 Introduction

Osteoblasts play a pivotal role in the regulation of bone quality as they are the bone forming cells and the directors of bone resorption by osteoclasts. Osteoblasts are derived from mesenchymal stem cells and undergo a complex differentiation process regulated by many endocrine and autocrine factors [1].

Glucocorticoid steroids (GCs) such as cortisol are crucial initiators of human osteoblast differentiation [2, 3]. At the molecular level GC signaling is mediated via the GC receptor (GR). The GR is expressed in various cell-types including osteoblasts [4], where it regulates gene expression by binding to GC responsive elements in the regulatory regions of target genes. Despite clear stimulation of bone formation by GCs *in vitro*, GCs are still regarded as negative regulators of bone formation. This is mainly based on clinical use of GCs, which frequently results in bone loss and increased risk of fractures (GC-induced osteoporosis) [5]. It should, however, be considered that this negative effect on bone is caused by high pharmacological levels of GCs for longer periods and might not reflect the physiological role of GCs in bone. Furthermore, most *in vitro* studies on GC-induced osteoporosis are performed using mouse osteoblasts that lack the need for GC-treatment to induce differentiation and mineralization [6]. This latter is in great contrast to human osteoblasts for which GCs are a prerequisite for proper differentiation.

In human osteoblasts cortisol triggers the production of a mature extracellular matrix (ECM) that eventually becomes mineralized. Initiation of mineralization requires the precipitation and attachment of hydroxyapatite crystals ($Ca_{10}[PO_4]_6[OH]_2$) to the ECM [7]. However, the exact mechanism is poorly understood. The osteoblast marker alkaline phosphatase (ALPL) is suggested to play a role in matrix mineralization, where it hydrolyzes organic phosphatase to release free inorganic phosphate [8]. Osteoblasts produce numerous ECM proteins including the most abundant, such as collagen type-I, osteopontin and osteocalcin. This results in a complex mixture of proteins constituting the ECM of which the composition acts as an important determinant of initiation and progression of mineralization [7, 9].

Matrix mineralization is not unique for bone tissue demonstrated by ectopic mineralization in pathological conditions. Vascular smooth muscle cells (VSMCs) are believed to be primarily involved in vascular mineralization. These cells can undergo an osteoblast-like differentiation process expressing osteoblastic factors including ALPL and runt-related transcription factor 2 (RUNX2) [10, 11].

Despite the established requirement of GCs for osteoblast differentiation, little is known about the genes and molecular networks underlying GC-induced osteoblast differentiation and mineralization. Only a few GC target genes are described that are involved in osteoblast function and differentiation. New insights into GC-induced osteoblast differentiation and matrix mineralization might lead to additional bone-building reagents. To identify novel targets to control bone formation and bone quality we generated genome-wide expression profiles of mineralizing cultures and compared it with that of nonmineralizing cultures.

4.2 Results

Glucocorticoids are essential for ECM mineralization

GC-induced osteoblast differentiation and mineralization was studied in detail by using the human pre-osteoblast model SV-HFO, which is a well-characterized osteoblast cell-line showing a controlled GC-induced differentiation process in a 3-week period. In the presence of 10^{-7} M dexamethasone (DEX) or cortisol, an ECM is formed and the process of mineralization is initiated around day 12 [12]. Besides induction of mineralization (Fig. 4.1 A), GC-treatment also strongly induced the osteoblast differentiation marker alkaline phosphatase (ALPL) in SV-HFO osteoblasts (Fig. 4.1 B).

In addition to SV-HFO cultures, GC-induced differentiation and mineralization was studied using primary NHOst osteoblasts. These cells reflected more mature staged

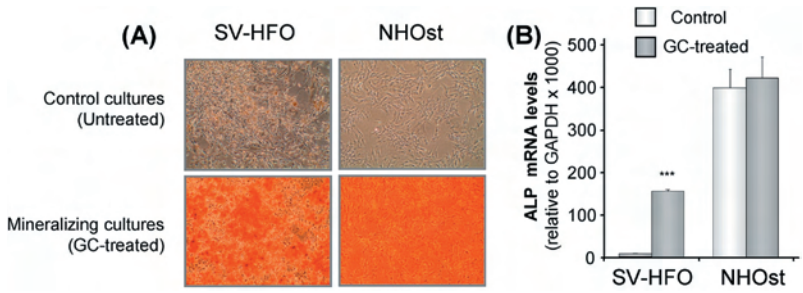

Fig. 4.1. Glucocorticoid-induced osteoblast differentiation and matrix mineralization. Calcium staining **(A)** and ALPL mRNA levels **(B)** of control and mineralizing (GC-treated) SV-HFO (day 19) and NHOst (day 14) cultures. Calcium was stained using Alizarin Red. ***p<0.001 compared to control cultures.

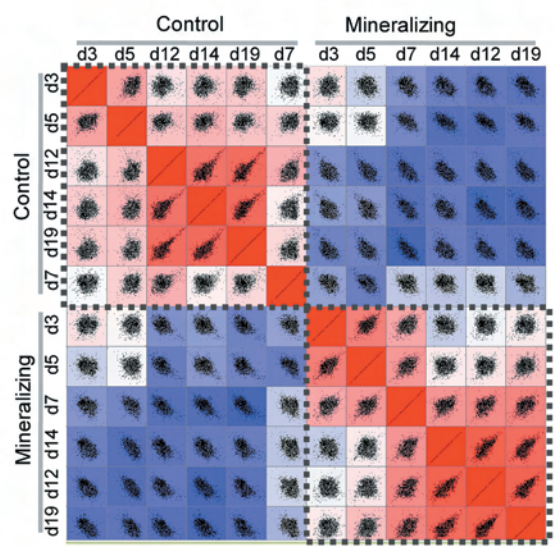

Fig. 4.2. Gene profile correlation plot of control and mineralizing cultures. Correlation plots of Affymetrix HG-U133 Plus 2.0 expression data of control and mineralizing osteoblasts (SV-HFO) at six time-points of culture. Red color indicate positive correlation, blue color indicates negative correlation.

osteoblasts compared to SV-HFO pre-osteoblasts. NHOst had higher basal ALPL mRNA levels compared to non-treated SV-HFO cultures. In contrast to SV-HFO, basal ALPL levels in NHOst were not further enhanced by GC-treatment (10^{-7} M DEX) (Fig. 4.1 B). But importantly, GC-treatment was still essential for the induction of mineralization in NHOst cultures (Fig. 4.1 A). These findings show that GC-treatment triggers a series of molecular events in human osteoblasts that eventually lead to mineralization of the ECM. During the remainder of this study GC-treated and non-GC-treated cultures are designated as mineralizing and control cultures, respectively.

Gene profiling in control and mineralizing osteoblasts

Gene expression profiling experiments were performed to assess the difference in gene expression between osteoblasts that are capable of matrix mineralization and nonmineralizing control osteoblasts. Affymetrix GeneChips were used to analyze both mineralizing and control cultures (SV-HFO) throughout the culture period (days 3, 5, 7, 12, 14, and 19).

These gene expression profiles of control and mineralizing cultures of each analyzed day were compared and ordered based on their correlation (Fig. 4.2). In Figure 4.2 a red color indicates high correlation between samples and a blue color low correlation. Two major blocks can be identified representing control and mineralizing cultures (dotted squares in Fig. 4.2). This demonstrated that control and mineralizing cultures have strongly different gene profiles.

In this paper we focused on the genes that distinguish these mineralizing cultures from control cultures. Relevant genes were identified by selecting probe sets (Affymetrix IDs) that had throughout culture time on average >2-fold higher or lower intensities in mineralizing cultures compared to control cultures. In other words, the average intensities of day 3 to day 19 in mineralizing cultures divided by the average intensities of day 3 to day 19 in control cultures should differ more then 2-fold. By doing this we included genes that were always up- or down-regulated throughout culture time but also genes that were specifically up- or down-regulated in the early or later stages but that reach overall an average a 2-fold difference. This selection query resulted in the identification of 740 probe sets, representing 489 unique genes and 64 non-annotated probe sets. From the 740 selected probe sets, 364 probe sets were up- and 376 probe sets were down-regulated in mineralizing cultures compared to control cultures (data not shown).

Validity of the GeneChip analysis

Validity of the GeneChip data was assessed by comparative quantitative PCR (qPCR) analyses of 34 genes. For this we analyzed RNA isolated from independent experiments not used for the initial GeneChip analysis. The difference in gene expression was assessed as fold-difference (average day 3 to day 19) between control and mineralizing cultures. This fold-difference was calculated following both GeneChip and qPCR analysis and this revealed that a 2-fold change is a robust cut-off. In general, the fold-difference found by qPCR was higher compared to the fold-difference calculated by GeneChip (Fig. 4.3).

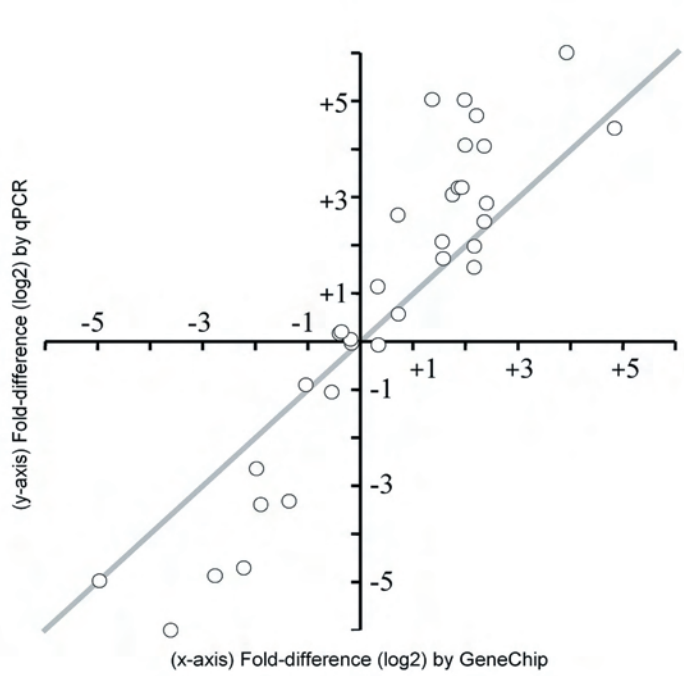

Fig. 4.3 Validity of the GeneChip data. mRNA expression of 34 genes was quantified in control and mineralizing cultures following qPCR and GeneChip. Fold-difference (log2 transformed) between control (average expression of d3 to d19) and mineralizing cultures (average expression of d3 to d19) is plotted. Grey line indicates 100 % correlation between qPCR and GeneChip.

Regulation of bone-related genes

Within the 489 genes being up- or down-regulated in mineralizing cultures compared to control cultures, numerous genes were identified with already known functions in bone biology or which are involved in bone metabolism related pathways or processes. In Table 4.1 a selection of these bone-related genes is presented. The strongest regulated osteoblast-related gene in this list was zinc finger and BTB domain containing 16 (ZBTB16). ZBTB16 was recently identified as a stimulator of osteoblast differentiation and mineralization acting upstream of RUNX2 [13]. qPCR in SV-HFO and NHOst cultures confirmed the GeneChip data and showed that this gene was undetectable in non-mineralizing control cultures. GC-treatment, however, strongly increased ZBTB16 mRNA expression. Moreover, during osteoblast differentiation and mineralization the expression progressively increased (Fig. 4.4 A).

Novel osteoblast-related genes

To identify novel genes involved in osteoblast differentiation and mineralization we selected genes having similar expression profiles as ZBTB16 and had an unknown function in osteoblasts. Six other genes were selected that showed stable low expression in control osteoblasts and, like ZBTB16, were strongly induced in mineralizing osteoblasts. These genes were 1] transmembrane and tetratricopeptide repeat containing 1 (TMTC1), 2] glycoprotein M6B (GPM6B), 3] zinc finger protein 36 (ZFP36 also known as TTP or GOS24),

Table 4.1. Genes differentially expressed in control and mineralizing osteoblasts cultures. Selection of genes, which have to our knowledge known functions in bone biology or which are involved in bone related pathways or processes. Fold difference indicates average intensities of mineralizing cultures (d3 to d19) over average intensities of control cultures (d3 to d19). Genes are presented with their affymetrix ID by which they were identified. (*) The direction of regulation was analyzed and confirmed by qPCR.

	Gene Symbol	Fold Difference
Osteoblast markers		
Alkaline phosphatase, liver/bone/kidney [215783_s_at; 1557924_s_at]	ALPL*	↑ 3.7
Runt-related transcription factor 2 [232231_at]	RUNX2*	↑ 2.1
Osteopontin [209875_s_at]	SPP1*	0.49 ↓
Extracellular matrix regulation		
Matrix metalloproteinase 1 [204475_at]	MMP1*	0.032 ↓
Matrix metalloproteinase 2 [201069_at]	MMP2	0.27 ↓
Matrix metalloproteinase 3 [205828_at]	MMP3	0.053 ↓
Matrix metalloproteinase 14 [217279_x_at; 202827_s_at]	MMP14	0.39 ↓
Matrix metalloproteinase 19 [204575_s_at]	MMP19	↑ 2.2
ADAM metallopeptidase with thrombospondin type 1 motif 2 [236901_at; 214454_at]	ADAMTS2	↑ 2.5
Tissue inhibitor of metalloproteinase 3 [201149_s_at]	TIMP3	0.42 ↓
Tissue inhibitor of metalloproteinase 4 [206243_at]	TIMP4*	↑ 28
Growth factor related		
Insulin-like growth factor binding protein 2 [202718_at]	IGFBP2	↑ 5.2
Insulin-like growth factor binding protein 5 [211958_at; 203425_s_at; 1555997_s_at; 211959_at]	IGFBP5	0.20 ↓
Transforming growth factor, beta 2 [228121_at; 209909_s_at; 220407_s_at]	TGFB2	↑ 2.6
BMP-binding endothelial regulator precursor protein [241986_at]	BMPER	0.37 ↓
Noggin [231798_at]	NOG	0.34 ↓
Interleukin 1, alpha [210118_s_at]	IL1A	0.074 ↓
Interleukin 1, beta [205067_at; 39402_at]	IL1B	0.053 ↓
Interleukin 6 (interferon, beta 2) [205207_at]	IL6	0.076 ↓
Wnt signaling		
Low density lipoprotein receptor-related protein 6 [205606_at]	LRP6	↑ 2.2
Frizzled homolog 8 [227405_s_at; 224325_at]	FZD8*	0.26 ↓
Secreted frizzled-related protein 1 [202037_s_at; 202035_s_at; 202036_s_at]	SFRP1*	0.21 ↓
Prostaglandin related		
Prostaglandin E receptor 2 [206631_at]	PTGER2*	↑ 5.3
Prostaglandin E receptor 4 [204896_s_at; 204897_at]	PTGER4*	↑ 4.5
Prostaglandin-endoperoxide synthase 2 (COX-2) [204748_at; 1554997_a_at]	PTGS2*	0.15 ↓
Prostaglandin E synthase [207388_s_at; 210367_s_at]	PTGES*	0.22 ↓
Prostaglandin D2 synthase [211748_x_at]	PTGDS*	↑ 3.51
Others		
Leptin receptor [209894_at; 227095_at; 211354_s_at; 211356_x_at; 211355_x_at]	LEPR	↑ 2.7
Natriuretic peptide receptor C/guanylate cyclase C [219790_s_at; 219789_at]	NPR3	↑ 9.5
Zinc finger and BTB domain containing 16 [205883_at]	ZBTB16*	↑ 15
Hydroxysteroid (11-beta) dehydrogenase [205404_at]	HSD11B1*	0.26 ↓

4] eyes absent homolog 2 (EYA2), 5] dishevelled associated activator of morphogenesis 2 (DAAM2) and 6] the cannabinoid receptor 1 (CNR1). Next, mRNA expression was quantified by qPCR in control and mineralizing osteoblast cultures (SV-HFO and NHOst). This showed that TMTC1 and GPM6B had similar expression profiles as ZBTB16 in both osteoblast models (Figs. 4.4 B and 4.4 C). The ZFP36 expression pattern was less clear, but it was increased at all time points compared to the control cultures (Fig. 4.4 D). Expression of EYA2 and DAAM2 increased strongly in mineralizing SV-HFO cultures; however, in the NHOst cultures no induction was observed (Figs.4.4 E and 4.4 F). The expression pattern of CNR1 could be confirmed in both osteoblast models. This gene was specifically up regulated during stages of mineralization, whereas the expression was low and stable in control osteoblasts (Fig. 4.5 A). Since several reports recently indicated a role of cannabinoid receptors 1 and 2 in bone [17-19], this gene was studied in more detail in human osteoblasts.

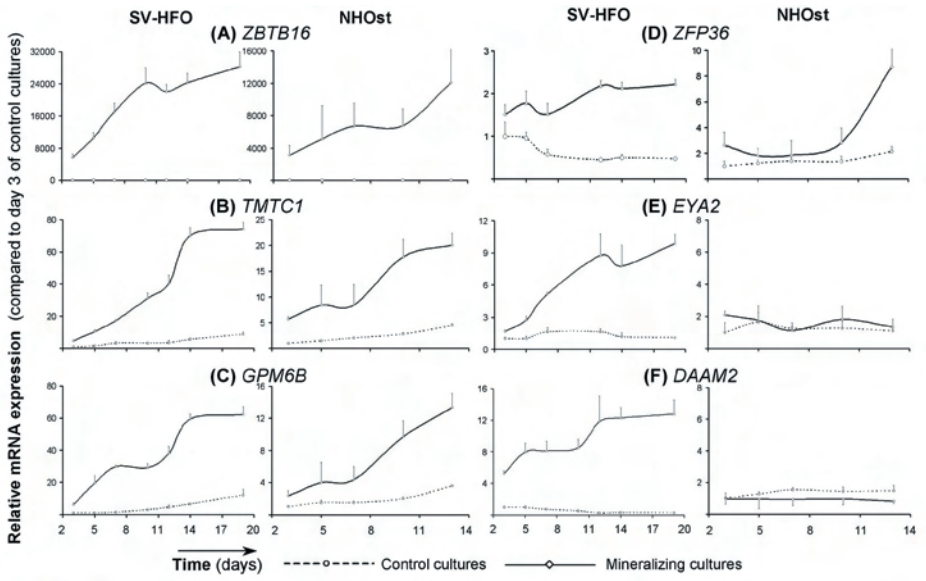

Fig. 4.4. Expression pattern of ZBTB16 and novel osteoblast genes during osteoblast differentiation. **(A)** ZBTB16 mRNA expression in control and mineralizing cultures of SV-HFO and NHOst. In addition, 5 other genes with similar expression patterns as ZBTB16 were analyzed in control and mineralizing cultures of SV-HFO and NHOst **(B-F)**. Graphs show relative mRNA expression compared to day 3 of control cultures determined by qPCR.

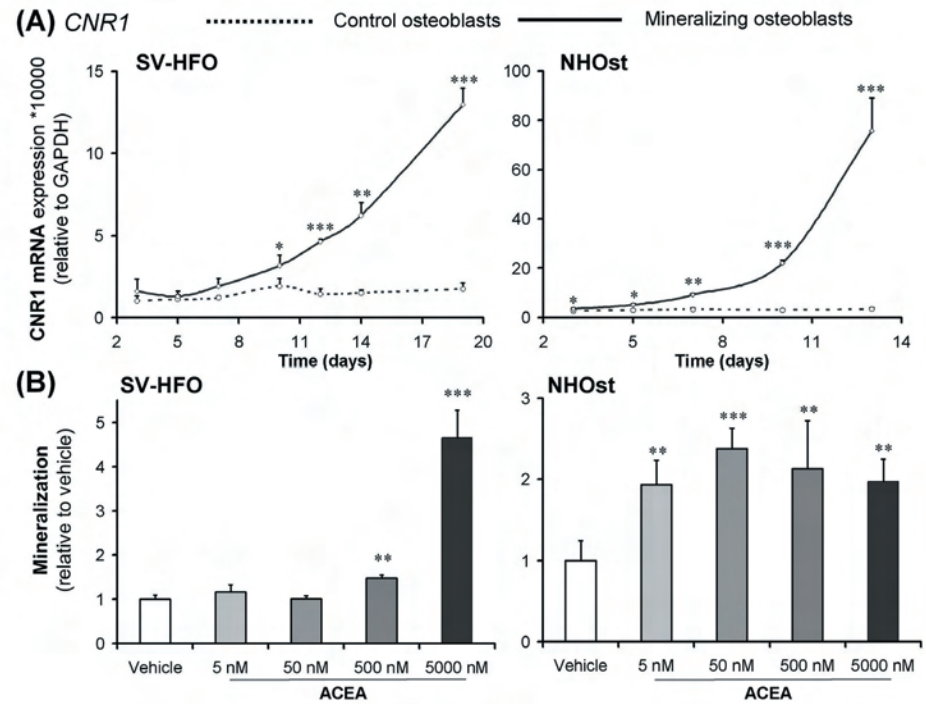

Fig. 4.5. CNR1 expression and functionality in human osteoblasts. **(A)** CNR1 mRNA expression in control and mineralizing osteoblasts cultures determined by qPCR. *p<0.05; **p<0.01; ***p<0.001 compared to control cultures at indicated time points. **(B)** Quantification of mineralization (day 16) in osteoblasts cultures that were continuously treated with various concentrations of the specific CNR1 agonist ACEA. *p<0.05; **p<0.01; ***p<0.001 compared to vehicle.

Table 4.2. List of networks derived using Ingenuity Pathway Knowledge database. Input genes were 98 genes that were differentially expressed in control and mineralizing osteoblast cultures and were annotated with the GO term development. Score: indicates the enrichment of the network. Top functions: indicates the kind of processes these genes have been shown to be involved in. Data based on ingenuity version 4.0. ↑ > 2.0-fold up-regulated, ↓ > 2.0-fold down-regulated in mineralizing cultures compared to control cultures.

	Genes	Score	Top functions
1	**ANGPT1**↑, **ANKH**↓, **CCL2**↓, **COL8A1**↑, **CSF2**↓, **DCN**↑, **ENG**↓, **FOXC2**↑, **FZD8**↓, **HTRA1**↑, **IL6**↓, **IL8**↓, **IL11**↓, **INHBA**↓, **JAG1**↑, **KLF6**↑, **LIF**↓, **LRP6**↑, **NOG**↓, **NRP2**↓, **PPARG**↑, **PTGS2**↓, **RUNX2**↑, **SEMA3C**↓, **SFRP1**↓, **SMTN**↓, **SNAI2**↑, **SPP1**↓, **TGFB2**↑, **THBS1**↑, **TIE1**↓, **TLR4**↑, **VEGF**↓, **WISP1**↓, **ZBTB16**↑	75	Cellular Movement, Tissue Development, Organismal Development
2	**ADAM10**↑, AFP, ALOX5AP, **ALPL**↑, **ANGPTL4**↑, ARG1, CCR7, CTNNB1, **EDIL3**↑, **ESM1**↓, F2, FCGR1A, FGF18, **FOXE1**↓, FPRL1, HAS1, IL32, IL23A, IL3RA, INDO, **KRT34**↓, **LAMA4**↓, **LAMB3**↓, **LEPR**↑, **LRP6**↑, **MEOX1**↓, NLK, **NRCAM**↑, **SEMA3C**↓, **SMTN**↓ **SOCS4**↑, STAT3, TGFB1, TNF, **TNFAIP2**↓	26	Cellular Movement, Connective Tissue Disorders, Inflammatory Disease
3	ALOX5, ANG, AQP4, **BHLHB3**↓, **CLEC3B**↑, **COL11A1**↑, **COL12A1**↑, DAD1, **DMD**↑, **EYA2**↑, G3BP, **HMGA1**↓, IDI1, **IER3**↓, **IGFBP2**↑, KCNJ4, **KLF6**↑, KRT19, **LDB2**↓, LIMK2, MBOAT5, MMP2, MYOD1, **NRG1**↓, PCSK6, PLG, RNF4, SIM1, **SIX1**↑, SLC2A3, SP1, **SPOCK1**↓, TGFB1, THBS2, **VEGFC**↓	22	Cancer, Cardiovascular System Development and Function, Tumor Morphology
4	**ADARB1**↑, **ARHGDIB**↓, CCNB2, CDC42, **CDC42EP3**↑, CEACAM5, COL5A1, **COL5A3**↑, CSDA, **CXCL1**↓, **EBF**↑, EP300, **FBN2**↑, FN1, GART, HMGN2, **ICK**↑, ISGF3G, LY6A, **MAFB**↓, MFAP2, **MMP19**↑, MYC, **NDRG1**↓, NFYB, NOSIP, PPP1R15A, PTTG1, RRM2, **SOX9**↓, TOP2A, TP53, UBE2C, YY2, **ZIC2**↓	18	Cell Cycle, DNA Replication, Recombination, and Repair, Cancer
5	**ADAM12**↓, AGT, CCR7, CD38, **CSF3**↓, **EHF**↓, **EPHB2**↓, **FGD4**↑, FPR1, **GPM6B**↑, HDC, HTRA2, IL4, IL25, **ITGA2**↓, **KCNMA1**↓, LTB, LY96, MAPK1, MAPK8, NCF1, **NOTCH3**↑, **NOV**↓, **NPR3**↑, PLA2G10, PLSCR1, **PTPRR**↓, SH3PXD2A, **SPHK1**↓, SRC, ST5, TFF3, TIMP1, TNFRSF11A, TPSD1	18	Cellular Movement, Cellular Growth and Proliferation, Immune Response

Cannabinoid receptor 1

The functionality of CNR1 in osteoblasts was tested using the specific CNR1 agonist ACEA (Arachidonyl-2'-chloroethylamide) [20]. SV-HFO and NHOst osteoblasts were cultured in the presence of 5, 50, 500 and 5000 nM ACEA. Mineralization and ALPL activity was measured at day 16 of culture. ACEA-treatment stimulated matrix mineralization in osteoblasts cultures at concentrations higher than 5 nM (Fig. 4.5 B). ALPL activity and cell number (DNA content) remained unchanged after treatment (data not shown).

Gene Ontology and gene network analysis

The biological relevance of the 489 selected genes was examined by Gene Ontology (GO) enrichment analysis. The 489 genes were categorized for: GO Biological Process (Fig. 4.6 A), GO Molecular Function (Fig. 4.6 B), and GO Cellular Component (Fig. 4.6 C) [21]. In total, we identified 106 biological processes, 28 molecular functions, and 9 cellular components being significantly over-represented compared to what would

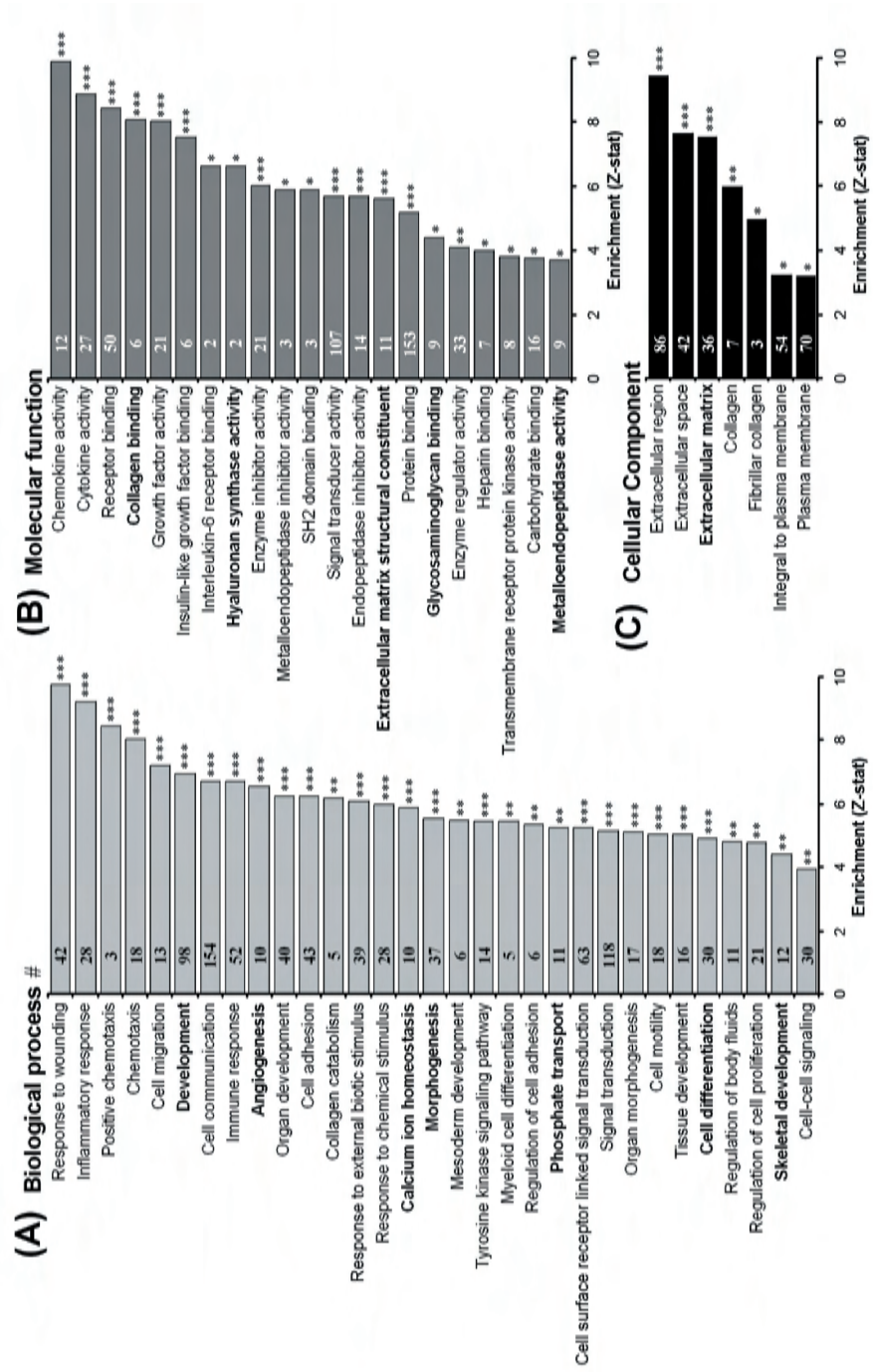

Fig. 4.6. Gene Ontology (GO) enrichment analysis. Enriched GO terms found within the set of 489 differentially expressed genes (between control and mineralizing cultures) for GO Biological Process **(A)**, GO Molecular Function **(B),** and GO Cellular Component **(C)**. The z-score indicates the standardized enrichment score. Numbers inside the bars indicate number of genes from the set of 489 differentially expressed genes determining the GO terms. # Number of GO terms was reduced by exclusion of several overlapping GO terms. *p<0.05; **p<0.01; ***p<0.001

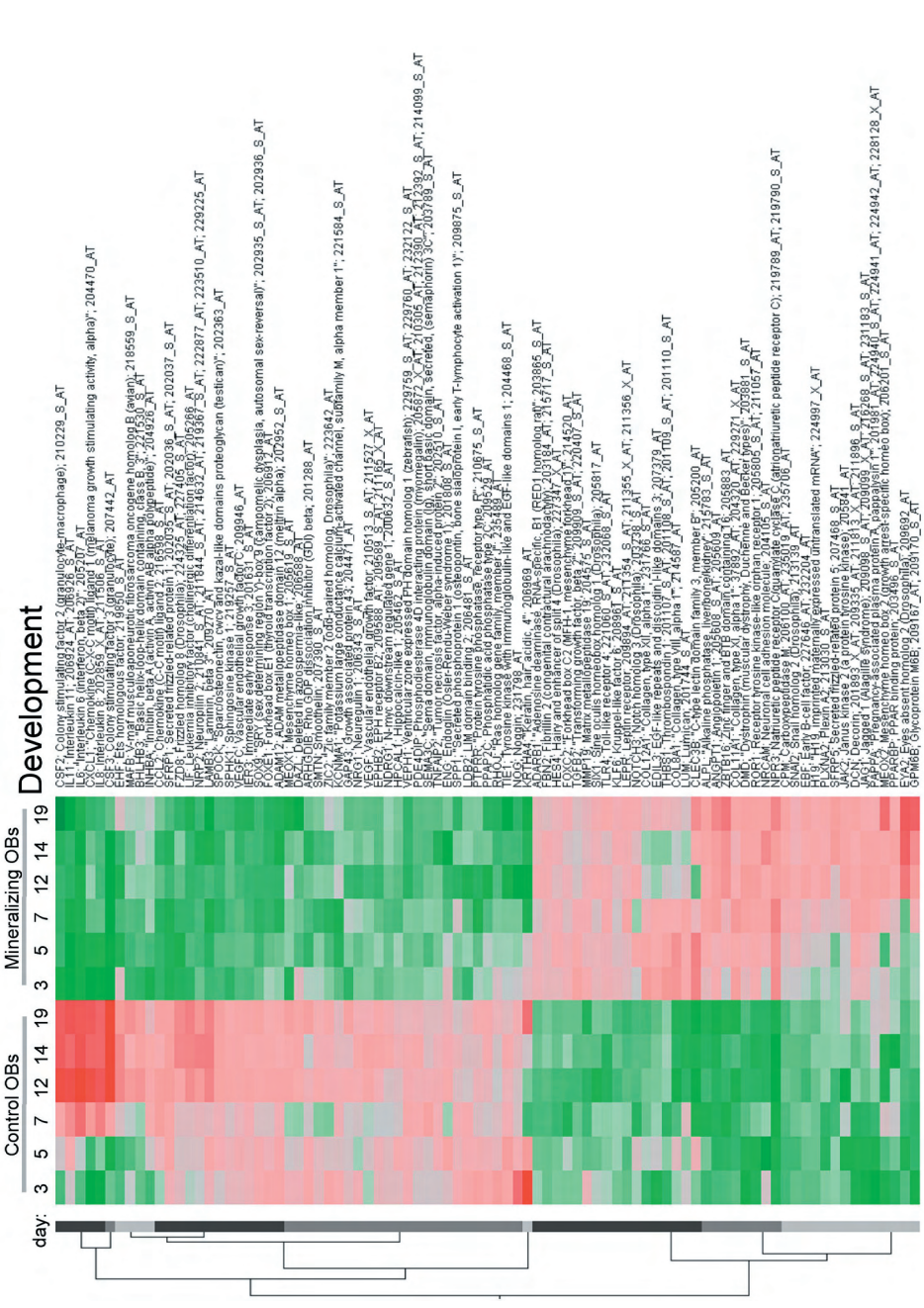

Fig. 4.7. Developmental genes regulated by GC-induced differentiation. Hierarchical clustering of 98 genes annotated with the GO-term development that were differentially expressed in control and mineralizing cultures. Red color indicates increased expression and green color indicates decreased expression compared to the geometric mean. Genes are annotated with their gene symbol, gene name and probe sets by which they were identified.

be expected if 489 randomly selected genes were analyzed.

The identification of differentiation-related processes including development, morphogenesis, angiogenesis and cell differentiation are intuitively logical considering the cell models used as are the processes calcium ion homeostasis and phosphate transport (Fig. 4.6 A). They are of great interest as these processes might contain novel important regulatory genes for osteoblast differentiation. Within the 489 differentially expressed genes 98 genes were annotated with the GO term development, which are depicted in Fig. 4.7 The relationship and interaction between the 98 development genes was analyzed and visualized using Ingenuity Pathway analysis. This software

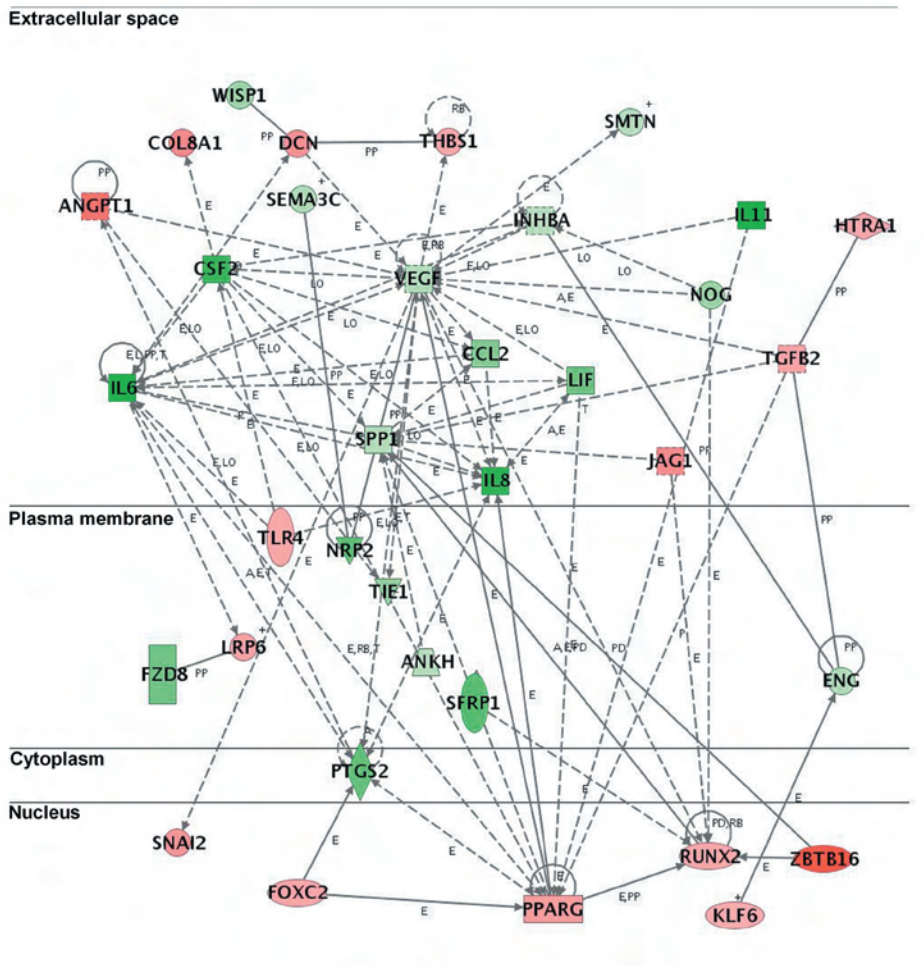

Fig. 4.8. Gene network 1 identified by Ingenuity Pathway Analysis based on the 98 development genes (annotated with GO biological process term development) differentially expressed in control andmineralizing cultures. All genes within this gene-network are differentially expressed indicated by their red (up in mineralizing cultures) or green (down in mineralizing cultures) color. Solid lines = direct interaction. Dotted lines = indirect interaction. A = activation / deactivation; RB = regulation of binding; PR = protein mRNA binding; PD = protein DNA binding; B = binding; E = expression; I =inhibition; L = proteolysis; M= biochemical modification; O = other; P= phosphorylation /desphosphorylation; T = transcription; LO = localization

tool generates gene networks based on known gene-to-gene interactions. Analysis of the 98 development genes resulted in de identification of five enriched gene networks that are shown in Table 4.2. Gene network 1 was strongly enriched since all 35 genes in this network were differentially expressed in control and mineralizing cultures. This gene network is demonstrated in Fig. 4.8 and contains genes such as RUNX2, ZBTB16, interleukin 6 and 8 (IL6/8), vascular endothelial growth factor (VEGF), low density lipoprotein receptor-related protein 6 (LRP6), decorin (DCN), and transforming growth factor beta 2 (TGFB2) in interaction with other known but also many novel bone-related genes. In addition to these developmental genes, the complete data set of 489 genes was used to generate interacting gene networks. In total, eight strongly enriched gene networks were identified which are shown in Table 4.3.

The GO Molecular Function analysis identified several enriched functions intuitively logical and of interest for bone formation such as collagen binding, hyaluronan synthase activity, metalloendopeptidase activity, extracellular matrix structural constituent, and glycosaminoglycan binding (Fig. 4.6 B). Following this the analysis for GO Cellular Component showed that, genes located in the extracellular region, space or matrix were significantly enriched (Fig. 4.6 C). In total, 36 genes annotated with ECM were differentially expressed in control and mineralizing cultures (Fig. 4.9 A).

Regulation of ECM genes

We analyzed 5 up-regulated and 5 down-regulated ECM genes in more detail by qPCR in control and mineralizing cultures of SV-HFO and NHOst (Fig. 4.9 B). First, this showed that the expression of ECM genes like; metallopeptidase inhibitor 4 (TIMP4); C-type lectin domain family 3, member B (CLEC3B, also known as tetranectin), laminin beta 3 (LAMB3) and matrix metallopeptidase 1, 2 and 3 (MMP1/2/3) were strongly regulated (> 10-fold) in mineralizing osteoblasts. Secondly, it showed that the majority of the ECM genes were similarly regulated in NHOst and SV-HFO osteoblasts. The regulation of matrix metallopeptidase 3 (MMP3) and hyaluronan synthase 1 (HAS1), however, showed discrepancies between SV-HFO and NHOst cultures. This might be explained by the already low expression of HAS1 and MMP3 in control NHOst cultures, whereas in SV-HFO the basal expression was higher but strongly decreased by GC-treatment (Fig. 4.9 C). This supports the notion of a more mature phenotype of NHOst compared to SV-HFO, which is in line with our conclusion on basis of difference in basal ALPL activity.

Direct DEX gene regulation

All genes discussed in this paper were identified according to their differential expression between control and mineralizing cultures upon continuous treatment with DEX. Using this selection, the difference in gene expression might be caused by two processes; 1] direct gene regulation by DEX-treatment or 2] indirect as a consequence of the GC-induced osteoblast differentiation program. It is difficult to discriminate between these two processes since continuous presence of DEX is necessary to induce differentiation and mineralization. Nevertheless, several culture conditions were created in which we aimed to reveal whether a gene is directly regulated by DEX or indirectly as a consequence of GC-induced differentiation (Fig 4.10 A).

Table 4.3. List of networks derived using Ingenuity Pathway Knowledge database. Input genes were the 489 genes that were differentially expressed in control and mineralizing osteoblast cultures. Score: indicates the enrichment of the network. Top functions: indicates the kind of processes these genes have been shown to be involved in. Data based on ingenuity version 4.0. ↑ > 2.0-fold up-regulated, ↓ > 2.0-fold down-regulated in mineralizing cultures compared to control cultures.

	Genes	Score	Top functions
1	**CCL2**↓, **CCL3**↓, **CCL7**↓, **CCL8**↓, **CTSS**↓, **CXCL3**↓, **CXCL5**↓, **CXCL6**↓, **EDG7**↓, **F2RL1**↓, **FZD8**↓, **GBP2**↓, **HTRA1**↑, **IGFBP2**↑, **IGFBP5**↓, **IL6**↓, **IL1A**↓, **IL1RN**↓, **LIFR**↑, **LRP6**↑, **MMP1**↓, **MMP2**↓, **MMP3**↓, **MMP14**↓, **NFKBIZ**↓, **PAPPA**↑, **PROS1**↑, **PTGES**↓, **SAA1**↑, **SOD2**↓, **SPP1**↓, **STOM**↑, **THBS2**↓, **TIMP3**↓, **TIMP4**↑	53	Immune Response, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
2	**AMPD3**↓, **ANGPT1**↑, **AREG**↑, **BDKRB1**↓, **BDKRB2**↓, **CCL20**↓, **CD44**↓, **CSF3**↓, **EDN1**↓, **ESM1**↓, **FOXC2**↑, **G0S2**↓, **GCNT1**↑, **H19**↑, **HAS1**↓, **HSD11B1**↓, **ICAM1**↓, **IL8**↓, **IL24**↓, **IL1B**↓, **IL1RAP**↑, **KLF2**↓, **ODC1**↓, **PBEF1**↓, **PTGDS**↑, **PTGER2**↑, **PTGER4**↑, **PTGS2**↓, **PTPRB**↓, **SERPINB2**↓, **SPINT1**↓, **TEK**↓, **TIE1**↓, **TNFAIP2**↓, **VEGFC**↓	53	Cardiovascular System Development and Function, Organismal Injury and Abnormalities, Cell-To-Cell Signaling and Interaction
3	**C5ORF13**↑, **DCN**↑, **DPP4**↓, **EDNRA**↑, **ENG**↓, **FAP**↓, **FBLN1**↑, **FN1**↑, **INHBA**↓, **ITGB8**↓, **JAG1**↑, **KLF6**↑, **LAMA4**↓, **MMP19**↑, **NID2**↑, **NOG**↓, **NOV**↓, **NRP2**↓, **PDGFRB**↑, **PLAU**↓, **PLAUR**↓, **RUNX2**↑, **SEMA3C**↓, **SFRP1**↓, **SMTN**↓, **SNAI2**↑, **STC1**↓, **TFPI2**↓, **TGFB2**↑, **TGFBI**↓ **THBS1**↑, **TNC**↓, **TNFAIP6**↓, **VEGF**↓, **WISP1**↓	53	Cellular Movement, Cellular Development, Cellular Growth and Proliferation
4	**ANGPTL4**↑, **BCL2A1**↓, **BHLHB3**↓, **CCND1**↓, **CDCP1**↓, **CDKN2B**↑, **COL8A1**↑, **CSF2**↓, **F2RL2**↓, **FGF7**↓, **HAS2**↓, **HIPK2**↑, **HMGA1**, **IER3**↓, **IL11**↓, **KIT**↑, **KITLG**↑, **KLF9**↑, **KLHL24**↑, **LAMB3**↓, **MGLL**↓, **NF1**↑, **NRG1**↓, **PER1**↑, **PODXL**↓, **PPARG**↑, **PRKG2**↓, **PTPRR**↓, **RPS6KA2**↑, **SDC1**↓, **SOX9**↓, **TNFSF15**↓, **TSC22D3**↑, **ZFP36**↑, **ZFP36L2**↑	53	Cellular Growth and Proliferation, Cancer, Cell Cycle
5	**ABTB1**↓, **ALPL**↑, **ANGPT1**↑, CASP3, CASP4, CDH1, **COL11A1**↑, **CSRP1**↓, **DNAJB4**↑, DRAP1, DSG1, **FKBP5**↑, G3BP, GCLC, **IER3**↓, ITGAE, **JAG1**↑, **KCNIP3**↑, KRT19, MT2A, NEU3, **PBEF1**↓, **PKP2**↑, PLOD1, PTEN, **SEPT4**↑, **SFRS2IP**↑, SLC20A1, **SLC7A11**↑, SNRP70, **STARD10**↓, **TCF8**↑, TGFB1, TNFRSF10A, TP73L	16	Cancer, Cell Death, Hepatic System Disease
6	**CLEC3B**↑, **CXCL1**↓, **CXCL2**↓, **CXCL12**↓, CYP3A5, DIO1, **DPP4**↓, ↑**EBF**, EP300, ETV1, FPRL1, **GPM6B**↑, **HCLS1**↓, HGF, IL4, **ISG20**↓, KLKB1, LAMA3, **MAOA**↑, **MMP2**↓, MMP10, MN1, **NCOA3**↑, NCOA6IP, NR4A3, PCMT1, PIGR, **PKIB**↓, PLG, **PPARBP**↑, **PRNP**↓, S100A9, **SPOCK1**↓, **SPSB1**↓, TXK	16	Cell-To-Cell Signaling and Interaction, Cellular Movement, Hematological System Development and Function
7	**ADAM12**↓, AKT1, ARHGDIA, **C21ORF7**↓, CCL19, CDC42, **CDC42EP3**↑, CFL1, CTSL2, **CXCL1**↓, CYBB, **DOCK9**↑, **FGD4**↑, **FLJ20701**↓, **FNBP1L**↑, GAS6, HOMER2, IL13, IL8RB, **ITGA10**↑, ITGB1, ITGB1BP1, LGALS3, MUC5AC, **NEDD4L**↓, **NEXN**↑, **PLEK2**↓, **RGS3**↓, SEPT7, **SEPT11**↑, **SLC16A6**↓, SLC3A2, **TEK**↓, **TGM2**↓, YWHAZ	16	Cellular Movement, Cell-To-Cell Signaling and Interaction, Tissue Development
8	ADCYAP1, ANXA2, **AREG**↑, **CA12**↓, CFL1, **CNN1**↓, **CORO2B**↓, CTSL2, DACH1, **DYSF**↓, **EFEMP1**↑, FOS, **FOXD1**↓, **GAS1**↑, GPX1, **IER3**↓, **IL11**↓, INSR, MKL1, **MYOCD**↓, **NDRG1**↓, NMI, **PSG2**↓, **RGC32**↑, RHOB, RPS9, S100A10, SMAD4, TERT, TK1, **TNC**↓, VHL, XBP1, **ZFP36**↑, **ZNF33A**↑	16	Cell Death, Gene Expression, Cellular Development

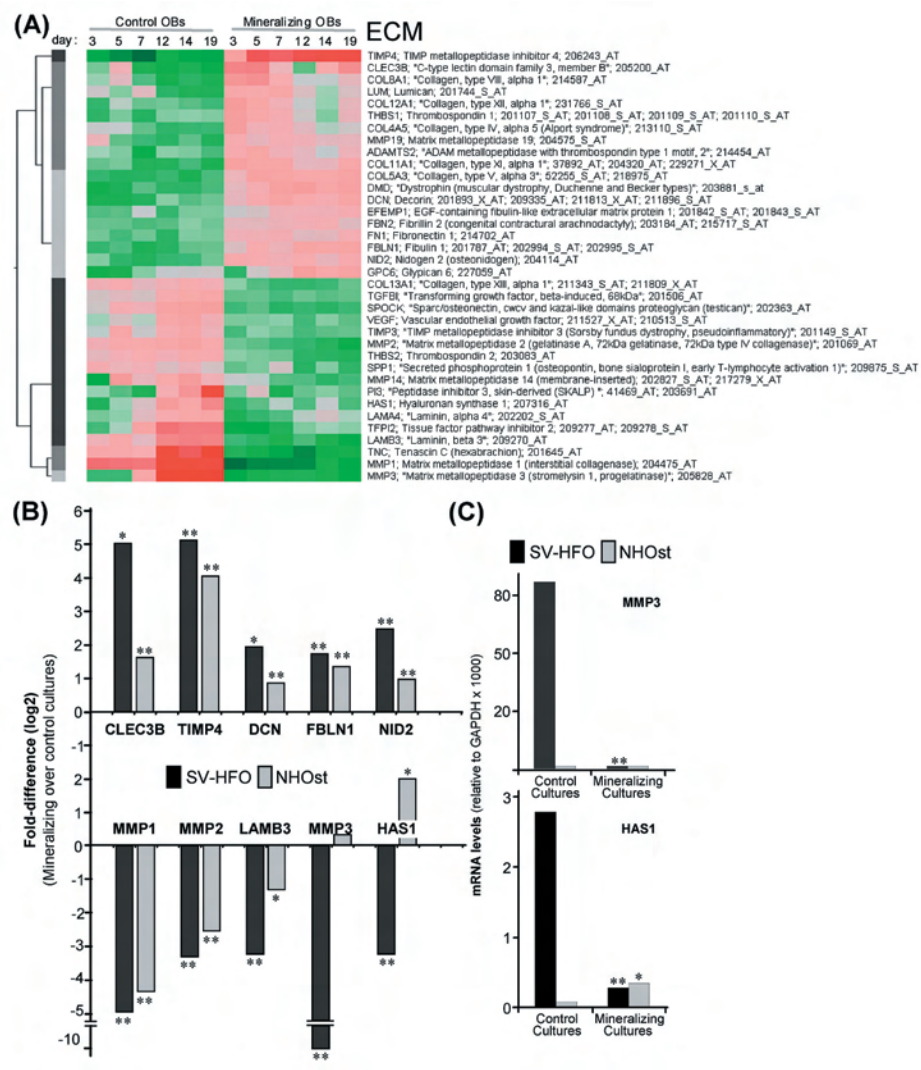

Fig. 4.9. ECM genes differentially expressed in control and mineralizing osteoblasts. **(A)** Hierarchical clustering of 35 genes annotated with ECM, which were differentially expressed in control and mineralizing cultures (SV-HFO). Red color indicates increased expression and green color indicates decreased expression compared to the geometric mean. Color intensities indicate the magnitude of regulation. Genes are annotated with their gene symbol, gene name and probe sets by which they were identified. **(B)** mRNA expression of a selection of ECM genes shown in Fig. 4.9 A.Gene expression was quantified by qPCR in control and mineralizing cultures of SV-HFO and NHOst. Difference in gene expression is presented as the fold-difference (log2 transformed) between mineralizing and control cultures (average expression of d5 to d19). **(C)** mRNA expression of HAS1 and MMP3 in control and mineralizing osteoblasts (SV-HFO and NHOst). *p<0.05; **p<0.01 compared to control cultures.

Gene expression was measured at day 14 in control cultures that were treated with DEX for 3, 6, or 24 h and at day 14 in cultures that were continuously treated with DEX (Figs. 4.10 B-E). In addition, gene expression was measured in cultures that were treated with DEX for 10 days to induce differentiation followed by exclusion of DEX for the final 4 days before RNA isolation at day 14 of culture (Fig. 4.10 F). This showed that several genes were direct DEX target genes showing up-regulation already after

3h of DEX-treatment. The most sensitive gene for DEX was ZBTB16, which showed a 83-fold increase after 3h of DEX-treatment increasing up to 2000-fold after 24h. Other genes were only significantly regulated after 6h or 24h of DEX-treatment. Excluding DEX for the final 4 days showed that the expression of the majority of genes was still changed compared to control cultures even in the absence of DEX. Together, these data demonstrated that we identified direct DEX target-genes (*e.g.*, ZBTB16, ZFP36 and

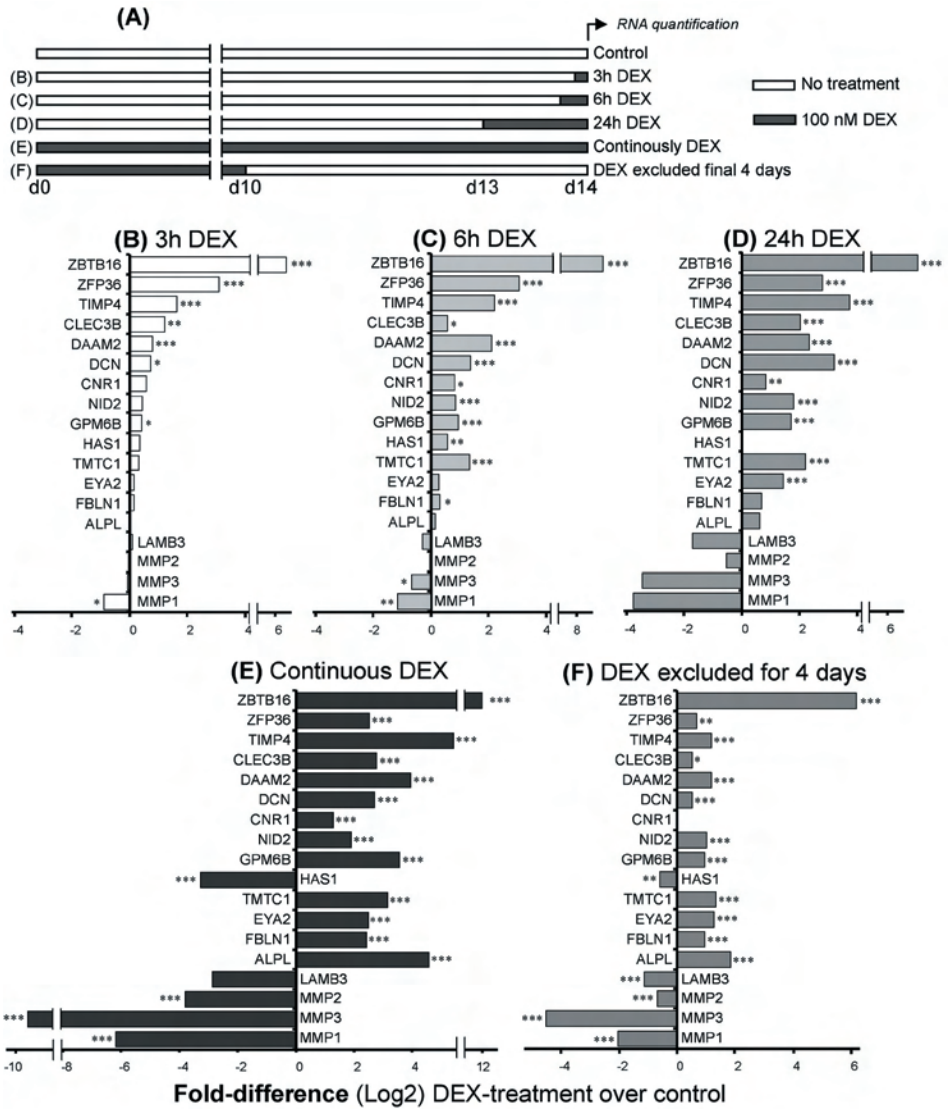

Fig. 4.10. mRNA expression after short and long term DEX-treatment. Gene expression at day 14 of osteoblast culture (SV-HFO) after DEX treatment quantified by qPCR. **(A)** DEX incubation scheme for figures 4.10 B-F. Cultures were treated for **(B)** 3 hours (3h), **(C)** 6 hours (6h), **(D)** 24 hours (24h) and **(E)** continuously with DEX at day 14 of culture. In addition, cultures were treated with DEX for 10 days to induce differentiation and subsequently DEX was excluded for 4 days before RNA isolation at day 14 **(F).** *p<0.05; **p<0.01; ***p<0.001 compared to control cultures.

TIMP4), genes that were primarily regulated in a differentiation dependent manner (*e.g.*, ALPL, EYA2 and MMP2) and genes that are probably regulated by combination of both processes (*e.g.*, HAS1).

Vascular smooth muscle cell induced ECM mineralization

To extend the observations beyond skeletal development we used a vascular smooth muscle cell-based model for vascular calcification [22, 23]. Human VSMCs were cultured in an

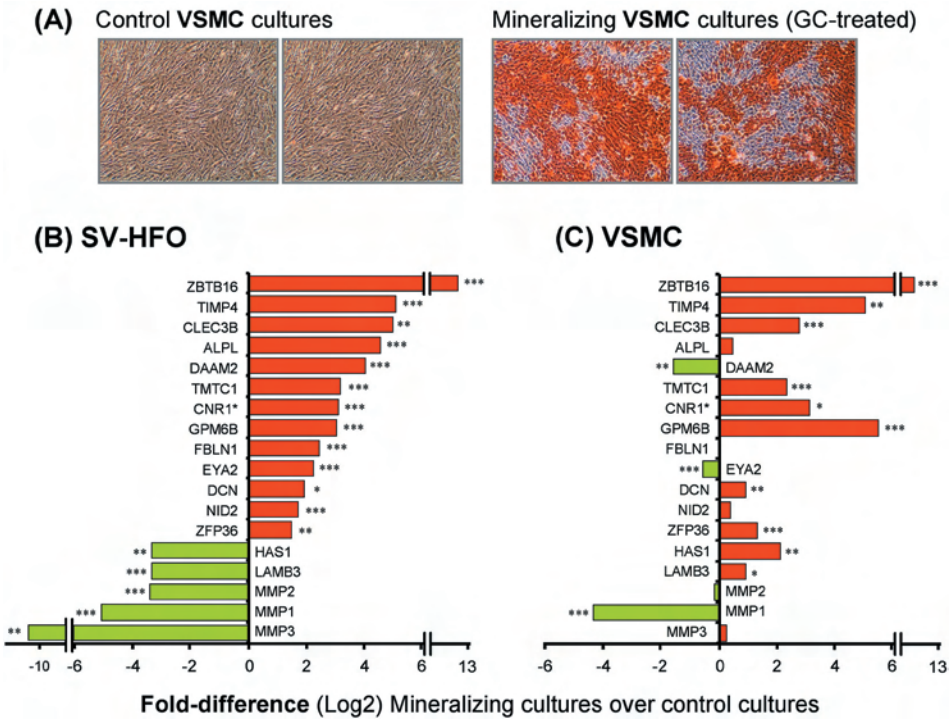

Fig. 4.11. Comparative analysis of osteoblast and VSMC induced matrix mineralization. **(A)** VSMCs were cultured in osteogenic medium with and without DEX. After three weeks of culture cells were stained for calcium using Alizarin Red. **(B-C)** Gene regulation in control and mineralizing osteoblast (SV-HFO) and VSMC cultures. Difference in gene expression is presented as the folddifference (log2 transformed) between mineralizing (average expression of d3 to d19) and control cultures (average expression of d3 to d19). *p<0.05; **p<0.01; ***p<0.001 compared to control cultures.

osteogenic medium containing DEX (10^{-7} M). Using this osteogenic medium mineralization of the ECM could be detected in the third or fourth week of culture (Fig. 4.11 A). Importantly, if DEX was excluded from the culture medium no mineralization of the ECM was observed. This suggest that matrix mineralization in VSMC is triggered via a similar mechanism as observed in osteoblasts. To reveal if a similar set of genes was differentially expressed in VSMC compared to osteoblasts, we quantified mRNA expression in control and mineralizing VSMCs (Fig. 4.11 C). In total, the mRNA expression of 18 genes was quantified using qPCR. This revealed a large overlap in gene regulation between osteoblasts and VSMCs. However, there were also some discrepancies in gene regulation between VSMCs and osteoblasts (*e.g.*, DAAM2, FBNL1, LAMB3 and MMP2).

4.3 Discussion

The current study identified processes, molecular networks and novel targets in the control of osteoblast differentiation and bone formation and identified novel GC target genes in osteoblasts. Comparative analyses demonstrated overlap and discrepancies between bone and atherosclerosis-related mineralization. Although GCs are crucial for human osteoblast differentiation and mineralization surprisingly little is known about the mechanism behind these processes. In relation to osteoblast differentiation several microarray studies have been performed, however, mainly in rodent models [21-24]. Murine osteoblast models are frequently used to study the effects of GCs in relation to GC-induced osteoporosis and data about GCs are therefore interpreted from a totally different point of view [25]. Only a limited number of microarray studies have been performed using human cells, which do not focus on GC-induced differentiation [13, 26]. The human pre-osteoblast SV-HFO model proved to be a good model to identify regulatory genes involved in bone formation. In order to confirm data found with SV-HFO cultures, human NHOst cultures were used. These osteoblasts represent a more mature staged osteoblast, exemplified by high basal ALPL activity. Nevertheless, GC-treatment was still necessary to induce matrix mineralization in these NHOst cultures. This indicates that GC-treatment triggers early osteoblast differentiation (SV-HFO) but is still required for matrix synthesis and mineralization by more mature staged osteoblasts (NHOst).

The presented study is unique in that it compares gene profiles of human osteoblast cultures induced by GCs to differentiate and mineralize with that of nonmineralizing control cultures. These two extreme conditions were exploited to select relevant genes involved in bone formation. This proved to be a powerful tool in selecting genes of biological relevance, as demonstrated by the identification of numerous known osteoblast-related genes such as RUNX2, ALPL and ZBTB16. ZBTB16 is a transcriptional repressor involved in cell cycle control and has been shown to have pleiotropic functions including limb development [27-29]. The identification of ZBTB16 was interesting since it was recently identified as a crucial factor for matrix mineralization in human mesenchymal stem cell cultures acting upstream of RUNX2 [13]. In lymphoblastic leukemia and in human stromal and smooth muscle cells ZBTB16 was shown to be a direct target of GCs [30, 31]. We showed that in human osteoblasts this gene is also a direct GC-target and might be an important mediator of GC-induced differentiation and mineralization.

Several genes that paralleled the expression pattern of ZBTB16 and that were so far unknown to be expressed in osteoblasts or to have a function in osteoblasts were studied in more detail. The expression patterns of TMTC1, GPM6B and ZFP36 in the SV-HFO model were also found in the more mature NHOst differentiation model, *i.e.* increase during differentiation and mineralization. Currently no data on expression of TMTC1 in other cells or on function of TMTC1 are available. GPM6B is only characterized regarding its expression in neurons and glia [32]. ZFP36 is a transcriptional regulator involved in the development of various organs and tissues. Mice deficient for ZFP36 have been generated and showed an autoimmunity phenotype including severe arthritis but no other clear skeletal phenotype [33]. For EYA2 and DAAM2 the expression pattern in NHOst

cells was different from that in SV-HFO. No increase in expression during mineralization was observed. Whether this is related to the initial difference in maturity between these osteoblast differentiation models is not clear. For DAAM2 no expression or functional data are available yet. EYA2 is a transcriptional regulator highly homologous to EYA1 which appears to be a part of the group of developmental genes such as PAX6, GLI3, SOX9 and sonic Hedgehog (SHH) that in case of disturbed expression levels lead to several clinical symptoms [34, 35]. Interestingly, disturbed expression of GLI3, SOX9 and SSH in mice leads to various disturbances in skeletal development and mineralization [36-38]. Based on this it is tempting to postulate that EYA2 plays an important role in skeletal development and bone formation. Despite that so far little direct information on the function of these genes in bone is present, their strong up-regulation during stages of mineralization makes them interesting novel candidates for the control of bone formation. Proof of principle is given by targeting CNR1 which activation increased matrix mineralization. The role of CNR1 in bone is currently confined to osteoclasts [14]. Our data now implicate also a significant role of CNR1 in bone formation. Together with the data on CNR2 [15, 16] it substantiates a role for cannabinoid-related ligands in bone metabolism.

The biological relevance of the 489 identified genes was further substantiated by GO en gene network enrichment analysis. Identification of GO biological process terms like development, morphogenesis and cell differentiation is in line with the experimental setup of GC-induced osteoblast differentiation. In the GO cellular component analyses ECM genes were identified as strongly enriched, which emphasized the importance of matrix composition for matrix mineralization. Currently, little is known about the role of the majority of these ECM genes in matrix mineralization, which makes it yet difficult to interpret the significance of these ECM genes. Nevertheless, the observed direction of regulation coupled to the mineralization phenotype may already hint at the function of these ECM genes. In addition, as shown in Figure 4.6, numerous other GO terms were identified as strongly enriched, however, most of these processes are still unknown in relation to osteoblast differentiation and bone metabolism. Presently it is too premature to discuss these processes extensively but their identification opens up the opportunity to unravel these processes in osteoblast differentiation.

Together, the current approach of a differential screen of mineralizing and nonmineralizing cultures delivered a relatively small set of 489 genes which, aside already known bone-related genes, will contain other novel osteoblast regulatory genes. The identified gene networks are good leads to select appropriate candidate genes for the control of bone formation. In these networks genes with unknown function in bone co-localize and interact with genes known to be functional in bone, indicating the potential importance for the novel bone related genes. However, to pinpoint precisely their functional role elaborate studies are needed. This is a major challenge, as these genes should not only be studied independently but importantly also in interaction with each other. This latter is directly related to the fact that metabolic bone diseases such as osteoporosis are complex diseases in which not a single gene but a multitude of interacting genes are at the basis of the disease.

To extend the observations beyond skeletal development and mineralization we studied VSMCs as a model of atherosclerosis. GCs also triggered VSMC-induced

matrix mineralization and regulated a similarly subset of genes in VSMCs compared to osteoblasts. Using this model and the comparative analyses, genes can be identified that are specifically involved in the mineralization process in a cell type independent manner, while on the other hand genes can be identified which are specific for either bone or vascular mineralization. This approach and the currently identified genes are therefore also of great importance for cardiovascular disease, since vascular mineralization is a risk factor for cardiovascular failure [39, 40].

In conclusion, a unique aspect of this study was that we compared mineralizing human osteoblast cultures with their nonmineralizing counterparts. This led to the identification of numerous potential targets to control bone formation and bone quality. Novel genes were identified that are either directly or indirectly regulated by GCs including ZBTB16, CNR1 and multiple ECM genes. Together these data substantially add to the understanding of cellular processes, molecular networks and genes in osteoblast differentiation and mineralization and form a solid start to further unravel the molecular mechanism behind human osteoblast differentiation and bone formation.

4.4 Materials and Methods

Cell culture

SV-HFO cells were cultured as previously described [12]. After seeding, cells were incubated for two days before they were put on mineralizing or nonmineralizing medium (indicated as day 0). Medium was supplemented with freshly added 10 mM b-glycerophosphate (Sigma), 100 nM dexamethasone (DEX) (Sigma) or other additives and replaced every 2-3 days. The CNR1 agonist ACEA was purchased from Tocris.

Normal human osteoblasts (NHOst) (Cambrex Bio Science; CC-2538) and vascular smooth muscle cells (Coronary artery smooth muscle cells; Cambrex Bio Science; CC-2583) were cultured similar as SV-HFO cells only with the following adjustments. NHOst were used between passage 3 and 6 and were seeded in a density of 5×10^3 vital cells per cm^2. NHOst cultures were induced to mineralize in similar medium as SV-HFO, except that 10 % charcoal-treated heat-inactivated FCS (Gibco BRL) was used. VSMCs were used between passage 3 and 7 and seeded in a density of 5×10^3 vital cells per cm^2. Expansion of VSMCs was performed in smooth muscle cell medium (Cambrex Bio Science) supplemented with Clonetics Sm-GM-2 Bulletkit (Cambrex Bio Science). VSMCs were induced to mineralize in DMEM (Gibco BRL; with 4500 mg/l glucose, L-glutamine and pyruvate) supplemented with 100 nM DEX, 0.1 mM ascorbic acid (Sigma), 10 mg/ml insulin (Sigma), 1 mM $CaCl_2$ (final concentration of 2.8 mM), 10 mM b-glycerophosphate and 10 % FCS (Gibco BRL).

DNA, alkaline phosphatase activity, and mineralization assays

At the end of cultures cells were scraped in PBS-Triton X-100 and stored at -80° until analysis. ALPL, DNA and calcium measurements were performed as previously described [41]. Shortly, DNA content was measured fluorimetrically using ethidium bromide solution. Alkaline phosphatase activity was measured by determining the release of

Table 4.4. Primer and probe sequences and concentration used for qPCR analysis. Primer sets without a fluorescence probe were used in combination with the SYBR green QPCR kit (Eurogentec).

	Forward primer nM, 5'-3'	**Reverse primer** nM, 5'-3'	**Probe** nM, 5'-FAM – 3' TAMRA
ALPL	300 gacccttgacccccacaat	300 gctcgtactgcatgtcccct	50 tggactacctattgggtctcttcgagcca
CNR1	100 gggatgcgaagggattgc	100 ggccatctaggatcgacttcat	
CLEC3B	100 caccaccgagccaccaa	100 ggtgtccagacggctcttga	
DAAM2	250 gcctaccagaggcaccagaa	250 cctccggccatcaatgac	100 atctacctggcttcccgcaaggtcaaa
DCN	100 taaccgaaatcaaagatggagactt	100 tccaggactaacttgctaatttattg	
EYA2	400 gtgggacttggatgagacaataatt	400 cttccatcataaggccaatgc	100 atccagatacgggaaggacaccacgac
FBLN1	200 ggagaccggagatttggatgt	200 tcagatatgggtcctcttgttcct	
GAPDH	150 atgggaaggtgaaggtcg	150 taaaagcagccctggtgacc	150 cgcccaatacgaccaaatccgttgac
GPM6B	400 agtgaaagaactgcacggtgagt	400 aagcacatagtgaggaaaacga	100 caaccgcttgtggccgatgcat
HAS1	100 aactgctgcaagaggttattcctatat	100 gcgggcttgtcagagctact	
LAMB3	200 caggcagcctcacaactactaca	200 cagagagacaggttcacatcatt	
MMP1	900 ctcaatttcacttctgttttctg	900 catctctgtcggcaaattcgt	100 cacaactgccaaatgggcttgaagc
MMP2	200 tcaagttcccggcgat	200 tgttcaggtattgcactgcca	100 tcgcccccaaaacggacaaaga
MMP3	200 ttttggccatctcttccttca	200 tgtggatgcctcttgggtatc	100 aacttcatatgcggcatccacgcc
NID2	100 ctgaacagtctgtgaaaaatctctatc	100 gcagctggcctgacattgt	
TIMP4	100 cagggctcgatgtagttgtgca	100 cagccagaagcagtatctcttgac	
TMTC1	400 ccactacagaacagctctcaagttgta	400 tctcgtcagttgttccaaggttgt	100 ccacgccatgcaagtgcgctc
ZBTB16	400 gcggttcctg gatagtttgc	400 tgatcacagacaaaggctttgg	100 atgcacttactgcgtcattcagcggg
ZFP36	125 gacttcagcgctcccactct	125 agcgacaggaggctctcgta	125 acacccctcatggccaaccgttaca

paranitrophenol from paranitrophenylphosphate. Calcium content was colorimetrically determined after addition of 1M ethanolamine buffer (pH 10.6) 0.35 mM o-cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline and 0.6 mM hydrochloric acid at 595 nm. For Alizarin Red S staining cell cultures were fixed for 60 min with 70 % ethanol on ice. After fixation, cells were washed twice with PBS and stained for 10 min with Alizarin Red S solution (saturated Alizarin Red S in demineralized water adjusted to pH 4.2 using 0.5 ammonium hydroxide). Alizarin Red solution was removed and cultures were washed with demineralized water.

Quantitative PCR analysis (qPCR)

RNA isolation, cDNA synthesis and QPCR were performed as described previously [12]. Primer and probes sequences and concentrations are shown in Table 4.4. The expression of genes for which no probe sequence is given are analyzed using the SYBR green I or qPCR core kit (Eurogentec)

Affymetrix GeneChip-based gene expression

Purity and quality of isolated RNA was assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). Per analyzed condition total RNA of 3 different biological samples was pooled. Synthesis of first and second strand cDNA from total RNA was performed according to the One-Cycle Target Labeling protocol (Affymetrix; 701024 Rev. 3). In total, 4.0 µg of total RNA was reverse transcribed using Superscript ds-cDNA Synthesis Kit according to the manufacturer's description (Invitrogen). Subsequently, double-stranded cDNA was purified using GeneChip Sample Cleanup Module (Affymetrix) and served as a template in the *in vitro* transcription reaction using BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). Next, the RNA (cRNA) was purified using GeneChip Sample Cleanup Module. In total, 20 µg of biotin-labeled cRNA was fragmentized by metal-induced hydrolysis at a final concentration of 0.5 µg/µL for 35 minutes at 94°C. Fragmentation was checked on an Agilent 2100 Bioanalyzer confirming an average size of approximately 100 nt. In total, 15 µg fragmented biotinylated cRNA was hybridized to GeneChip Human Genome U133 Plus 2.0 oligonucleotide GeneChips (Affymetrix) according to the manufacturer's protocol (Affymetrix, 701025 Rev 5). Staining, washing and scanning procedures were carried out as described in the GeneChip Expression Analysis technical manual (Affymetrix). Data acquisition was performed using the GeneChip Scanner 3000.

GeneChip data analysis

In order to examine the quality of the different arrays, measured intensity values were analyzed using the GeneChip Operating Software (GCOS, Affymetrix). The percentage of present calls (about 40 %), noise, background, and ratio of GAPDH 3' to 5' (<1.4) all indicated high quality of the samples and an overall comparability. Probe sets that were never present (according to Affymetrix MAS5.0 software) in any of the GeneChips were omitted from further analysis. Raw intensities of the remaining probe sets (30336) of each chip were log2 transformed and normalized using quantile normalization. After normalization the data was back transformed to normal intensity values. Data analysis

and visualization were done using the OmniViz package, version 3.6.0. For each probe set, the geometric mean of the hybridization intensities of all samples was calculated. The Pearson's correlation and visualization tool of OmniViz was used to study the results of unsupervised clustering of the samples (based on the log2 geometric mean values). In the correlation plot samples are ordered by correlation (samples with high positive correlations or high negative correlations are putted into separate blocks) and the cells are color-coded by correlation coefficient values. For the correlation plots analysis genes were used that were differentially expressed (>2-fold difference from geometric mean) at one of the analyzed days.

To select differentially expressed probe sets the average intensity value of all 6 time-points was calculated for both control and mineralizing cultures using an cut off of 30 (intensity value). Probe sets were selected that showed at least a 2.0-fold difference between control and mineralizing cultures.

For GO enrichment analysis the 740 selected probe sets were analyzed using Ontology Transfer [14]. Ingenuity Pathways Analysis 4.0 was used to identify gene networks. The 740 probe sets with corresponding fold differences (log2 transformed) were uploaded into the application. Each Affymetrix ID was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these focus genes were then algorithmically generated based on their connectivity.

Gene nomenclature

Gene names and gene symbols were used as provided by HUGO Gene Nomenclature Committee [43].

Statistics

Data were only presented if multiple independent experiments showed similar results. Experiments were performed at least in triplicate. Values are the means ± SEM. Significance was calculated using the Student's *t*-Test. To calculate significance for GO analyses Benjamini and Hochberg false discovery rate was used [42].

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Chapter 5

The activin A-follistatin system: potent regulator of human extracellular matrix mineralization

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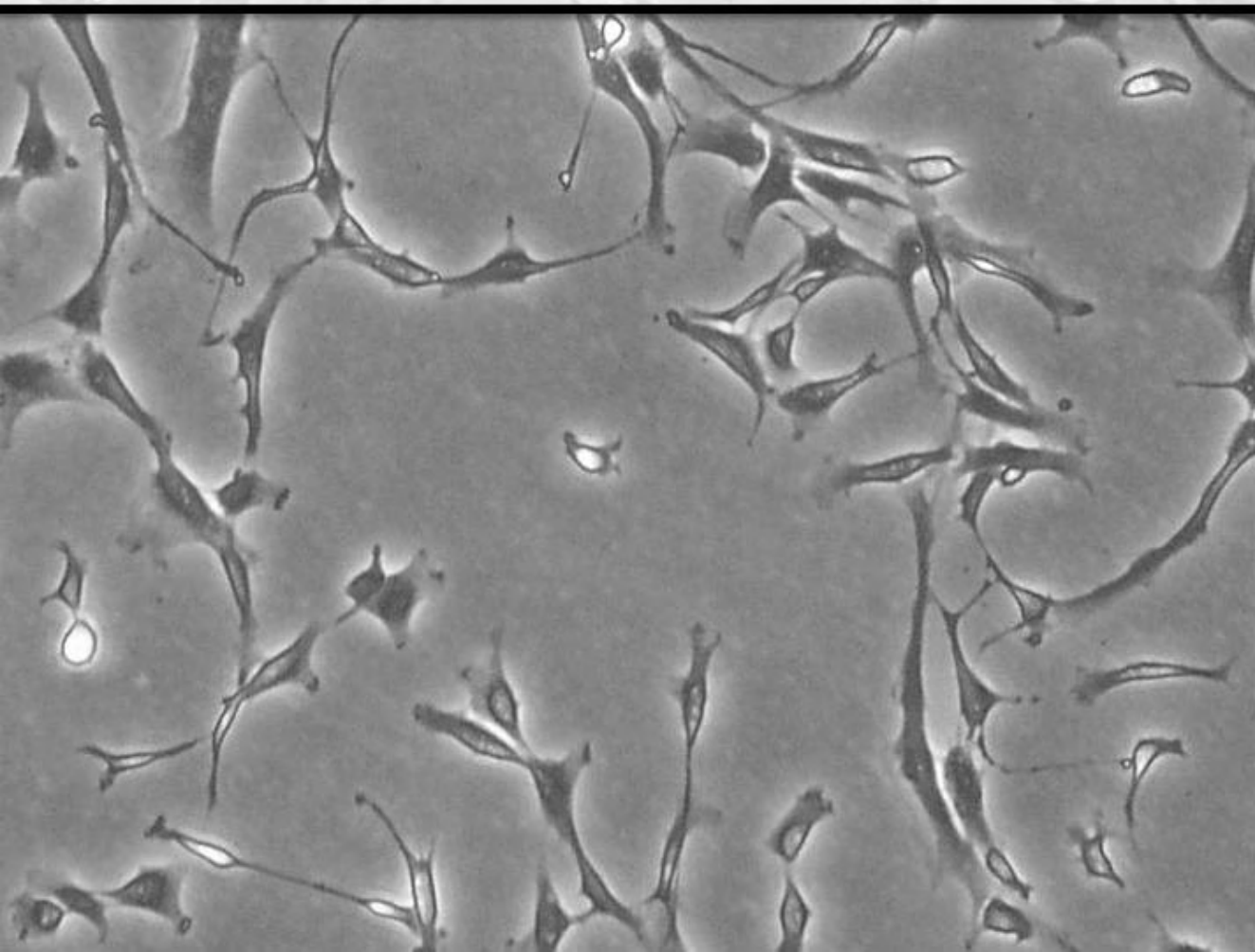

Abstract

Bone quality is an important determinant of osteoporosis, and proper osteoblast differentiation plays an important role in the control and maintenance of bone quality. We investigated the impact of activin signaling on human osteoblast differentiation, extracellular matrix formation, and mineralization. Activins belong to the transforming growth factor-β superfamily and activin A treatment strongly inhibited mineralization in osteoblast cultures, whereas the activin antagonist follistatin increased mineralization. Osteoblasts produced activin A and follistatin in a differentiation-dependent manner, leading to autocrine regulation of extracellular matrix formation and mineralization. In addition, mineralization in a vascular smooth muscle cell-based model for pathological calcification was inhibited. Comparative activin A and follistatin gene expression profiling showed that activin signaling changes the expression of a specific range of extracellular matrix proteins prior to the onset of mineralization, leading to a matrix composition with reduced or no mineralizing capacity. These findings demonstrate the regulation of osteoblast differentiation and matrix mineralization by the activin A-follistatin system, providing the possibility to control bone quality as well as pathological calcifications such as atherosclerosis by using activin A, follistatin, or analogs thereof.

5.1 Introduction

Decreased bone quality is an important determinant of osteoporosis. Bone quality is determined largely by a combination of protein composition and mineralization of the extracellular matrix (ECM) [1]. Osteoblasts play a pivotal role in regulating bone quality, as they are the bone-forming cells and the directors of bone resorption by osteoclasts. Osteoblasts are of mesenchymal origin and undergo a complex differentiation process regulated by multiple endocrine, paracrine, and autocrine factors. During differentiation and bone formation, osteoblasts produce a complex ECM that eventually starts to mineralize. Initiation of mineralization requires the precipitation and attachment of calcium phosphate crystals to the ECM, although the exact mechanism is poorly understood [2]. The process of mineralization is not unique for bone cells demonstrated by ectopic mineralization in pathological conditions. Vascular smooth muscle cells (VSMCs) are thought to be primarily involved in vascular mineralization. These cells undergo an osteoblast-like differentiation process expressing osteoblastic factors, including alkaline phosphatase (ALPL) and runt-related transcription factor 2 [3-5].

Several members of the transforming growth factor-β (TGFβ) superfamily, including TGFβ and bone morphogenic proteins (BMPs), are well-known regulators of bone formation. Both TGFβ and BMPs promote bone development by stimulating the differentiation of osteoblast progenitors [6, 7]. In addition, TGFβ seems to inhibit later phases of osteoblast differentiation and mineralization [8-10]. Activins belong to the TGFβ superfamily, but their precise role in bone formation is unknown. The structure of activins is closely related to that of TGFβ, and activins act via similar intracellular signaling molecules [11]. Activins and their relatives, inhibins, were initially purified from gonadal fluids and characterized on the basis of their ability to modulate FSH secretion from pituitary gonadotropes [12, 13]. Besides this classical role of activins, activins can affect the function of other cell types and tissues (*e.g.*, the adrenal gland, liver, neurons, pancreas, and bone) [14-18].

Activins and inhibins are composed of the inhibit subunits α, βA, and βB. Heterodimerization of the α and βA, or βB subunit forms inhibin A or inhibin B. Homo- and heterodimerization of the βA, and βB subunits result in formation of activin A, activin AB, or activin B [12]. Activins need type I and type II activin receptors for signal transduction. Activins bind to the activin type IIA or type IIB receptors, leading to recruitment and phosphorylation of the activin type IB receptor (ALK4). The phosphorylated type I receptor, in turn, phosphorylates intracellular signaling proteins known as Smads. Smad2 or 3 are phosphorylated by activins and TGFβ, whereas Smad1, 5, or 8 are phosphorylated by the BMP-like ligands [11]. Activin signaling is inhibited by the extracellular action of inhibins or follistatin [19]. Follistatin is a soluble protein that functions as an activin binding protein preventing activin from interacting with its receptor [20]. Inhibins need the presence of a coreceptor, betaglycan, to inhibit activin signaling via competitive binding to the activin type II receptor, preventing recruitment of the activin type I receptor [21, 22].

Several studies demonstrated a role for activins in bone metabolism. Large quantities of activin are found in the ECM of bovine bone [23]. Activin A enhances osteoclast-like cell formation in murine bone marrow cultures [24, 25]. Activin A promotes osteoblastogenesis

in murine bone marrow cultures [25] and, *in vivo*, promotes bone formation and fracture healing in rodents [23-27]. However, other reports demonstrated an inhibitory effect of activin on osteoblast differentiation in rat and murine osteoblasts [28, 29].

The aim of this study was to assess the impact of activin signaling on osteoblast differentiation related to human bone formation and bone quality. The effect of activin A on matrix mineralization was measured together with the endogenous production of activin A and activin antagonists during osteoblast differentiation. Moreover, genome-wide expression profiling in osteoblasts was performed to reveal downstream mediators of activin signaling in relation to matrix mineralization.

5.2 Results

Activin A inhibits matrix mineralization in human osteoblast cultures

Activin signaling in osteoblasts was studied in detail using the SV-HFO osteoblast differentiation model. This human osteoblast model produces an ECM in culture, which eventually is mineralized in a 2 to 3 wk period [30]. Activin A treatment strongly inhibited the mineralization process (Fig. 5.1 A) (up to 50 – 100 %). Three other mineralization models were treated with activin A to demonstrate that inhibition of matrix mineralization by activin A is not unique for the SV-HFO osteoblast model. Activin A was tested in another human osteoblast model (NHOst) and in human mesenchymal stem cell (MSC) cultures that were induced to differentiate toward osteoblasts (Fig. 5.1 B, C). Similar to SV-HFO cells, mineralization in both human osteoblast models was inhibited by activin A. In addition, human VSMCs were treated with activin A. VSMCs can be used as a model for vascular mineralization since they can form a mineralized ECM *in vitro* [31]. Activin A treatment strongly inhibited matrix mineralization in these VSMC cultures (Fig. 5.1 D).

The effect on matrix mineralization was dose dependent, showing significant inhibition after treatment with activin A concentrations of 5 ng/ml and higher (Fig. 5.1 E). Together with decreased matrix mineralization, activin A also decreased the activity of the osteoblast differentiation marker ALPL dose dependently, although the magnitude of inhibition (10–30 %) was smaller than that of mineralization (data not shown).

Osteoblasts produce activin A in a differentiation dependent manner

To reveal whether activin can act in an autocrine manner, we measured mRNA levels of the activin subunits inhibin-βA (INHBA) and inhibin-βB (INHBB) together with activin A protein production in human osteoblast cultures. QPCR demonstrated that INHBA was expressed abundantly compared with the INHBB subunit. In contrast, inhibin-α (INHA) mRNA was almost undetectable (Fig. 5.2 A). This suggests that osteoblasts mainly produce activin A. In addition, Fig. 5.2 A shows mRNA expression of the activin type I receptor (ACVR1B) and the activin type II receptors (ACVR2A/2B) in osteoblasts.

Quantification of activin A protein showed that osteoblast cultures secrete biologically relevant amounts of activin A (up to 5 ng/ml), whereas inhibin A protein could not be detected (data not shown). Activin A production corrected for cell number (DNA content) was measured at different stages of differentiation (days 5, 12, and 19).

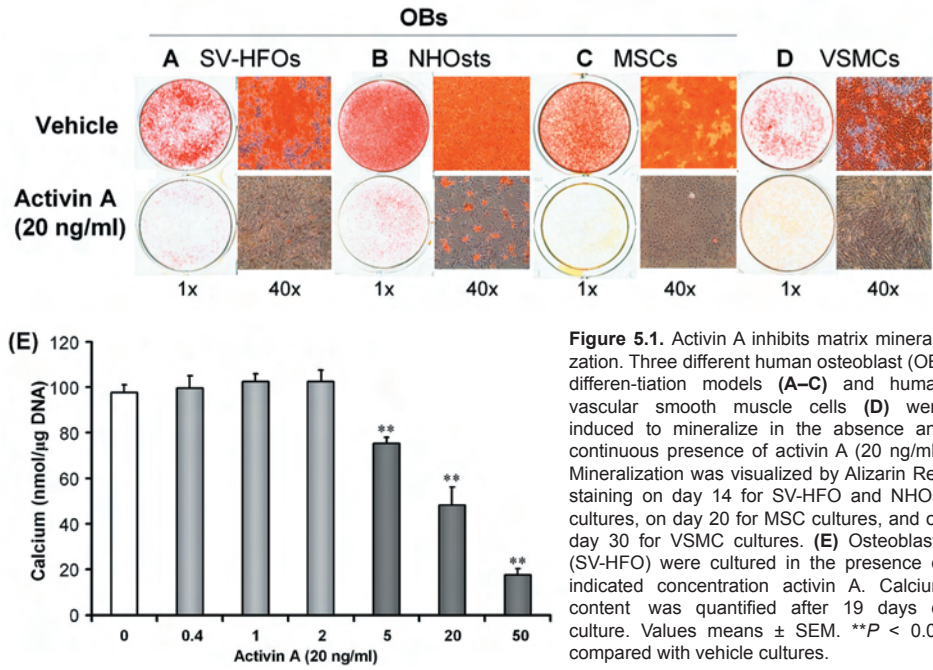

Figure 5.1. Activin A inhibits matrix mineralization. Three different human osteoblast (OB) differen-tiation models **(A–C)** and human vascular smooth muscle cells **(D)** were induced to mineralize in the absence and continuous presence of activin A (20 ng/ml). Mineralization was visualized by Alizarin Red staining on day 14 for SV-HFO and NHOst cultures, on day 20 for MSC cultures, and on day 30 for VSMC cultures. **(E)** Osteoblasts (SV-HFO) were cultured in the presence of indicated concentration activin A. Calcium content was quantified after 19 days of culture. Values means ± SEM. $^{**}P < 0.01$ compared with vehicle cultures.

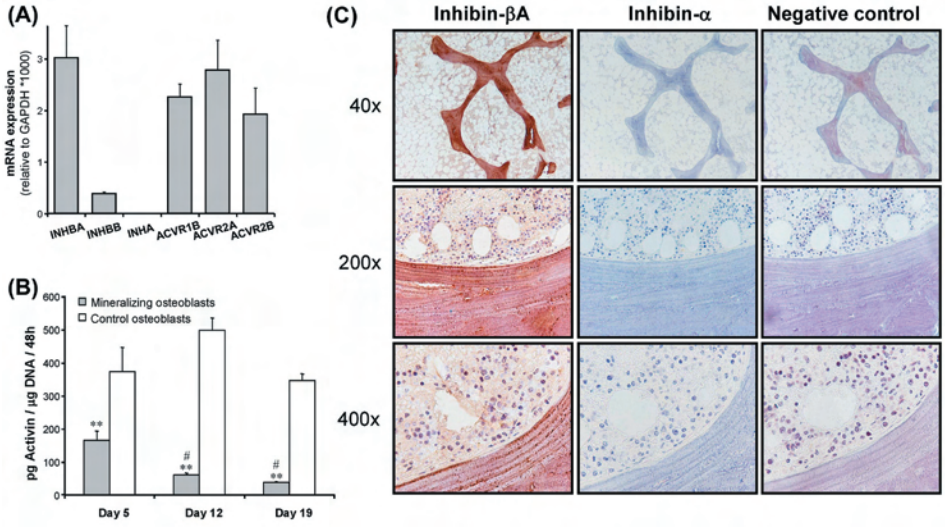

Figure 5.2. Production and localization of activin A in human bone. **(A)** mRNA expression of inhibin/activin subunits (*INHA, INHBA, INHBB*) and activin type I (*ACVR1B*) and type II receptors (*ACVR2A, ACVR2B*) in osteoblast cultures (SV-HFO). Values means ± SEM. **(B)** Production of activin A protein by human osteoblasts (SV-HFO). Activin A levels were measured in control osteoblast cultures (open bars) and osteoblast cultures that were induced to mineralize (gray bars). Production was corrected for the culture DNA content. Values means ± SEM. $^{**}P < 0.01$ compared with control osteoblasts; $\#P < 0.01$ compared with day 5 of mineralizing cultures. **(C)** Immunohistological staining of inhibin-βA subunit (left panels) and inhibin-α subunit (center panels) in human bone tissue. Mouse IgG2b was used as a negative control (right panels).

Activin A production decreased during differentiation and was lowest during stages of matrix mineralization (days 12 and 19) (Fig. 5.2 B, mineralizing osteoblasts). To explore this differentiation-dependent regulation, activin A production was also measured in preosteoblast cultures (SV-HFO) that did not undergo differentiation into fully functional and mineralizing osteoblasts. Nonmineralizing osteoblasts (control osteoblasts) were created by excluding glucocorticoids (DEX) from the culture medium. These osteoblast cultures had low ALP activity and did not show *in vitro* mineralization [32]. Control osteoblasts had significantly higher activin A production than their mineralizing counterparts (Fig. 5.2 B), demonstrating that, in differentiated osteoblasts, activin A production is suppressed during mineralization.

In addition, immunohistology on human bone biopsies showed that human bone tissue contained high levels of activin A. The inhibin-βA subunit was detected in mineralized bone matrix, but inhibin-α subunit could not be detected (Fig. 5.2 C).

Activin A inhibits mineralization in an autocrine manner

The impact of endogenously produced activins on osteoblast function was measured by neutralizing activin signaling by the addition of the activin binding protein follistatin. First we measured whether follistatin was able to neutralize activin signaling in osteoblast cultures. An activin-signaling luciferase reporter construct (CAGA-Luc) was used to measure activin signaling. Activin A treatment increased CAGA-Luc activity dose-dependently, which could be inhibited by coincubation with follistatin (Fig. 5.3 A). Follistatin also reduced basal CAGA-Luc signaling, which further supports endogenous

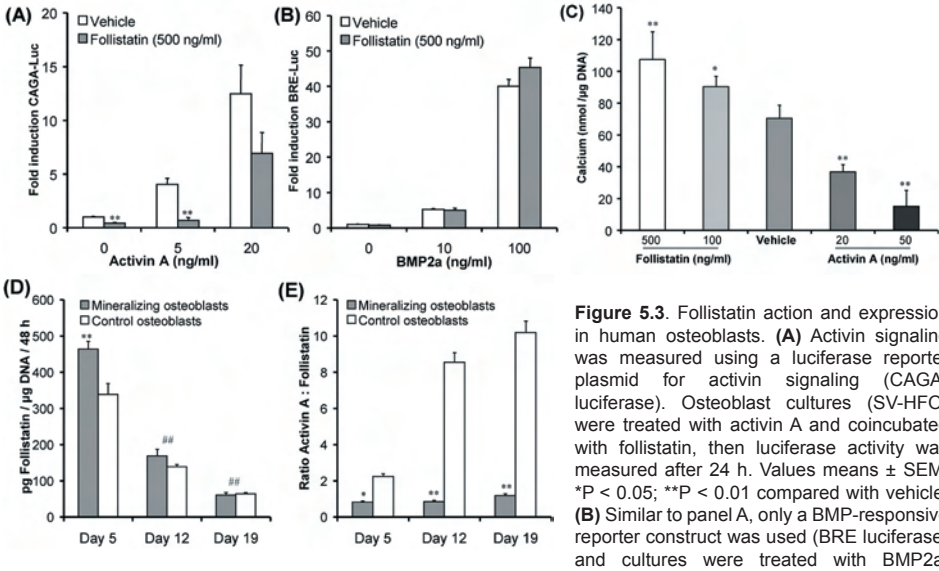

Figure 5.3. Follistatin action and expression in human osteoblasts. **(A)** Activin signaling was measured using a luciferase reporter plasmid for activin signaling (CAGA-luciferase). Osteoblast cultures (SV-HFO) were treated with activin A and coincubated with follistatin, then luciferase activity was measured after 24 h. Values means ± SEM. *P < 0.05; **P < 0.01 compared with vehicle. **(B)** Similar to panel A, only a BMP-responsive reporter construct was used (BRE luciferase) and cultures were treated with BMP2a. **(C)** Osteoblast cultures were treated with follistatin or activin A for 19 days. Calcium content was measured on day 19. Values means se. *P < 0.05; **P < 0.01 compared with vehicle. **(D)** Follistatin production by human osteoblasts (SV-HFO) on days 5, 12, and 19 in control and mineralizing conditions. Values means ± SEM. **P < 0.01 compared with control osteoblasts, ##P < 0.01 compared with day 5. **(E)** The molar ratio between activin A and follistatin of control and mineralizing osteoblast cultures on days 5, 12, and 19. Values means ± SEM. *P < 0.05; **P < 0.01 compared with control osteoblasts.

production of activin and activation of activin signaling in osteoblasts. It has been suggested that follistatin also neutralizes BMP action [33, 34]. To study this in osteoblasts, a BMP-responsive luciferase reporter construct was used (BRE-Luc). Addition of 10 and 100 ng/ml BMP2a strongly induced BRE-Luc activity. In contrast to activin signaling, addition of follistatin had no effect on either basal or BMP2a-induced BMP signaling (Fig. 5.3 B). This suggests that, under these conditions, follistatin specifically neutralizes activin signaling in osteoblasts.

Neutralization of endogenous activins by follistatin treatment (100 and 500 ng/ml) clearly increased matrix mineralization (Fig. 5.3 C) together with a minor increase in ALPL activity (data not shown). This shows that osteoblasts secrete activin A, which inhibits mineralization and ALPL activity in a paracrine and/or autocrine way. In these experiments, osteoblasts were also treated with activin A to compare the impact of activin A with that of follistatin. These incubations demonstrated a dose-dependent effect of activin signaling on mineralization, where follistatin- (500 ng/ml), vehicle-, and activin A-treated cultures (50 ng/ml) represent neutralized, endogenous, and high activin signaling, respectively (Fig. 5.3 C).

Activin A-to-follistatin ratio is decreased in mineralizing cultures

Besides activin A production by osteoblasts, we demonstrated that osteoblasts secrete biologically relevant amounts of follistatin. On day 12 of culture, maximum levels of follistatin (up to 1–3 ng/ml) could be measured in the culture supernatants. This indicates that, in osteoblasts, endogenous activin signaling is regulated by the expression of activin A as well as by expression of follistatin. The production of follistatin was measured (corrected for DNA content) throughout culture in mineralizing and control osteoblasts (Fig. 5.3 D). Follistatin production was higher at the beginning of culture (day 5) in mineralizing osteoblast compared with control osteoblasts and decreased during culture in both conditions. For the eventual action of activin A, it is important to take into account the molar ratio of activin A and follistatin. This demonstrated that mineralizing cultures have a lower activin A-to-follistatin ratio compared with control cultures, indicating suppressed activin signaling in mineralizing conditions (Fig. 5.3 E).

Activin signaling is most effective prior to the onset of mineralization

We investigated whether activin signaling inhibits mineralization in a specific time window of osteoblast differentiation by treating cultures with activin A during specific periods. First, cultures were treated in the premineralization period (up to day 10 of culture) or during the mineralization period (from day 10 of culture onward) with a moderate dose of activin A (20 ng/ml). Subsequently, mineralization was measured on day 19 (Fig. 5.4 A). Treatment preceding the mineralization period most effectively inhibited mineralization, whereas treatment in the period when mineralization was ongoing appeared to be ineffective. To zoom in on this premineralization period, more specific incubations were performed, as depicted in Fig. 5.4 B. Activin A was most effective when present in the final 7 days before the onset of mineralization. Even when activin A was present only during the final 2 days (days 10–12) before mineralization, a significant decrease in mineralization was measured (Fig. 5.4 B).

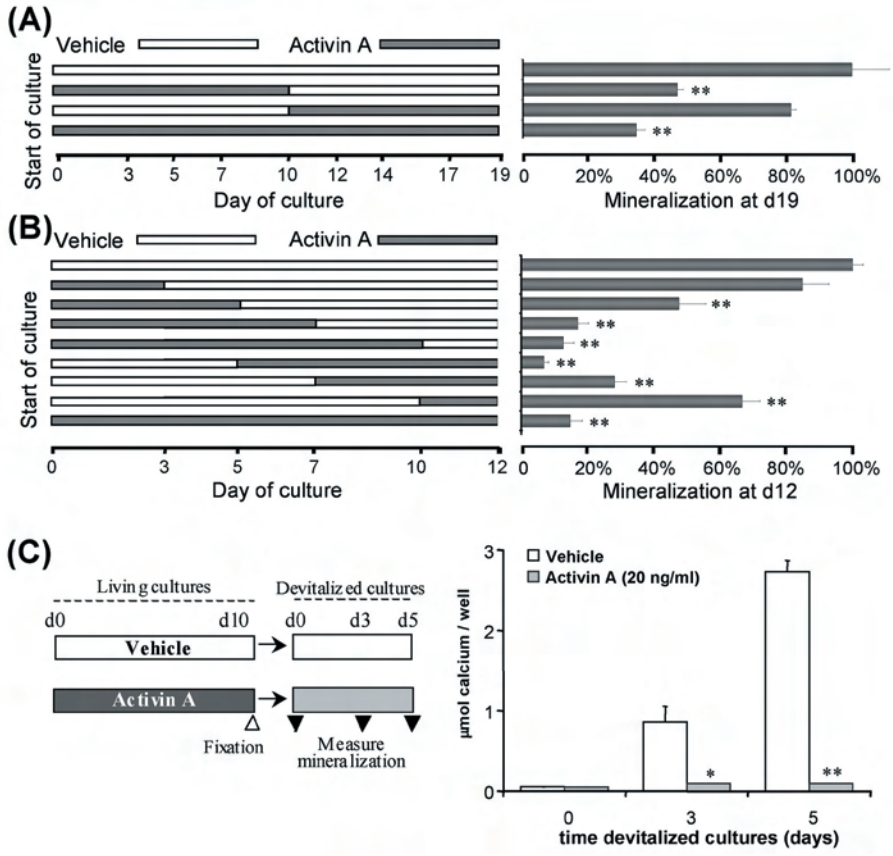

Figure 5.4. Activin A treatment during different stages of differentiation. **(A)** Osteoblasts (SV-HFO) were treated with activin A before the onset of mineralization (day 0 to 10), after the onset of mineralization (day 10 to 19), or during the whole culture period (day 0 to day 19). Calcium content was measured at the end of culture (day 19). **(B)** Osteoblasts (SV-HFO) were treated with activin A in a specific period before the onset of mineralization. Calcium content was measured on day 12. **(C)** Osteoblast cultures (SV-HFO) were cultured for 10 days in the presence or absence of activin A. On day 10, cultures were devitalized and subsequently cultured in normal culture medium. Calcium content was measured at the moment of devitalization (day 0) and 3 and 5 days after devitalization. Values means ± SEM. *P < 0.05; **P < 0.01 compared with vehicle cultures.

We hypothesized that activin signaling in this premineralization phase leads to an altered matrix composition, keeping it in an immature state not able to mineralize. To prove this, we exploited the fact that osteoblast cultures produce an ECM within the first 10 days of culture that is mature and can subsequently mineralize independent of the presence of additional living osteoblasts [35]. This effect was achieved by freezing osteoblast cultures to -20°C at the onset of mineralization (day 10). These cultures containing nonliving cells but an intact ECM were subsequently incubated for an additional 3 or 5 days with culture medium. Mineralization was quantified at the moment of fixation (day 10 living cultures=day 0 devitalized cultures) and after 3 and 5 days of additional incubation (see incubation scheme Fig. 5.4 C). No mineralization was measured on day 0 of devitalized cultures. However, after 3 and 5 days, mineralization of the ECM could be measured (Fig. 5.4 C, vehicle). In cultures that had been pretreated with activin A

prior to devitalization, no mineralization of the ECM could be detected. These findings support our hypothesis that the major impact of activin A occurs before the onset of mineralization and suggest an effect on matrix composition and maturation.

Gene profile analysis of follistatin- and activin A-treated osteoblast cultures

Gene profile experiments were performed to gain insight into the molecular action of activin A in osteoblasts using Affymetrix HG U133 Plus 2.0 Genechips. Gene expression profiles of follistatin- (500 ng/ml), vehicle-, and activin A- (50 ng/ml) treated osteoblasts were compared. These treatments created cultures having low, moderate (endogenous), and high activin signaling, resulting in three different levels of matrix mineralization as demonstrated in Fig. 5.3 C.

A phenotype-based query was designed to identify genes regulated by activin signaling in the premineralization period (days 5 and 12), which was shown to be important for inhibition of mineralization by activin A (Fig. 5.4 B). An important aspect in the selection query was that we searched for genes that were regulated in follistatin- and activin A-treated cells, since both conditions affected mineralization. Genes were selected that were up-regulated in activin A-treated cultures (low mineralization) and down-regulated in follistatintreated cultures (high mineralization) compared with vehicle cultures, or vice versa, on days 5 and 12 In addition, the difference in gene expression between activin A- and follistatin-treated cultures should be at least 2-fold on one of these 2 days (Fig. 5.5 A). In total, 520 Affymetrix IDs were selected representing 397 unique genes and 62 nonannotated Affymetrix IDs. The selected Affymetrix IDs are presented as a hierarchical clustering in Fig. 5.5 A; red and green indicate up- and down-regulation compared with vehicle cultures, respectively.

Gene ontology analysis

Next, GO enrichment analysis was used to categorize the 397 selected genes for the GO categories: biological process (Fig. 5.5 B), molecular function (Fig. 5.5 C), and cellular component (Fig. 5.5 D). In total, we identified 47 biological processes, 13 molecular functions, and 4 cellular components as significantly over-represented compared with what would be expected if 397 random genes were analyzed. Several GO terms for biological process shared high similarity and were identified by similar groups of genes. In Fig. 5.5 B, we reduced the number of biological process terms to 20 by excluding these overlapping GO terms.

QPCR showed that mRNA expression of osteoblast differentiation markers like RUNX2, collagen type-I, secreted phosphoprotein 1 (osteopontin), and bone gamma-carboxyglutamate gla protein (osteocalcin) were unchanged by activin signaling (data not shown), suggesting no direct effect on the osteoblast differentiation process. However, GO biological process analysis demonstrated that differentiation-related processes like development and morphogenesis were strongly enriched by activin signaling (Fig. 5.5 B), including the osteoblast transcription factor MSX2 [36]. QPCR confirmed GeneChip analyses that MSX2 mRNA expression was significantly inhibited by activin A in osteoblasts (SV-HFO) as well as in VSMCs (Fig. 5.5 E).

The GO enrichment analysis for cellular component showed that genes related to

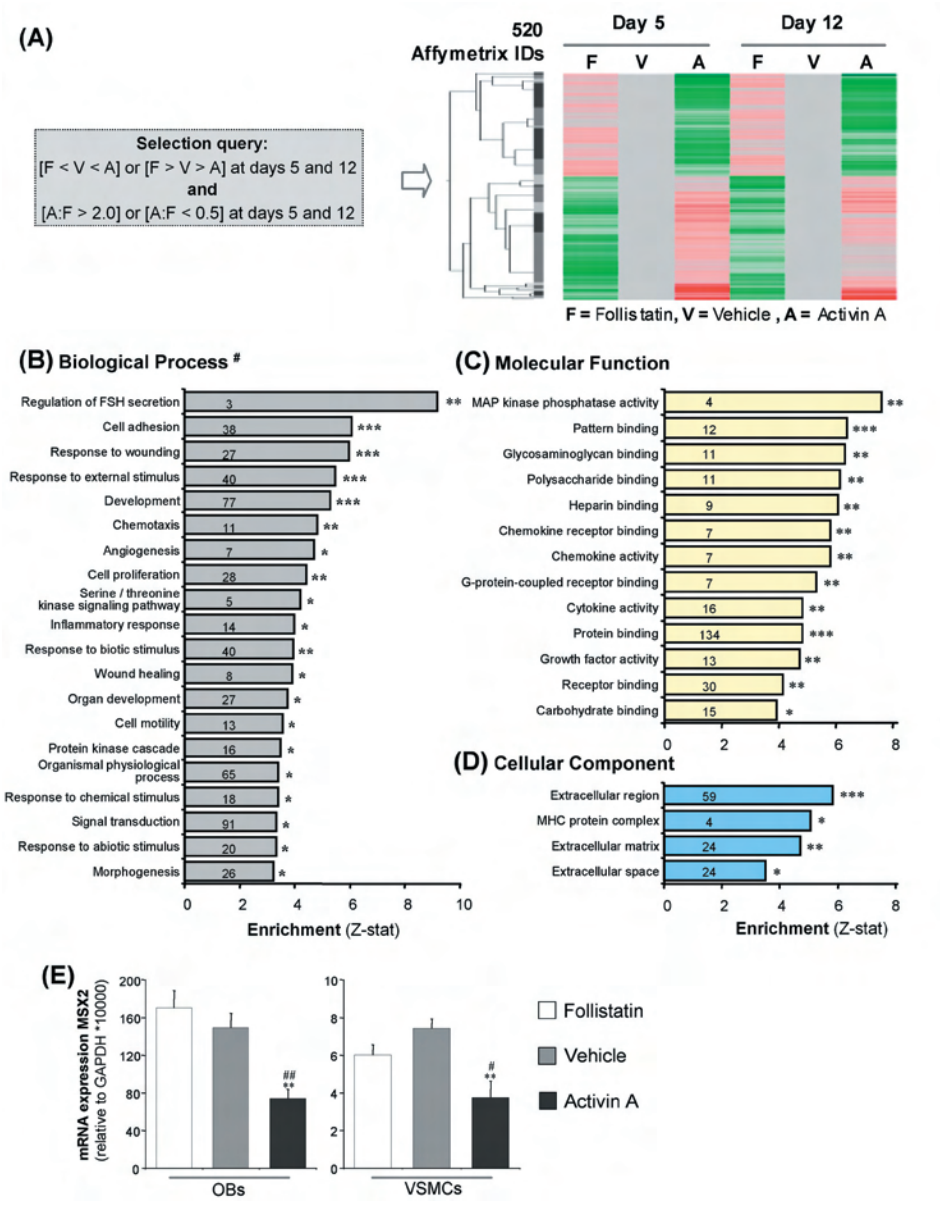

Figure 5.5. Gene ontology (GO) enrichment analysis of activin signaling-regulated genes. **(A)** Phenotype-based selection query based on Affymetrix Genechip data of vehicle-, activin A- (50 ng/ml), and follistatin- (500 ng/ml) treated osteoblast cultures (SV-HFO). In total, 520 affymetrix probe sets matched this selection query, representing 397 unique genes and 62 nonannotated probe sets. Affymetrix probe sets are hierarchically clustered; red indicates up-regulation and green indicates down-regulation compared with vehicle cultures. Color intensities indicate the magnitude of regulation. **(B–D)** Enriched GO terms within the set of 397-regulated genes for GO biological process **(B)**, GO molecular function (C), and GO cellular component **(D)**. Enriched categories are those identified as significantly enriched ($P < 0.05$) after multiple testing. Numbers inside the bars indicate the number of genes from the set of 397 selected genes determining the GO term. #Number of presented biological processes is reduced by exclusion of several overlapping GO terms. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **(E)** QPCR analysis of MSX2 expression in osteoblast (SV-HFO, day 7) and VSMC (day12) cultures after activin A (50 ng/ml) and follistatin (500 ng/ml) treatment. Values means ± SEM. **$P < 0.01$ compared with vehicle. #$P < 0.05$; ##$P < 0.01$ compared with follistatin.

localization in the extracellular region, space, or matrix were significantly enriched (Fig. 5.5 D). This result is in line with our hypothesis that activin A affects extracellular matrix formation and maturation (see Fig. 5.4 C). Although expression of the well-known and abundant ECM proteins in bone, collagen type-I, osteopontin, and osteocalcin was

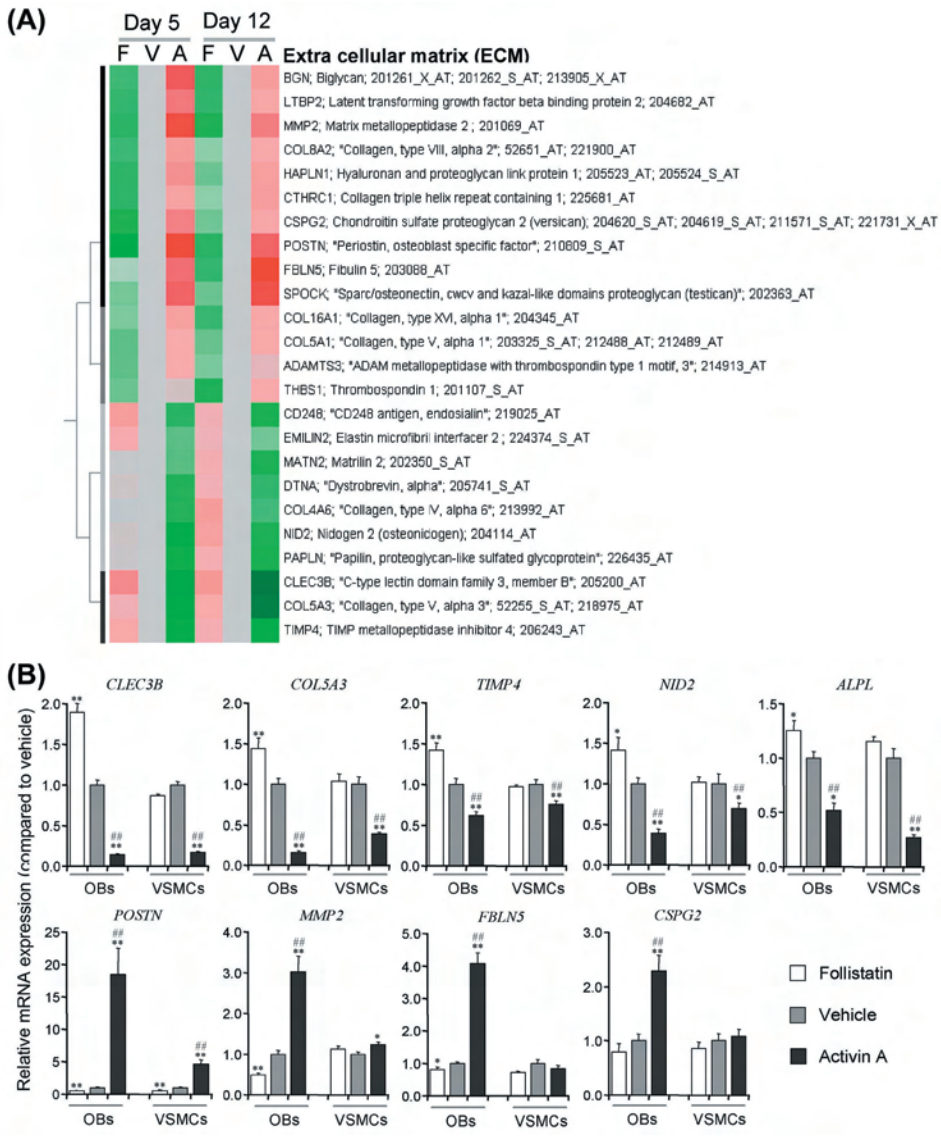

Figure 5.6. ECM genes regulated by activin signaling. **(A)** Hierarchical clustering of genes that matched the selection query (Fig. 5.5 A) and were annotated with the GO term ECM. Genes are annotated with their gene symbol, gene name, and Affymetrix IDs. Green indicates down-regulation and red indicates up-regulation compared with vehicle cultures. Color intensities indicate the magnitude of regulation (Genechip data). **(B)** QPCR analysis of nine selected ECM genes together with ALPL as a positive control. Expression was measured in follistatin-, vehicle-, and activin A-treated osteoblasts (SV-HFO, day 7) and VSMC cultures (day 12). The mRNA expression in vehicle cultures was set to 1 and used as a reference. Values means ± SE. **P* < 0.05; ***P* < 0.01 compared with vehicle. #*P* < 0.05; ##*P* < 0.01 compared with follistatin.

unchanged by activin signaling, these GeneChip analyses identified numerous ECM genes that were regulated by activin signaling (Fig. 5.6 A).

Activin signaling alters ECM composition in osteoblasts as well in VSMCs

A selection of ECM genes was analyzed in more detail using QPCR in follistatin-, vehicle-, and activin-treated osteoblasts and VSMCs (Fig. 5.6 B). The expression of ALPL was also quantified as a positive control. This experiment showed that CLEC3B, COL5A3, TIMP4, NID2, and ALPL, which were suppressed by activin A (upper 5 panels) in osteoblasts, were also suppressed by activin A in VSMC cultures. Activin signaling in osteoblasts increased POSTN and MMP2 expression, which was also demonstrated in VSMCs. In contrast, activin A increased FBLN5 and CSPG2 expression in osteoblasts, but the expression of these genes was unchanged in VSMCs.

Follistatin treatment did not alter gene expression in VSMCs. This result agrees with the expression data of INHBA and INHBB (mRNA) in VSMC, both of which had a low level compared with levels in osteoblasts (data not shown). Moreover, activin A production by VSMCs cultures was much lower than the production by osteoblasts (data not shown). In VSMCs, only POSTN mRNA expression was regulated by follistatin (0.5-fold). This gene was strongly induced by activin signaling and is probably already activated by a low concentration of activins (Fig. 5.6 B).

5.3 Discussion

This study shows that activin signaling controls human osteoblast differentiation and function, and inhibits matrix mineralization through mechanisms that include altered ECM composition and maturation. The presence and production of activin A in bone together with the strong effects of activin A on mineralization *in vitro* demonstrate a physiological impact of activin A on human osteoblast differentiation and bone metabolism. As a consequence, the control of activin signaling in bone might be crucial for proper bone formation. We showed that activin signaling in osteoblasts is tightly controlled in a differentiation-dependent manner. Mineralizing osteoblast cultures showed decreased activin A production and expression of the activin antagonist follistatin, resulting in a decreased activin A-tofollistatin ratio in mature osteoblasts. This indicates that osteoblasts autoinhibit matrix mineralization via expression of activin A, which in turn is controlled by its inhibitor follistatin. These control mechanisms have been shown for other regulatory growth factors in bone (*e.g.*, activities for both BMPs and insulin-like growth factors are regulated by various binding proteins in bone) [6, 37]. We demonstrated that, in osteoblasts, follistatin acts as a specific inhibitor of activin A and not of BMP2. This is in line with other data showing that follistatin does not inhibit BMP2 [38]. It is tempting to speculate that the function of the activin-follistatin mechanism is to control the extent of mineralization and, for example, to prevent overmineralization of bone tissue, which can result in brittle bones that are too stiff and unable to reform during loading [1].

Suppression of osteogenesis by activins was also found by others using murine and

rat osteoblasts [28, 29]. Other studies performed in rodent models showed stimulation of osteogenesis, bone formation, and fracture healing by activins [25-27, 39, 40]. Mice deficient for the inhibin-A subunit and follistatin have been generated and have shown abnormal tooth and craniofacial development after birth, but no other peculiar skeletal phenotypes [41, 42]. Unfortunately, both of these available systemic knockout mouse models die within 24 h of birth, making it impossible to study postnatal bone development and bone remodeling. Follistatin-overexpressing transgenic mice are viable, but no skeletal phenotype has yet been described in these mice [43]. Whether the lack of effects or opposite effects in murine models reflect yet-unexplained species differences is not clear. However, these studies lack detailed analysis of bone tissue (*e.g.*, bone mineral content, bone mineralization rate, and bone microarchitecture).

It has been shown that TGFβ, a structural relative of activin, can inhibit matrix mineralization in osteoblast cultures [8-10]. At the signal transduction level, activins and TGFβ both stimulate Smad2/3 phosphorylation [11], which might explain the overlapping effects of activin and TGFβ *in vitro*. Even though the effects of TGFβ on matrix mineralization are similar to the effects we describe for activin A, the physiology might differ. TGFβ is present as a latent complex in the bone matrix, and accumulated TGFβ is activated only during bone resorption by osteoclasts [44, 45]. In contrast, activins are produced and released directly in an active form. Therefore, activin and TGFβ differ in bioavailability during osteoblast development *in vivo*. TGFβ may activate osteoblasts (precursors) during bone resorption when inactive TGFβ becomes activated, whereas activin and follistatin control osteoblast differentiation and mineralization in a mechanism independent of a direct coupling to resorption.

The current mineralization data demonstrate that, in osteoblasts, activin treatment before the initiation of mineralization is crucial for strong inhibition of mineralization. On the basis of these data, it can be hypothesized that activin causes an altered ECM composition in such a way that the mineralization potential of the ECM is strongly inhibited. This hypothesis is substantiated by gene expression studies. The unbiased gene expression profiling approach demonstrated an altered expression of numerous ECM genes by activin signaling. Little is known about the role of the majority of these ECM proteins in matrix mineralization, which makes it difficult to interpret the full extent and significance of these ECM proteins alone or in combination. Only a limited number of deficient mice have been generated for these ECM genes, of which the majority lack a bone phenotype. Although for functional matrix protein analysis, knocking out or changing the expression of only a single ECM might not be sufficient to alter matrix mineralization. The combination of matrix proteins (*i.e.*, the ECM composition) might be of greater importance for the eventual matrix quality and mineralization capacity. Nevertheless, the observed direction of regulation coupled to the mineralization phenotype may hint at the function of these ECM genes. ECM genes that are up-regulated by activin signaling (low mineralization) may act negatively on mineralization, whereas ECM genes that are downregulated may act positively. In support of this is the observation that C-type lectin domain family 3 member B (CLEC3B, also known as tetranectin), down-regulated by activin signaling, has been shown to be positively involved in mineralization [46], whereas periostin (POSTN) and chondroitin

sulfate proteoglycan 2 (CSPG2), up-regulated by activin signaling, are negatively implicated in mineralization [47-49]. The direction of the regulation of genes involved in ECM turnover (MMP2, ADAMTS3, and TIMP4) is also in line with this hypothesis.

To extend the observation beyond skeletal development and mineralization, we showed that in a VSMCbased model for vascular mineralization, matrix mineralization was also inhibited by activin A treatment. This indicates that, besides osteoporosis, these findings can also be of great importance for vascular mineralization, which is a major risk factor for cardiovascular failure [50, 51]. Other studies have shown that activin A is expressed in VSMC cultures and in atherosclerotic lesions [52, 53]. Moreover, it was demonstrated that activin A promotes the contractile phenotypes of VSMC [52]. Promotion of this VSMC phenotype might simultaneously inhibit VSMC osteogenesis and matrix mineralization. On the other hand, activin A might inhibit VSMC-induced matrix mineralization via a mechanism similar to that in osteoblasts. This is supported by our observations that several ECM genes were similarly regulated by activin A in VSMCs and osteoblasts. Shao and colleagues showed that Msx2 promotes cardiovascular calcification by activating paracrine Wnt signaling [54]. We showed that activin signaling suppressed MSX2 expression in VSMCs as well as osteoblasts. According to GeneChip data, paracrine Wnt signaling seemed to be unaffected, with the exception of WNT2B. QPCR analyses demonstrated that activin signaling significantly decreased WNT2B mRNA expression in osteoblast and VSMC cultures (data not shown).

In summary, the key findings are 1) osteoblasts express activin A and its natural inhibitor follistatin to control activin signaling in a differentiation-dependent manner, 2) activin inhibits mineralization in a human bone formation model as well as in a model for vascular mineralization, and 3) activin does so by changing the expression of a wide range of matrix proteins before the onset of mineralization leading to a matrix composition with no or reduced mineralizing capacity. This led to the conclusion that activin signaling is a potent regulator of bone matrix formation and mineralization, and thereby an interesting mechanism in the control and maintenance of bone quality. Mineralization can be controlled in two directions using activin A, follistatin, or analogs of these compounds. As a consequence, activin signaling and activin target genes are important therapeutic targets for controlling matrix mineralization in bone as well as mineralization in pathological conditions.

5.4 Materials and methods

Cell culture

SV-HFO cells were cultured as described previously [30]. Medium was supplemented with freshly added 10 mM β-glycerophosphate (Sigma, St. Louis, MO, USA), 100 nM dexamethasone (DEX) (Sigma), or other additives (activin and follistatin) and replaced every 2 or 3 days. Follistatin was purchased from PeproTech and activin A from R&D Systems (Minneapolis, MN, USA). Normal human osteoblasts (NHOst) (Cambrex Bio Science CC-2538; East Rutherford, NJ, USA), vascular smooth muscle cells (coronary artery smooth muscle cells; Cambrex Bio Science; CC-2583), and human

Table 5.1. Primer and probe sequences and concentration used for qPCR analysis. Primer sets without a fluorescence probe were used in combination with the SYBR green QPCR kit (Eurogentec).

	forward primer nM 5'-3'		reverse primer nM 5'-3'		**Probe** nM 5'-FAM – 3' TAMRA	
ACVRIB	300	catcattgtttccttgtcattaactatc	300	cttgccaataatcttgtaaaacga	200	agcgcacagtggcccgaacc
ACVR2A	300	ttctcgctgtactgctgcagat	300	cttcctgcatgtcttcaagagatg	200	tggccaatttcctcctcaaatggca
ACVR2B	300	tcagcacacctggcatgaag	300	agttcgttccatgtgatgatgttc	200	acaagggctccctcacggattacctca
ALPL	300	gacccttgacccccacaat	300	gctcgtactgcatgtcccct	50	tggactacctattgggtctcttcgagcca
CLEC3B	100	caccaccgagccaccaa	100	ggtgtccagacggctcttga		
COL5A3	200	cctgcctctatcccgacaag	200	gcgtccacgtaggagaaacttct		
CSPG2	600	tggaatgatgttccctgcaa	600	aaggtcttggcatttctacaacag	200	ctggccgcaagcaactgttccttt
FBLN5	200	aaccgaagctgccaagacat	200	agccccctgtaaattgtagca		
GAPDH	150	atgggaaggtgaaggtcg	150	taaaagcagccctggtgacc	150	cgcccaatacgaccaaatccgttgac
INHA	300	ccgaggaagaggaggatgtct	300	cggtgacagtgccagcag	200	tgacttcagcccagctgtggttcca
INHBA	300	cctcggagatcatcacgtttg	300	ggcggatggtgactttggt	200	ctgacaggtcactgccttccttggaaatct
INHBB	300	acggccgcgtggagat	300	ggacgtaggcaggagtttca	200	tccgaaatcatcagcttcgccga
MMP2	200	tcaagttccccggcgat	200	tgttcaggtattgcactgcca	100	tcgcccccaaaacggacaaaga
MSX2	100	aggaacccggccgatattc	100	cgaggagctgggatgtggta		
NID2	100	ctgaacagtctgtgaaaaatctctatc	100	gcagctggcctgacattgt		
POSTN	200	tgtggacagaaaacgactgtgtta	200	cgatgcccagagtgccata		
TIMP4	100	cagggctcgatgtagttgca	100	cagccagaagcagtatctcttgac		

mesenchymal stem cells (Cambrex Bio Science) were cultured in a manner similar to that of SV-HFO cells, with only the following adjustments. NHOsts and hMSCs were used between passages 3 and 6 and seeded in a density of 5 x 10^3 and 10 x 10^3 vital cells per cm^2, respectively. NHOst and hMSC cultures were induced to mineralize in a medium similar to SV-HFO, except that 10 % charcoal-treated, heatinactivated FCS (Gibco BRL, Carlsbad, CA, USA) was used. VSMCs were used between passages 3 and 7 and seeded in a density of 5 x 10^3 vital cells/cm^2. Expansion of VSMCs was performed in smooth muscle cell medium (Cambrex Bio Science) supplemented with Clonetics Sm-GM-2 Bulletkit (Cambrex Bio Science). VSMCs were induced to mineralize in DMEM (Gibco BRL; with 4500 mg/l glucose, l-glutamine, and pyruvate) supplemented with 100 nM DEX, 0.1 mM ascorbic acid (Sigma), 10 µg/ml insulin (Sigma), 1 mM $CaCl_2$ (final concentration of 2.8 mM), 10 mM β-glycerophosphate, and 10 % FCS.

DNA, alkaline phosphatase activity, and mineralization assays

ALPL, DNA, and calcium measurements were performed as described previously [32].

Quantitative PCR analysis (QPCR)

RNA isolation, cDNA synthesis, and QPCR were performed as described previously [30]. Primer and probe sequences and concentrations used for QPCR are listed in Table 5.1.

Quantification of activin A, follistatin, and inhibin A

At various days during culture, medium was collected for activin A and follistatin measurements. Medium was collected from the cultures after 48 h incubation. Medium was centrifuged (5 min, 500 g) and stored at -20°C for further analysis. Cell lysates were also prepared so as to analyze DNA content of the corresponding cultures. Activin A was measured using 50–100 µl medium and the activin A DuoSet ELISA kit. Follistatin was measured using 100 µl medium and the follistatin quantikine ELISA kit (R&D Systems). Inhibin A was measured using the inhibin A ELISA kit purchased from Serotec (Raleigh, NC, USA).

Immunohistology

Plastified bone sections were deacrylated in a 1:1 mixture of xylene and chloroform for 30 min, then dipped in xylene, rehydrated, and rinsed twice with distilled water. Slides were pretreated with Tris-EDTA buffer, pH 9.0, for 15 min at 100°C and cooled down for 15 min, followed by rinsing in tap water. Endogenous peroxidase activity was inhibited by a 10:1 mixture of PBS and H_2O_2 for 10 min, followed by two water washes and one Tris-HCl pH 8.0 wash. The following steps were carried out in a humidified chamber at room temperature. Primary antibodies were diluted 1:50 in normal antibody diluent (Skytek Laboratories, Logan UT, USA; code ABB999) and incubated for 60 min. Inhibin-βA, inhibin-α, and IgG2b negative control antibodies were purchased from Serotec (MCA950 ST, MSA951S, MCA691). Slides were rinsed twice in Tris-HCl, pH 8.0. Immunoreactivity was detected with the Dako REALtm Envisiontm Detection System, peroxidase/ DAB +, rabbit/mouse (Dako, Carpinteria, CA, USA; code K5007). After DAB detection, slides were rinsed in tap water and counterstained in Harris hematoxylin for 1 min, rinsed in tap water for 2 min, dehydrated in ascending ethanol steps, rinsed in xylene, and

coverslipped with Pertex mounting medium (Histolab, Västra Frölunda, Sweden).

Luciferase reporter assays

On day 5 of culture, cells were transfected with 200 ng reporter plasmids per well (12-well plate) using Fugene6 (Roche, Nutley, NJ, USA). After 24 h, medium was replaced by fresh medium containing low serum (0.2 % FCS) and incubated for 3 h. After 3 h the medium was refreshed for the second time with medium containing low serum, but this time supplemented with additives as described in Results. After 24 h, cells were lysed by incubating for 20 min in 100–200 µl lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured using 25 µl cell lysate and the Steady-Glo Luciferase Assay System (Promega). Activin signaling was measured using pGL3(CAGA)12-lux (CAGA-Luc) [55] and BMP signaling was measured using pGL3-BRE-luc (BRE-Luc) [56].

Devitalization of osteoblast cultures

On day 10 of culture, medium was removed and the cultures were washed once with PBS (Gibco BRL). Cultures were air dried and frozen overnight at -20°C. Next, devitalized cultures were incubated normally as described in the cell culture methods (indicated as day 0 for devitalized cultures).

Affymetrix Genechip-based gene expression

Purity and quality of isolated RNA were assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA of three different biological samples was pooled per condition analyzed. Synthesis of first strand second strand cDNA from total RNA was performed according to the One-Cycle Target Labeling protocol (Affymetrix, Santa Clara, CA, USA; 701024 Rev. 3). In total, 4.0 µg of total RNA was reverse transcribed using a Superscript ds-cDNA Synthesis Kit according to the manufacturer's description (Invitrogen, Carlsbad, CA, USA). Subsequently, double-stranded cDNA was purified using GeneChip Sample Cleanup Module (Affymetrix) and served as a template in the *in vitro* transcription reaction using BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). The amplified biotinylated complementary RNA (cRNA) was purified using a GeneChip Sample Cleanup Module. In total, 20 µg of biotinlabeled cRNA was fragmentized by metal-induced hydrolysis at a final concentration of 0.5 µg/µl for 35 min at 94°C. Fragmentation was checked on an Agilent 2100 Bioanalyzer, confirming an average size of ≈100 nt. Fifteen micrograms of fragmented biotinylated cRNA was hybridized to GeneChip Human Genome U133 Plus 2.0 oligonucleotide Genechips (Affymetrix) according to the manufacturer's protocol (Affymetrix, 701025 Rev 5). Staining, washing, and scanning procedures were carried out as described in the GeneChip Expression Analysis technical manual (Affymetrix). Data acquisition was performed using the GeneChip Scanner 3000.

Genechip data analysis

To examine the quality of the various arrays, measured intensity values were analyzed using GeneChip Operating Software (Affymetrix). The percentage of present calls (≈40 %), noise, background, and ratio of GAPDH 3' to 5' (1.4) all indicated a high quality

of samples and an overall comparability. Probe sets that were not present (according to Affymetrix MAS5.0 software) in any of the Genechips were omitted from further analysis. Raw intensities of the remaining probe sets of each chip were log2 transformed and normalized using quantile normalization. After normalization, the data were back-transformed to normal intensity values. Data analysis was carried out using OmniViz software, version 3.6.0. For gene ontology (GO) analysis, the 520 selected affymetrix IDs were analyzed using OntologyTraverser [57].

Gene nomenclature

Gene names and gene symbols were used as provided by HUGO Gene Nomenclature Committee [58].

Statistics

Data were presented only if multiple independent experiments showed similar results. Experiments were performed at least in triplicate. Values are the means ± SEM. Significance was calculated using the Student's *t* test. The Benjamini and Hochberg false discovery rate was used to calculate significance for the GO analyses [57].

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Chapter 6

Wnt signaling acts and is regulated in a human osteoblast differentiation dependent manner

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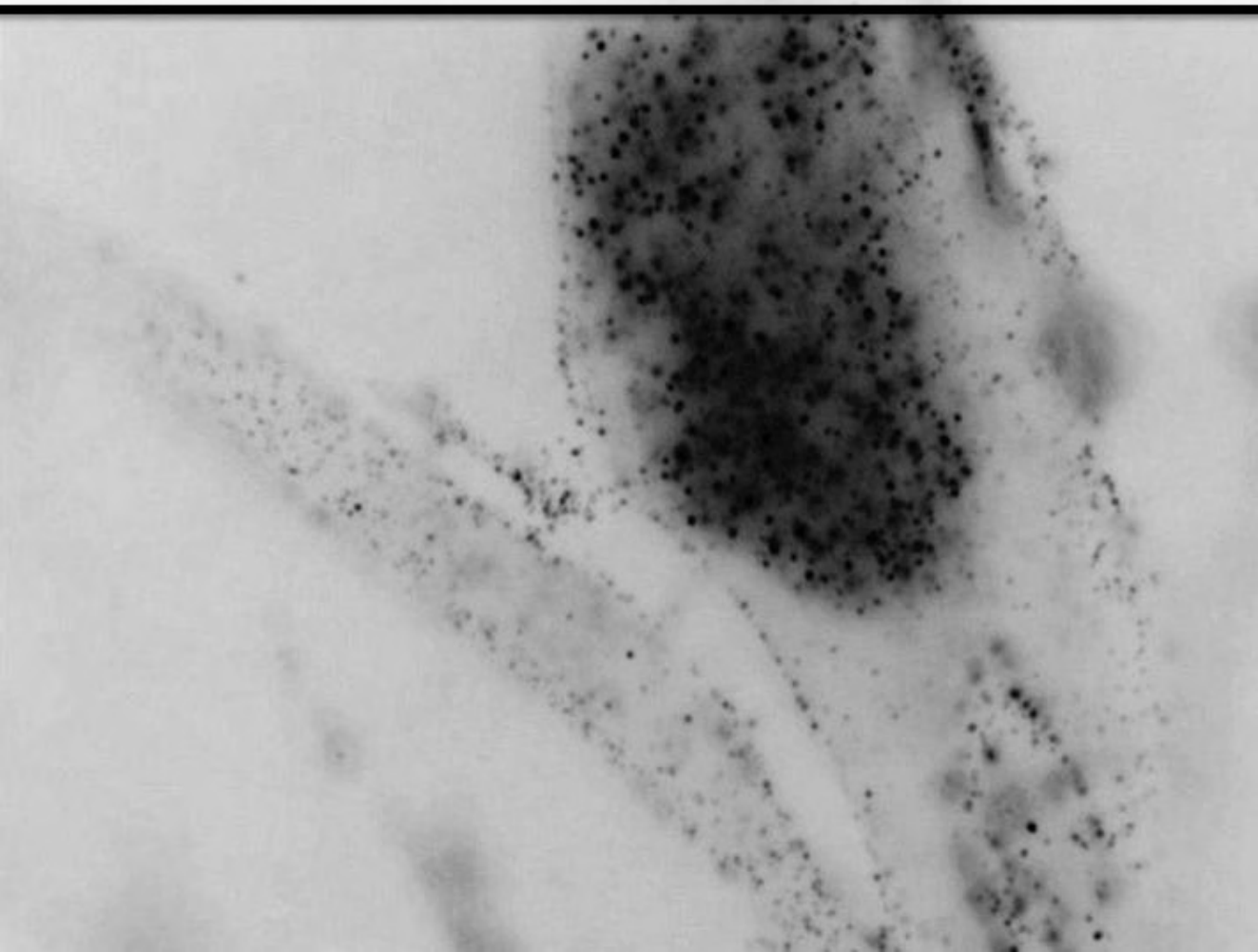

Abstract

The Wnt signaling pathway is an important regulator of cellular differentiation in a variety of cell types including osteoblasts. In this study we investigated the impact of Wnt signaling on the function of human osteoblasts in relation to the stage of differentiation. Differentiating osteoblasts were created upon glucocorticoid (GC) treatment, whereas nondifferentiating osteoblasts were created by excluding GC from the culture medium. GC-induced differentiation caused suppression of endogenous β-catenin levels and transcriptional activity. During glucocorticoid (GC)-induced osteoblast differentiation Wnt signaling activity slightly decreased alkaline phosphatase activity in differentiating osteoblasts, but strongly suppressed matrix mineralization. In addition, mRNA expression of several Wnt signaling genes was strongly regulated during GC-induced osteoblast differentiation, including frizzled homolog 8, dickkopf homolog 1 and secreted frizzled-related protein 1. In contrast, in the absence of GC-induced differentiation, Wnt signaling acted positively by stimulating basal alkaline phosphatase activity. Interestingly, pre-stimulation of Wnt-signaling in osteoblasts enhanced their differentiation capacity later on during the GC-induced differentiation process. In conclusion, we showed a differentiation-dependent effect of Wnt signaling in osteoblasts. Wnt signaling stimulated early osteoblasts in their capacity to differentiate, whereas mature osteoblasts were strongly inhibited in their capacity to induce mineralization. Moreover, osteoblast differentiation suppressed endogenous Wnt signaling and changed expression of multiple Wnt signaling genes.

6.1 Introduction

The Wnt signaling pathway is an important regulator of cellular differentiation in a variety of cell types [1]. It plays a widespread role in skeletogenesis from embryonic skeletal patterning, through fetal skeletal development, and bone remodeling in adults [2, 3].

In humans 19 different Wnt proteins have been identified. Wnt proteins activate Wnt/β-catenin signaling upon binding to the frizzled receptor and a co-receptor LRP 5 or 6. During activation of Wnt signaling degradation of β-catenin is prevented, which results in β-catenin accumulation, its nuclear transport and regulation of several target genes. In the absence of Wnt signal, the level of β-catenin is kept low through degradation of cytoplasmic β-catenin. A multiprotein complex containing glycogen synthase kinase 3β and scaffolding proteins axin and adenomatosis polyposis coli (APC) mediate the degradation of β-catenin by phosphorylation of specific amino acid residues. Wnt signaling is under the control of several extracellular factors that inhibit Wnt signaling. Secreted frizzled related proteins (SFRPs) and Wnt inhibitory factor (WIF) bind directly to Wnt proteins thereby preventing receptor activation. Dickkopf (Dkk) proteins inhibit Wnt signaling *via* binding to LRP5 or 6 and the associated protein Kremen, which dissociates LRP5 or 6 from frizzled preventing formation of an active Wnt receptor complex [1, 4].

Multiple skeletal defects have been described in mice deficient for distinct Wnt signaling genes [5-11]. In humans, loss-of-function mutations in LRP5 are associated with osteoporosis-pseudoglioma syndrome, which is characterized by low bone mass and skeletal fragility [12]. In contrast, gain of function mutations in LRP5 are associated with a high bone mass phenotype [13, 14]. Moreover, various genetic variants of LRP5 are associated with BMD and risk of fractures in humans [15-18]. Despite all these data, the precise mechanism of Wnt signaling in bone biology remains unclear. The skeletal defects are at least partially accomplished by regulating osteoblast differentiation. Several reports show that Wnt signaling contributes to osteoblast differentiation [19-21], whereas other reports show the ability of Wnt signaling to inhibit osteoblast differentiation [22-24].

The conflicting data on whether Wnt signaling is stimulating or inhibiting osteoblast differentiation is likely to reflect the diverse function of Wnt signaling at different stages of osteoblast development. To address this we exploited a human pre-osteoblast cell line, SV-HFO, that dependent on the presence or absence of GCs either differentiates in a full active bone forming osteoblast or does not differentiate, respectively. We studied the endogenous Wnt signal and expression of Wnt signaling related genes in differentiating and nondifferentiating osteoblasts. In addition, the impact of Wnt signaling was studied on osteoblast function in both differentiation conditions.

6.2 Results

Glucocorticoid induced human osteoblast differentiation

Wnt signaling was studied in human osteoblasts representing two functionally different

stages of differentiation. To create these two different staged osteoblasts the human pre-osteoblast cell line SV-HFO was treated with or without 100 nM dexamethasone (DEX). DEX treatment induced osteoblast differentiation and created cultures having high alkaline phosphates activity (ALPL) that initiated matrix mineralization after 2 weeks of culture. Cultures that were not treated with DEX showed low ALPL activity and showed no matrix mineralization [25]. Throughout the remainder of the study non-DEX-treated and DEX-treated cultures will be referred as nondifferentiating and differentiating cultures, respectively.

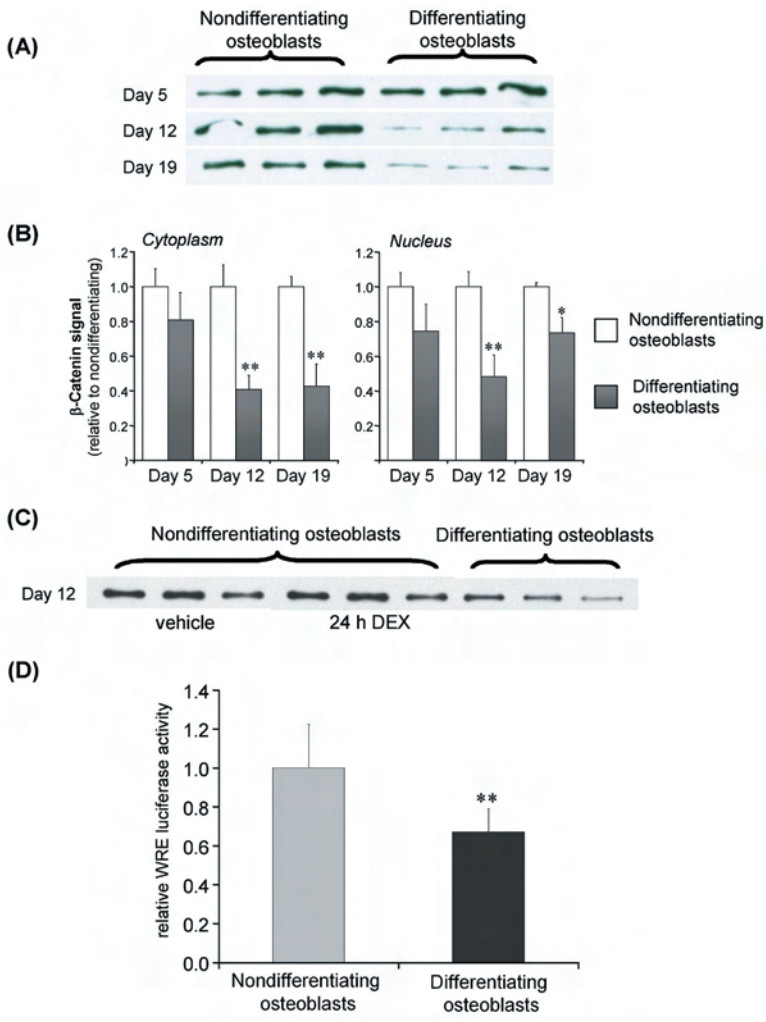

Figure 6.1. Endogenous Wnt signaling in differentiating and nondifferentiating osteoblast cultures. **(A)** β-catenin levels (cytosolic fraction) at day 5, 12 and 19 of culture. **(B)** Quantification of β-Catenin in the cytosolic and nuclear fraction in nondifferentiating and differentiating osteoblast cultures at day 5, 12 and 19 of culture. **(C)** β-Catenin levels (cytosolic fraction, day 12) of nondifferentiating cultures, nondifferentiating cultures that were treated for 24 h with DEX and differentiating osteoblast cultures. **(D)** Wnt signaling measured by a Wnt responsive luciferase reporter construct in nondifferentiating and differentiating cultures at day 7. * $p<0.05$, ** $p<0.01$ compared to nondifferentiating OBs.

Endogenous Wnt signaling is reduced in differentiating osteoblasts

To explore the relationship between differentiation and Wnt signaling, endogenous Wnt signaling was measured in differentiating and nondifferentiating osteoblasts. β-catenin levels were measured in both the cytosolic and nuclear fraction of osteoblast cultures at days 5, 12 and 19 of culture. This showed that differentiated osteoblasts had clearly lower β-catenin levels compared to their nondifferentiating counterparts in both cellular fractions. Quantification of multiple independent Western blot assays showed that β-catenin was significant reduced in differentiating cultures in both the cytosolic and the nuclear fraction at day 12 and 19 (Fig. 6.1 B). In addition, short-term DEX treatment (10^{-7}M, 24 h) did not change β-catenin levels (Fig. 6.1 C), demonstrating that the difference found in β-catenin levels is a consequence of the DEX-induced differentiation program.

Reduced β-catenin levels suggest reduced endogenous Wnt signaling. A Wnt signaling luciferase reporter construct was used (WRE-Luc) to measure Wnt signaling in osteoblasts. Indeed, this showed that differentiating osteoblasts had lower WRE-promoter activity compared to nondifferentiating cultures (Fig. 6.1 D).

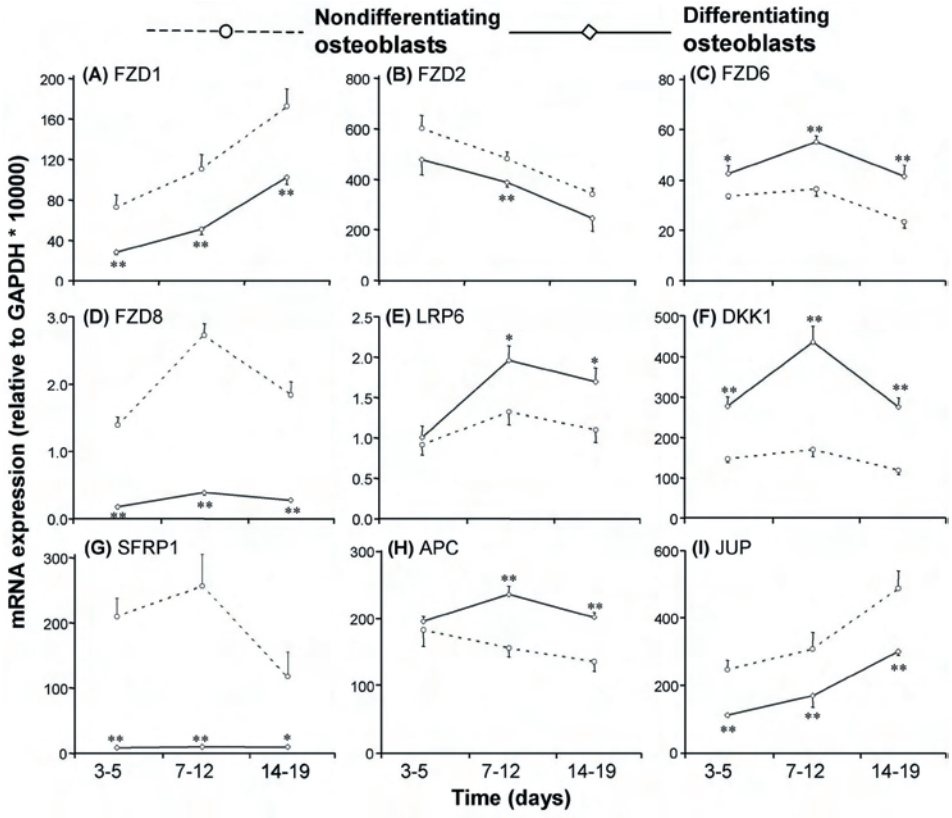

Figure 6.2. Gene expression of various Wnt signaling genes during osteoblast differentiation. **(A-I)** qPCR data of 9 Wnt signaling related genes that were differentially expressed between nondifferentiating and differentiating osteoblast cultures. * p<0.05, ** p<0.01 compared to nondifferentiating osteoblasts.

Regulation of Wnt related genes during osteoblasts differentiation

Affymetrix GeneChips were used to identify the expression of a broad range of Wnt signaling related genes in human osteoblasts. A gene was selected as a Wnt signaling gene on basis of data on http://www.stanford.edu/~rnusse/wntwindow.html. First, genes were identified as expressed in human osteoblast if they were detected by multiple independent GeneChip experiments. An overview of the presence of various Wnt signaling related genes in human osteoblasts is shown in Table 6.1. Secondly, Wnt signaling genes were selected that showed differential expression between nondifferentiating and differentiating osteoblasts. Genes were selected if the expression was 1.6 fold-different between nondifferentiating and differentiating cultures at at least one of the time points analyzed. Since a 1.6-fold difference is a relative mild cut-off, these genes were

Table 6.1. Wnt signaling related genes expressed in human osteoblast identified by Affymetrix GeneChips.
According to GeneChip data using a cut off value of 1.6-fold difference. * No probeset was present on the GeneChip to detect these genes.

Gene symbol	Detected by GeneChip	Regulated #	qPCR	Gene symbol	Detected by GeneChip	Regulated #	qPCR
Wnt proteins				**Inhibitors**			
WNT1	N	-		DKK1	Y	Y	Fig. 6.2 F
WNT2	N	-		DKK2	Y	N	
WNT2B	Y	N		DKK3	Y	N	
WNT3	Y	N		DKK4	N	-	
WNT3A*	-*	-*		SFRP1	Y	Y	Fig. 6.2 G
WNT4	N	-		SFRP2	N	-	
WNT5A	Y	N		FRZB	Y	N	
WNT5B	Y	-		SFRP4	Y	N	
WNT6	Y	Y		SFRP5	N	-	
WNT7A	N	-		WIF	N	-	
WNT7B	N	-		**Nuclear regulators**			
WNT8A	Y	N		CTNNB1	Y	N	
WNT8B	N	-		JUP	Y	Y	Fig. 6.2 I
WNT9A	Y	N		LEF1	Y	N	
WNT9B	N	-		TCF7	Y	N	
WNT10A	N	-		TCF7L1	Y	N	
WNT10B	N	-		TCF7L2	Y	N	
WNT11	N	-		**Cytoplasmic regulators**			
WNT16	Y	N		GSK3A	Y	N	
Receptors				GSK3B	Y	N	
FZD1	Y	Y	Fig. 6.2 A	APC	Y	Y	Fig. 6.2 H
FZD2	Y	Y	Fig. 6.2 B	APC2	Y	N	
FZD3	Y	N		DVL1	Y	N	
FZD4	Y	N		DVL2	Y	N	
FZD5	N	-		DVL3	Y	N	
FZD6	Y	Y	Fig. 6.2 C	DVL1L1*	-*	-*	
FZD7	Y	N		AXIN1	N	-	
FZD8	Y	Y	Fig. 6.2 D	AXIN2	Y	N	
FZD9	N	-		FRAT1	Y	N	
FZD10	N	-		FRAT2	Y	N	
KREMEN1	Y	N		NKD1	N	-	
KREMEN2	N	-		NKD2	N	-	
LRP5	Y	N		PORCN	N	-	
LRP6	Y	Y	Fig. 6.2 E				

only identified as potentially regulated. Quantitative real time PCR (qPCR) was used to confirm the differential mRNA expression of these genes.

The following 10 Wnt signaling related genes were identified as potentially regulated and analyzed using qPCR (Fig. 6.2); wingless-type MMTV integration site family member 6 (WNT6), frizzled homolog 1/2/6 and 8 (FZD1/2/6/8), low density lipoprotein receptor-related protein 6 (LRP6), dickkopf homolog 1 (DKK1), secreted frizzled-related protein 1 (SFRP1), adenomatosis polyposis coli (APC) and junction plakoglobin (JUP).

In total, 5 Wnt receptors were differentially expressed by GC-induced differentiation. FZD1, 2 and 8 were all suppressed by GC-induced differentiation, whereas FZD6 together with LRP6 were up regulated during GC-induced differentiation. The Wnt inhibitor DKK1 was stimulated by GC-induced differentiation at all days of culture, whereas another inhibitor of Wnt signaling SFRP1 was strongly suppressed by GC-induced differentiation. APC, which also acts negative on Wnt signal increased during the final stages of GC-induced differentiation. The β-catenin homolog JUP [26] was decreased by GC-induced differentiation. Differential expression of WNT6 could not be confirmed using qPCR (data not shown).

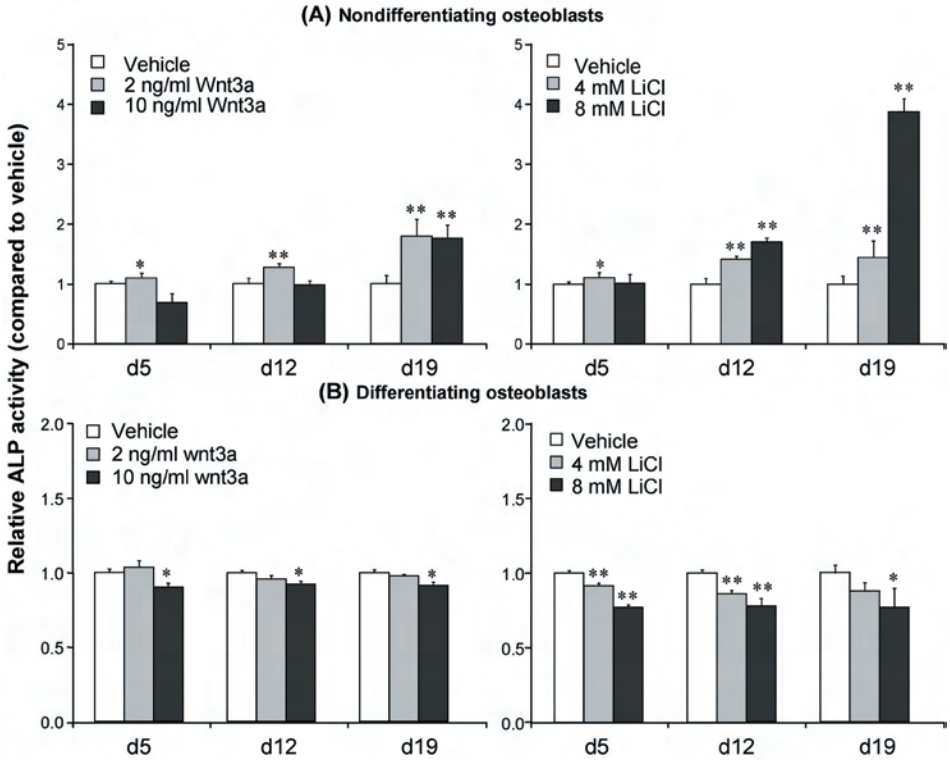

Figure 6.3. Effect of Wnt signaling on osteoblast differentiation. **(A)** Nondifferentiating osteoblasts were continuously treated with LiCl or Wnt3a to induce Wnt signaling, subsequently ALPL activity was measured at day 5, 12 and 19**. (B)** Differentiating osteoblasts (DEX treated) were continuously treated with LiCl or Wnt3a to induce Wnt signaling, subsequently ALPL activity was measured at day 5, 12 and 19. * p<0.05, ** p<0.01 compared to vehicle.

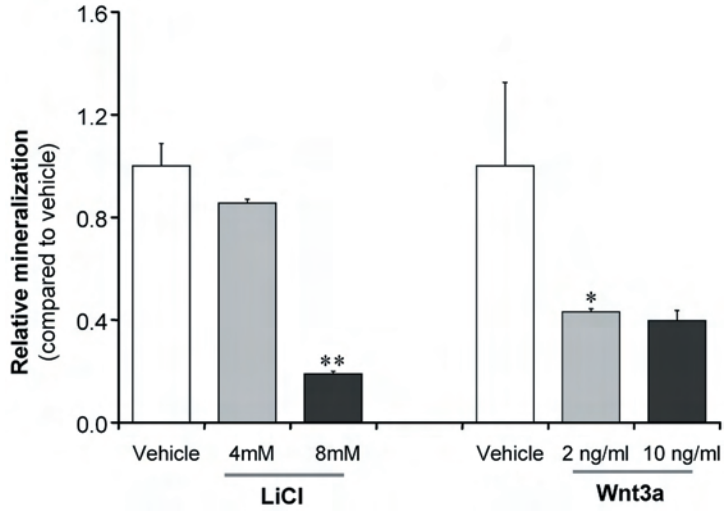

Figure 6.4. Effect of Wnt signaling on matrix mineralization. Differentiating osteoblasts (DEX treated) were continuously treated with LiCl or Wnt3a to induce Wnt signaling, subsequently matrix mineralization was measured at day 19. * p<0.05, ** p<0.01 compared to vehicle.

Wnt signaling controls osteoblast differentiation and function

Together these data show that during GC osteoblast differentiation, osteoblast lower their endogenous Wnt signal and change the expression of several genes involved in Wnt signaling. To reveal the impact of enhanced Wnt signaling in both differentiating and nondifferentiating osteoblasts Wnt signaling was activated in both conditions. Wnt signaling was induced by culturing SV-HFO cells in the presence of recombinant Wnt3a or lithium chloride (LiCl), which stimulates Wnt signaling by inhibiting β-catenin degradation [27]. First, Wnt signaling was activated in nondifferentiating pre-osteoblast cultures. These cultures were continuously treated with LiCl (4 and 8 mM LiCl) or Wnt3a (2 and 10 ng/ml), and subsequently ALPL activity was measured at days 5, 12 and 19 of culture. This showed that activation of Wnt signaling significantly induced basal ALPL activity with the most prominent effect after 19 days of culture (Fig. 6.3 A). Although ALPL activation was increased, this stimulation of ALPL in the absence of DEX was not sufficient to induced mineralization (data not shown). Secondly, Wnt signaling was activated in differentiating osteoblast cultures. Remarkably, in these differentiating cultures an opposite effect was observed. LiCl and Wnt3a treatment slightly decreased ALPL activity (Fig. 6.3 B). Moreover, in this condition we measured the effects of Wnt signaling on matrix mineralization. This showed that Wnt signaling in differentiating osteoblasts strongly inhibited matrix mineralization (day 19, Fig. 6.4).

In order to exclude any discrepancy in β-catenin activation between nondifferentiating and differentiating osteoblasts, β-catenin activation by LiCl treatment was measured in both differentiating conditions. This showed that 8 mM of LiCl clearly induced β-catenin levels in both differentiation conditions (data not shown). Moreover, in all experiments NaCl (4 and 8 mM) was used as a negative control for LiCl, which showed no effect on β-catenin levels, ALPL activity, and mineralization irrespective of the differentiation condition.

Wnt signaling controls osteoblast function in an differentiation dependent manner independent of glucocorticoids

Differentiating osteoblasts were created upon continuous treatment with DEX. Using this method the observed differentiation dependent effects of Wnt signaling on ALPL activity might be caused by two different processes; 1] direct interaction with glucocorticoid signaling or 2] as a consequence of the osteoblast differentiation program.

In order to study the role of DEX in more detail, experiments were performed in which LiCl treated cultures were co-incubated with DEX for 48 hours (Fig. 6.5). This should reveal whether the presence of DEX results in a direct inhibitory effect of Wnt signaling as observed during DEX-induced differentiation. SV-HFO cultures were cultured for 10 days in nondifferentiating conditions. Subsequently, these cultures were treated for 48 hours with LiCl (8 mM), DEX (100 nM) or a combination of both. DEX and LiCl treatment independently stimulated ALPL activity. Importantly, Wnt signaling-stimulated ALPL activity was independent of the presence or absence of DEX (Fig. 6.5). This proves that the different effects of Wnt signaling as shown in Figures 6.3 A and B are due to differences in osteoblast differentiation between the two conditions. In other words, Wnt signaling enhances ALPL activity when pre-osteoblasts are still undifferentiated, whereas Wnt signaling inhibits ALPL activity in differentiated osteoblasts.

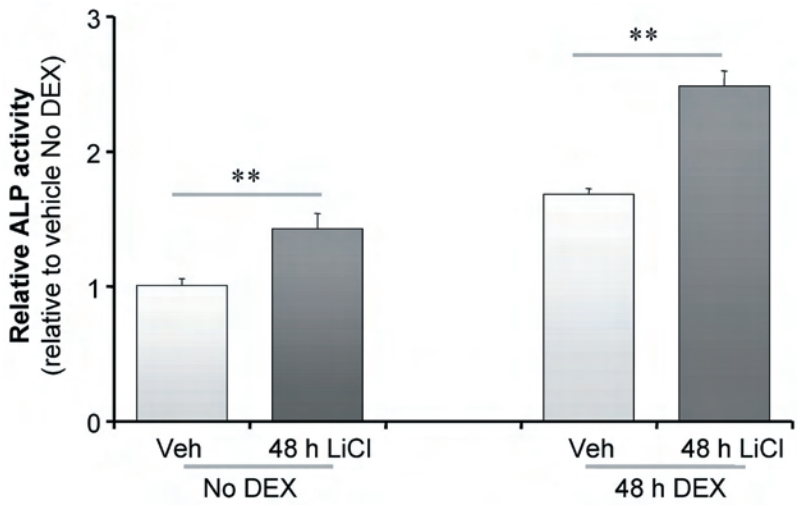

Figure 6.5. Short term LiCl and DEX treatment in nondifferentiating osteoblast cultures. Nondifferentiating osteoblasts were cultured until day 10. Subsequently, these cultures were treated for 48 hours with LiCl (8 mM), DEX (100 nM) or a combination of both. ** $p<0.01$

These data led to the hypothesis that activation of Wnt signaling in the early stages of osteoblast differentiation will enhance differentiation later on. In order, to test this hypothesis we activated Wnt signaling in nondifferentiating osteoblasts before they were induced to differentiate. This was achieved by pre-treating the cultures with LiCl (8 mM) for 5 days. After these 5 days the cultures were induced to differentiate by DEX

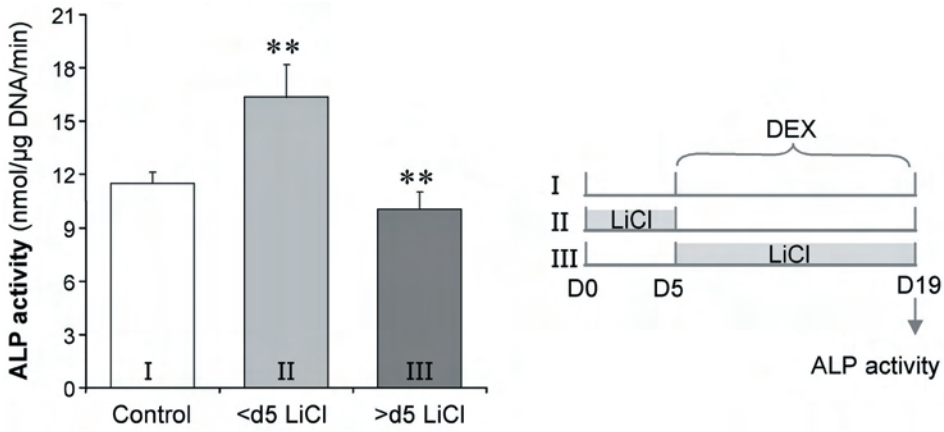

Figure 6.6. Activation of Wnt signaling in early osteoblasts. Nondifferentiating cultures were pre-treated with 8 mM LiCl for 5 days (II). After these 5 days differentiation was initiated by DEX treatment. In contrast, osteoblast cultures were treated with 8 mM LiCl only during DEX-induced differentiation from day 5 onwards (III). ALPL activity was measured at day 19 of culture. ** p<0.01compared to control (I).

treatment (Fig. 6.6 condition II). As a control, pre-osteoblast cultures were treated with LiCl (8 mM) only during DEX-induced differentiation from day 5 onwards (Fig. 6.6 condition III). At day 19 we quantified the level of differentiation by measuring ALPL activity. This revealed that pre-treatment with LiCl until day 5 enhanced ALPL activity at day 19 compared to the non pre-treated controls (Fig. 6.6 condition I). In contrast, cultures that were treated with LiCl only during DEX-induced differentiation had slightly reduced ALPL activity (Fig. 6.6 condition III).

6.3 Discussion

Conflicting data have been published whether Wnt signaling acts stimulatory or inhibitory on osteoblast differentiation. Using human pre-osteoblasts we showed that Wnt signaling acts either negative or positive on osteoblast function. Most importantly, these effects were dependent on the differentiation stage of the osteoblast. In human osteoblasts, differentiation can be initiated after exposure to GCs. During this GC-induced osteoblast differentiation process Wnt signaling strongly inhibited matrix mineralization. This negative effect of Wnt signaling on osteoblast differentiation and matrix mineralization was also demonstrated by other studies using osteoblasts from various origin [22-24]. In contrast, Wnt signaling acted positive on osteoblasts when, in the absence of GCs, they were not induced to differentiate. Smith and colleagues demonstrated that GCs can directly influence Wnt signaling by inhibiting the transcriptional activity of LEF/TCF in differentiating murine osteoblasts [28]. However, we showed that the effects of Wnt signaling in differentiating osteoblasts are not due to a direct effect of GCs on Wnt signaling but the result of an altered cellular response to Wnt signaling as a consequence of the GC-induced differentiation program.

We hypothesized that Wnt signaling stimulates early osteoblasts in their capacity to differentiate and subsequently to progress to mature matrix synthesizing osteoblasts.

This is proven by our pre-treatment studies in which Wnt signaling was induced for 5 days in the early stage of culture and next induced to differentiate by GCs. The differentiation capacity of the Wnt signaling pre-treated cells was higher than the non-pretreated cells. However, other additional signals, *i.e.*, GCs were still necessary to induce full differentiation. This positive property of Wnt signaling on osteoblast differentiation capacity is supported by LRP5 deficient mice that showed a reduced number of osteoblasts and by reports showing stimulation of osteoblast differentiation by Wnt signaling *in vitro* [5, 19-21]. These early effects together with the observations of a controlling or limiting effect on the final differentiation and mineralization of osteoblasts shows a dual control mechanism of osteoblast differentiation by Wnt signalling. Such a dual control mechanism might act as an elegant feedback mechanism to control proper bone formation by controlling the number of osteoblast precursors that will become mature osteoblasts and at the same time controlling the activity of the mature osteoblasts. In case this control mechanism is strongly out of balance this results in impaired bone formation, as demonstrated by various mice models that are deficient for distinct Wnt signaling genes [5, 7-11].

The diverse function of Wnt signaling at different stages of osteoblast development makes the control of Wnt signaling during these different stages of great importance to ensure proper osteoblast function. Suppressed β–catenin levels in differentiating osteoblasts points to such a control mechanism. In mouse ES cells β–catenin levels are correlated with the differentiation capacity of these cells [29]. Suppressed β–catenin levels in differentiating osteoblasts might help to facilitate osteoblast differentiation and matrix mineralization. One candidate to suppress Wnt signaling during human osteoblast differentiation is the Wnt signaling inhibitor DKK1. DKK1 was significantly up-regulated during GC-induced differentiation and is a well-known inhibitor of Wnt signaling. A functional role of Dkk proteins in osteoblasts has been described for Dkk2 in murine osteoblasts. Dkk2 expression is strongly increased during murine osteoblast differentiation and silencing of Dkk2 clearly reduced matrix mineralization [24]. Moreover, Dkk2 deficient mice show reduced matrix mineralization, supporting the negative effects of Wnt signaling on mature osteoblasts *in vivo* [11]. However, it is likely that several other regulators are involved as well in suppressing Wnt signaling in mature osteoblasts.

The fact that there are multiple Wnt proteins, Wnt inhibitors and Wnt receptors makes it yet difficult to unravel the role, regulation and function of Wnt signaling in osteoblasts. More information about which Wnt signaling genes are expressed and functional in osteoblasts is important for better understanding the role and complexity of Wnt signaling in bone. One challenge is to reveal the impact of endogenous expression of Wnt proteins, *e.g.*, which Wnt proteins activate early differentiation and which Wnt proteins inhibit later stages of differentiation and *via* which Frizzled or LRP receptors these actions take place.

Our data show that several Wnt signaling genes were differentially expressed between nondifferentiating and differentiating osteoblasts. A similar kind of study was performed by Boland and colleagues in human mesenchymal stem cells (MSCs) focusing on FZD on WNT genes [23]. They found differential mRNA expression of FZD6, FZD7, WNT11 and WNT9A during osteoblast differentiation. We found differential expression of four

Frizzled receptors, LRP6, and the Wnt signaling inhibitors DKK1 and SFRP1. SFRP1 was the most strongly regulated Wnt signaling gene found in our study. Interestingly, in mice this Wnt antagonist has been shown to be a negative regulator of trabecular bone formation [10]. Although we cannot explain the precise consequence of all the differentially expressed genes for osteoblast function we can correlate them with decreased β–catenin levels and decreased Wnt signal. Moreover, it might well be that due to the differentially expressed Wnt receptors and Wnt antagonists osteoblasts have a changed sensitivity and specificity for the various Wnt proteins during differentiation.

It is still unclear, however, how the differentiation dependent effects of Wnt signaling are mediated. In this paper we excluded a direct interaction of GCs. Moreover, LiCl experiments showed that the differentiation dependent effects were independent of any Wnt signaling receptor, Wnt protein, or Wnt antagonist. Therefore, it is tempting to speculate that differentiation-dependent epigenetic activation and silencing of Wnt signaling-regulated genes takes place. However, to answer this question detailed target gene promoter analyses should be performed in Wnt activated early and mature osteoblast cultures.

In conclusion, we showed that Wnt signaling stimulates the early steps of osteoblast differentiation and inhibits more mature osteoblasts. Importantly, we demonstrated these contradicting findings within the same osteoblast differentiation model, precluding possible cell dependent or species dependent effects. Furthermore, we found that differentiating osteoblasts have suppressed Wnt signal and changed expression of various Wnt signaling genes, which might help facilitate the differentiation and mineralization process in osteoblasts.

6.4 Materials and methods

Cell Culture

SV-HFO cells were cultured in αMEM (Gibco BRL, Paisley, U.K) supplemented with 20 mM HEPES, pH 7.5 (Sigma, St.Louis, MO); streptomycin/penicillin; 1.8 mM $CaCl_2$ (Sigma); and heat-inactivated FCS (GIBCO) at 37°C and 5 % CO_2 in a humidified atmosphere. Thawed cells were precultured for 1 week in the presence of 10 % FCS. In this preculture, cells were seeded in a density of 5 x 10^3 vital cells per cm^2 and were subcultured every week. During this preculture, SV-HFO cells remained in an undifferentiated stage. After preculturing, cells were seeded in a density of 10 x 10^3 vital cells per cm^2 in 6- or 12-wells plates (Corning, NY, USA). After seeding, cells were incubated for two days before they were put on differentiating medium (indicated as day 0). Medium was supplemented freshly with 10 mM β–glycerophosphate (Sigma), 100 nM dexamethasone (DEX) (Sigma) or other additives and replaced every 2-3 days. Nondifferentiating cultures were similarly cultured as differentiating cultures, however DEX was excluded. LiCl and Wnt3a were purchased from Sigma and R&D systems (Minneapolis, MN, USA), respectively. For analysis, medium was collected and stored at -20°C and cells were harvested from the culture dish in PBS containing 0.1 % Triton X-100 and stored at -80°C. Cell lysates were sonicated on ice in a sonifier cell disrupter for 2 x 15 sec before analysis.

Alkaline phosphatase activity and mineralization assays

For DNA measurements, 100 µl SV-HFO cell lysates were treated with 200 µl heparin (8 IU/ml in PBS) and 100 µl ribonuclease A (50 mg/ml in PBS) for 30 min at 37°C. This was followed by adding 100 ml ethidium bromide solution (25 µg/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (PerkinElmer, Wellesley, MA) using an extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma) was used.

ALPL activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamine buffer supplemented with 1 mM MgCl2 at pH 9.8) in the SV-HFO cell lysates for 10 min at 37°C. The reaction was stopped by adding 0.06 M NaOH and measured at 405 nm. Results were adjusted for DNA content of the cell-lysates.

For quantification of the mineral content cell lysates were incubated overnight in 0.24 M HCl at 4°C. Calcium content was colorimetrically determined after addition of 1M ethanolamine buffer (pH 10.6) 0.35 mM o-cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline and 0.6 mM hydrochloric acid at 595 nm. Results were adjusted for DNA content of the cell-lysates.

β-catenin westernblot

From SV-HFO cultures, nuclear and cytoplasm fractions were isolated using NE-PER isolation kit (Pierce, Rockford, IL, USA) according to the manufacturer's description and stored at -80ºC. Before analysis, total protein concentration was measured using BCA protein assay reagent (Pierce) according to the manufacturer's description. Next, 10 µg protein was separated on a 10 % SDS-PAGE gel, followed by blotting on Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blotting, membranes were blocked for 2 hours in TBST (20 mM Tris, 137 mM NaCl, 0.1 % Tween-20, pH 7.6) containing 5 % milk powder. Next, membranes were incubated overnight at 4º C with monoclonal anti-β-Catenin antibody (1:2000; Cell signaling, Beverly, MA, USA) in TBST containing 5 % bovine serum albumin (Sigma). For detection, membranes were incubated for 2 hours with goat-α-rabbit-HRP (1:1000; DAKO, Carpinteria, CA, USA) in TBST containing 5 % milk powder. Next, the ECL detection system (Amersham Pharmacia Biotech) was used and immunoreactive bands were quantified using Quantity One software (Bio-Rad Hercules, CA, USA)

Luciferase reporter assays

At day 5 of culture cells were transfected with 200 ng Wnt signaling WRE-Luciferase reporter plasmid (TOPFLASH, [30]) (12 wells plate) using Fugene6 (Roche, Basel, Switzerland) according to the manufacturer's description. After 24 hours cells were lysed by incubating for 20 minutes in 100-200 µl lysis buffer (Promega, Madison, WI). Luciferase activity was measured using 25 µl cell-lysate and the Steady-Glo Luciferase Assay System (Promega). To correct for transfection efficiency between nondifferentiating and differentiating luciferase values were corrected for the luciferase activity of the pGL3-control vector (Promega).

RNA isolation and cDNA synthesis

Total RNA was isolated using RNA-Bee solution (Tel-Test, Friendwood, TX) according to the manufacturer's protocol. To remove calcium (derived from the extracellular matrix), RNA was precipitated by overnight incubation with 4 M LiCl and 50 mM EDTA at -20°C. After precipitation and centrifugation for 30 min at 14,000 rpm and 4°C, the RNA pellet was washed four times with 70 % EtOH and subsequently dissolved in H2O. The total amount of RNA was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). One microgram total RNA was reverse transcribed into cDNA using a cDNA synthesis kit and according to the protocol of the manufacturer (MBI Fermentas, St. Leon-Rot, Germany), using 0.5 µg oligo(dT)$_{18}$ and 0.2 µg random hexamer primers. When purified mRNA was used in the cDNA synthesis, 10 µg of total RNA was purified using a oliotex mRNA mini kit (Qiagen, Stanford, CA, USA) according to the manufacturer's protocol. RNA was eluted in 60 µl form which 10 µl was used in the cDNA synthesis.

Quantitative real time PCR (qPCR)

qPCR was carried out using an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in 25 µl volumes using a qPCR core kit (for assays using a probe) and qPCR kit for SYBR green I (for assays using SYBR green) (Eurogentec, Seraing, Belgium). Primer and probe sets were designed, using the Primer Express software (version 2.0; Applied Biosystems), amplicons overlapped at least one exon-exon boundary. Since the FZD1, 2 and 8 genes consist of a single exon these genes were also analyzed on cDNA derived from purified mRNA to avoid genomic DNA contamination. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Primer and probe concentrations are listed in Table 6.2. Genes consisting of a single exon were quantified using cDNA derived from purified mRNA.

Affymetrix GeneChip-based gene expression

Purity and quality of isolated RNA was assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). Per analyzed condition total RNA of 3 different biological samples was pooled. Synthesis of first and second strand cDNA from total RNA was performed according to the One-Cycle Target Labeling protocol (Affymetrix; 701024 Rev. 3). In total, 4.0 µg of total RNA was reverse transcribed using Superscript ds-cDNA Synthesis Kit according to the manufacturer's description (Invitrogen). Subsequently, double-stranded cDNA was purified using GeneChip Sample Cleanup Module (Affymetrix) and served as a template in the *in vitro* transcription reaction using BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). Next, the RNA (cRNA) was purified using GeneChip Sample Cleanup Module. In total, 20 µg of biotin-labeled cRNA was fragmentized by metal-induced hydrolysis at a final concentration of 0.5 µg/µl for 35 minutes at 94°C. Fragmentation was checked on an Agilent 2100 Bioanalyzer confirming an average size of approximately 100 nt. In total, 15 µg fragmented biotinylated cRNA was hybridized to GeneChip Human Genome U133 Plus 2.0 oligonucleotide GeneChips (Affymetrix) according to the manufacturer's protocol (Affymetrix, 701025 Rev 5). Staining, washing and scanning procedures were carried out as described in the GeneChip

Table 6.2. Primer and probe sequences and concentration used for qPCR analysis. Primer sets without a fluorescence probe were used in combination with the SYBR green QPCR kit (Eurogentec)

	forward primer nM5'-3'	**reverse primer** nM 5'-3'	**Probe** nM 5'-FAM – 3' TAMRA
APC	200 tttggaacagatacgcgcttac	200 agaacacacacagcaggacagatc	
DKK1	500 aatgtatcacaccaaaggacaagaag	500 gatcttggaccagaagtgtctagca	100 tctgtttgtctccggtcatcagactgtgc
FZD1	100 cactcccttctcccaccttagtt	100 caccctcaaaaattagacttcaaaa	
FZD2	200 tttctgggcgagcgtgat	200 aaacgcgtctcctcctgtga	
FZD6	600 gcggtggaaatggagcatt	600 atgctttgcagaggaaagtttca	100 ctctcgcaaatctggaatgttcaccaaaca
FZD8	200 aagacaggccagatcgctaact	200 cgctcgtcctggctgaa	
GAPDH	150 atgggaaggtgaaggtcg	150 taaaagcagccctggtgacc	150 cgcccaatacgaccaaatccgttgac
JUP	100 tcagcagcaagggcatca	100 ccctggctgttgtggacatc	
LRP6	200 gtgagaagagaacgcgagaag	200 gtcccgtctgtttgcataaagc	
SFRP1	200 cgtctgcatcgccatgac	200 atgttcaatgatggcctcagattt	

Expression Analysis technical manual (Affymetrix). Data acquisition was performed using the GeneChip Scanner 3000.

In order to examine the quality of the different arrays, measured intensity values were analyzed using the GeneChip Operating Software (GCOS, Affymetrix). The percentage of present calls (about 40 %), noise, background, and ratio of GAPDH 3' to 5' (<1.4) all indicated high quality of the samples and an overall comparability. Probe sets that were never present (according to Affymetrix MAS5.0 software) in any of the GeneChips were omitted from further analysis. Raw intensities of the remaining probe sets (30336) of each chip were log2 transformed and normalized using quantile normalization. After normalization the data was back transformed to normal intensity values.

Gene nomenclature

Gene names and gene symbols were used as provided by HUGO Gene Nomenclature Committee [31].

Statistics

Data were only presented if multiple independent experiments showed similar results. Experiments were performed at least in triplicate. Values are indicated as means ± SEM. Significance was calculated using the Student's *t*-Test.

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Chapter 7

General Discussion

7.1 The use of in vitro bone formation models

In the treatment of osteoporosis additional therapies that promote bone formation are highly desirable. To develop novel drugs that enhance bone formation, more knowledge about the biology of osteoblasts is required. To study complex processes such as osteoblast differentiation and matrix mineralization we used several *in vitro* osteoblast-based models of bone formation. In the presented studies three different human derived osteoblast models were used; 1] bone marrow derived mesenchymal stem cells (MSCs), 2] immortalized fetal pre-osteoblasts (SV-HFOs), and 3] primary committed osteoblasts (NHOsts). The SV-HFO osteoblast model was frequently used since it has been shown to be a robust model in timing of differentiation and formation of a mineralized extracellular matrix (ECM). This in contrast to the MSC and NHOst osteoblast models that showed much greater variations in timing of differentiation and mineralization. Nevertheless, the MSC and NHOst models are of great use for validation purposes. Moreover, using these three osteoblast models a broad window of the osteoblast differentiation process was covered.

7.2 Complexity in the control of osteoblast differentiation

In this thesis three signaling pathways were investigated in relation to bone formation; 1] glucocorticoid (GC) signaling, 2] activin signaling and, 3] Wnt signaling. GC signaling was shown to be essential for the initiation of human osteoblast differentiation, whereas the activin and Wnt signaling pathways strongly inhibited *in vitro* bone formation. Despite their negative effects, the Wnt and activin signaling pathways were shown to be endogenously active in osteoblasts. Although this seems illogical, it might function as a continuously break to control differentiation and (over) mineralization. Interestingly, in osteoblast cultures in which differentiation and mineralization were initiated the break was weakened as demonstrated by suppressed activin and Wnt activity (Chapter 5, Fig. 5.3 E; Chapter 6, Fig. 6.1). An opposite mechanism was observed for GC signaling, which was enhanced via expression of 11β-HSD1 in case of delayed differentiation (Chapter 3, Fig. 3.3). Together this demonstrates that osteoblasts intrinsically control multiple signaling pathways to facilitate and control their differentiation and mineralization in an optimal manner.

Despite the fact that the GC, activin, and Wnt signaling pathways were able to regulate osteoblasts, they are not the unique regulators of osteoblasts. Many other factors are known that regulate osteoblast differentiation and activity such as 1,25α(OH)$_2$D$_3$, parathyroid hormone, TGFβ ,insulin growth factor I and several BMPs [1-7]. It is likely that this high variety of endocrine, paracrine and autocrine factors regulating osteoblast differentiation is necessary to control the multiple steps involved in osteoblastogenesis (Chapter 1; Fig. 1.4). This indicates that during *in vitro* studies a certain hormone can be inactive in early stages of differentiation, whereas it has a major impact later on during differentiation and *vice versa*. This is emphasized by our findings, which showed that the GC, activin, and Wnt signaling all acted in a differentiation dependent manner.

It is likely that the steps in osteoblastogenesis are not controlled by one single factor but by a specific set of regulatory factors. Moreover, these various factors probably interact with each other in order to control osteoblast differentiation. The big challenge is now to map all factors that control human osteoblast function and differentiation. In addition, more knowledge is necessary about how, when and together with what these factors act on osteoblasts. Therefore, the following points should be addressed when studying regulatory factors in osteoblast cultures; 1] at which stage of osteoblast differentiation do these factors act, 2] do these factors interact with each other, 3] is there a certain hierarchy among the factors, 4] do they act in an endocrine, paracrine or autocrine manner, 5] are these factors controlled by osteoblasts, *i.e.*, can they be inhibited or activated by osteoblast themselves, and 6] can these factors be of physiological relevance, in other words can the factors reach an osteoblast *in vivo?*

7.3 Mouse, rat and human osteoblasts models are not one and the same

The studies concerning activin signaling in Chapter 5 were initiated because work done by Gaddy-Kurten and colleagues who showed that activins stimulated osteoblastogenesis in mice [8]. To our surprise we found a strong suppression of matrix mineralization in the human osteoblast models. This discrepancy between the two species is not a unique observation since various discrepancies have been described between murine and human osteoblasts. An example is given by GCs. In this thesis the importance of GCs for human osteoblast differentiation was emphasized (Chapters 2, 3 and 4). However, in murine osteoblast cultures, GCs are non-essential for initiation of differentiation [9, 10]. The osteocalcin gene is a good example of species dependent regulation of gene transcription. Osteocalcin is an important protein in bone and is used in the clinic as a serum marker for bone turnover. Remarkably, production of this protein in mice and humans is regulated in opposites ways by $1,25\alpha(OH)_2D_3$, which is a well-known hormone controlling calcium homeostasis and bone metabolism. $1,25\alpha(OH)_2D_3$ clearly enhances osteocalcin production in human (Chapter 2, Fig. 4) and rat osteoblasts [11], whereas it suppresses osteocalcin production in murine osteoblasts [12, 13]. Although rats are closely related to mice, rat osteoblasts show several discrepancies with murine osteoblasts, regarding $1,25\alpha(OH)_2D_3$ regulation of osteocalcin [11], the impact of activin A [14] and the requirement of GC for their differentiation [15, 16]. In these respects, rat osteoblasts show more similarities with human osteoblasts.

The combination of differentiation- and species-specific effects in osteoblasts complicates the comparison of distinct observations generated in different osteoblast models. For example, we believe that the discrepancies we found between murine and human osteoblasts are due to species-specific effects. However, since we compared two different osteoblast models we cannot completely exclude other factors explaining the contradicting effects of activin A. The stimulatory effects of activin A in the murine osteoblasts were based on directly isolated and cultured bone marrow cells. In these experiments adherent bone marrow cells were cultured and treated with activin A. In

our experiments, using human cells, we cultured only the MSC fraction of human bone marrow that was free from hematopoietic cells. Although unlikely, activin A might act differently in total bone marrow cultures compared to isolated MSC cultures.

7.4 Paradoxical effects of glucocorticoids in bone

Glucocorticiods are nowadays well-established factors that stimulate human osteoblasts *in vitro*. However, long term GC treatment frequently results in bone loss and increased risk of fractures [17-19]. These negative side effects of GC treatment on bone are still poorly understood, but are believed to be partially induced by reduced bone formation [18, 20, 21]. In the past murine osteoblast models were studied to unravel the mechanism underlying the negative side effects of GCs. These cells, however, do not require GCs to initiate their differentiation program *in vitro* [9, 10], which is in great contrast to human osteoblasts for which GCs are a prerequisite for proper differentiation. Using these murine osteoblast models, it was shown that GCs mediate their negative side effects directly via osteoblast by inducing apoptosis and inhibiting proliferation [20, 22, 23]. Nowadays, human osteoblast models are more frequently used, which lack the described negative side effects of GCs described in murine osteoblasts (Chapter 2, Fig. 2.1) [24].

Although GCs are essential for proper human osteoblast development, little was known about the molecular mechanism underlying GC-induced osteoblast differentiation and mineralization. Chapter 4 is the first study that describes the molecular consequence of GC-induced differentiation in human osteoblasts. In this study genome-wide gene expression microarrays were used to identify numerous genes that are under the control of the GC-induced differentiation program. Several transcription regulators were identified including ZBTB16 and ZFP36, which were shown to be direct GC target genes (Chapter 4, Fig. 4.10). RUNX2 was also found to be up-regulated during GC-induced differentiation (Chapter 2, Fig. 2.3). Although this gene is crucial for osteoblast differentiation and function [25, 26] it is not a direct target gene of GCs. Recently, ZBTB16 was found to be a crucial transcription factor for human osteoblast differentiation and matrix mineralization acting upstream of RUNX2. Therefore it was interesting to identify ZBTB16 as the strongest regulated gene in our human osteoblast models. Its expression was over 80-fold up-regulated after 3h of GC treatment and more than 20000-fold after 2 weeks of differentiation. It is tempting to speculate that ZBTB16 is the first transcriptional responder of GCs during GC-induced differentiation, activating many other secondary genes such as RUNX2 that initiate osteoblast differentiation.

The essential role of GCs for human osteoblast differentiation is emphasized by 11β-HSD1 activity in bone [27]. Why should such an enzyme, which converts inactive cortisone into cortisol, be expressed in bone if cortisol is detrimental for bone? In Chapter 3 (Figs. 3.3 and 3.4) we describe that 11β-HSD1 activity acts as a feedback mechanism for delayed osteoblast differentiation caused by low cortisol concentrations. Most of the cortisol in the circulation is bound to cortisol binding globulin (CBG) [28], which might limit the access of cortisol to the osteoblast microenvironment. In contrast, the 11β-HSD1 substrate cortisone has an approximately 10-fold lower affinity for CBG than

cortisol [29], resulting in relatively high levels of free cortisone, which can easily diffuse to the osteoblast microenvironment. In the latter situation 11β-HSD1 acts as a continuously paracrine activator of cortisol. Such a mechanism in bone is not unique for cortisol, paracrine activation of estrogens and $1,25\alpha(OH)_2D_3$ has also been demonstrated [30-32].

Although numerous studies show that GCs are non-essential for murine osteoblast differentiation, other studies show that physiological GC concentrations *in vitro* can be beneficial for murine osteoblast differentiation or activity [33-36]. These findings are supported by *in vivo* studies performed by Sher *et al*. [37] who demonstrated an anabolic effect of physiological levels of GCs on bone. They developed transgenic mice that have locally abolished corticosterone action in bone, achieved by specifically overexpressing 11β-HSD2 in osteoblasts. This enzyme converts corticosterone into inactive 11-dehydrocorticosterone to reduce active GC levels locally. These 11β-HSD2 transgenic mice showed reduced trabecular bone and lower basal collagen synthesis supporting the anabolic role of physiological GC concentrations in bone tissue.

All in all, it is valid to conclude that physiological concentrations of GCs are beneficial for bone, whereas high doses GCs initiate several secondary effects (*e.g.*, at the intestine, kidney, parathyroid glands) that act negatively on bone.

7.5 Microarray generated data

The microarray technology was used to find regulatory genes that control the process of differentiation and mineralization in osteoblasts. Gene profiles were generated in relation to; 1] GC-induced osteoblast differentiation and matrix mineralization (Chapter 4), 2] activin A suppression of matrix mineralization (Chapter 5), and 3] $1,25\alpha(OH)_2D_3$-enhanced osteoblast differentiation and matrix mineralization (data not shown in this thesis [38]). An important aspect of these studies was that we directly linked the microarray data to the phenotypic effects on the osteoblast (=matrix mineralization).

This approach identified many known osteoblast-related genes but also many genes were identified yet unknown to be expressed in osteoblasts. The identified genes were strongly enriched in specific Gene Ontology (GO) categories, like development, cell differentiation and skeletal development (Chapter 4, Fig. 4.6 A; Chapter 5, Fig. 5.5 B). The identification of these kinds of processes is in line with our experimental setup (osteoblast differentiation) and would be unlikely to happen if a biological incoherent set of genes had been selected. Moreover, this suggests that other expressed genes with yet unknown function have skeletal developmental-like functions. GO enrichment analyses are also of great use to confirm the biological validity of the selected genes. Technical validity of the microarrays was assessed by qPCR, which showed that the data obtained by our microarrays were reliable (Chapter 4, Fig. 4.3; and Chapter 5, Fig. 5.6 B).

Based on the findings described above, the set of genes found in our human SV-HFO osteoblast model is of great interest as the data is robust and contains many potential osteoblast regulatory genes. A great challenge will now be to study these genes in interaction with each other. A drawback of the gene profile studies is that despite the strict phenotype-based selection criteria we identified still too much genes. In

total, several hundreds of genes were identified, which cannot all be studied in detail. Publication of the identified genes, gene-networks and biological processes identified by our microarray approach in public databases will open up the functional scrutiny of the wide range of novel identified osteoblast genes by contributions of other researchers in the field.

To limit the number of identified genes, data obtained from distinct microarray experiments could be integrated. Using such an approach, genes are only selected if their expression is regulated by more than one osteoblast regulatory factor. In our case, we can merge the genes regulated by GC induced differentiation (Chapter 4) with the genes regulated by the activin-follistatin system (Chapter 5). This will drastically limit the number of genes and we believe that be doing this the most significant and relevant genes in osteoblasts are selected. An important aspect again, is to correlate the direction of gene regulation with the phenotype of the osteoblast cultures. In other words, if a gene is up-regulated by GC-induced differentiation (stimulator of mineralization) this gene should be down-regulated by activin A (inhibitor of mineralization) or the other way around. Optimally, more conditions can be included in such an approach. Preliminary analyses show that we indeed can select genes that are under control of more than two different osteoblast regulatory factors. Genes can be selected that are regulated by 1] GC-induced osteoblast differentiation, 2] the activin A-follistatin system, and 3] $1,25\alpha(OH)_2D_3$ (all potent regulators of mineralization). These genes have great potential and are of primary interest in our further studies.

7.6 Screening for potential therapeutic targets

To confirm whether we indeed successfully identified osteoblast regulatory genes in our microarray experiments the selected genes should be functionally characterized in relation to osteoblast function. Activating or inactivating target genes in osteoblasts is one way to reveal their functional role in bone formation. Genes representing a receptor-like protein are easy to target since most often various agonists or antagonists are available. Therefore, receptor-like genes are currently our primary targets to test in relation to bone formation. This approach already resulted in novel findings demonstrated by the proof of function of the cannabinoid receptor (CNR1) (Chapter 4, Fig. 4.5).

Nevertheless, the number of identified receptors is limited and other protein families are of equal interest. In order, to functionally screen a broad range of target genes an RNA interference (RNAi) approach should be used. This technique makes it possible to silence the mRNA expression of any gene in a cell [39-41] . In the past years various attempts have been made within our research group to develop RNAi-based gene silencing in human osteoblast cultures. Using plasmid based transient RNAi several target genes were successfully silenced (data not shown in this thesis), however, only for a limited period of time (several days). To mediate long-term gene silencing a lentiviral-based RNAi is currently under development [42], which will make it possible to characterize gene function during the entire osteoblast differentiation period.

To translate *in vitro* findings to physiological significance several approaches can be used. Population based association studies (*e.g.*, the Rotterdam study) [43] can be performed to reveal if gene-variants of the identified genes are associated with certain bone characteristics such as bone mineral density and fractures. To study genes in more detail, mice models can be developed that are deficient for certain genes. Subsequently, detailed micro CT bone analysis can be performed to analyze the phenotype of the bone [44]. Alternatively, the zebrafish model could act as an useful tool to screen gene function by using antisense RNA molecules (morpholinos). Zebrafish develop a mineralized skeleton in a 6 days period, which makes it possible to screen target genes in a high throughput manner *in vivo* [45].

Together, these screening methods should identify novel genes that regulate bone metabolism and act as novel targets for therapeutic intervention. In Figure 7.1 the process from *in vitro* bone formation to therapeutic drug envisaged by the Calcium and Bone research group of the Department of Internal Medicine, Erasmus MC is schematically depicted.

7.7 Extracellular matrix composition

In our studies we used human osteoblasts that produce a mineralized ECM. Interestingly, in Chapter 5 (Fig. 5.4 C) we showed that after 10 days of ECM synthesis the ECM seemed completely finished and ready for mineral deposition in the presence of Ca^{2+}, PO_4^{3-}, and a proper pH (pH 7.5). Importantly, mineralization could take place independent of the further presence of living osteoblasts. This phenomenon actually opened up the way to study the impact of ECM composition in more detail. We showed that treatment of osteoblasts with activin A only during ECM synthesis (treatment up to 10 day of culture) drastically decreased the mineralization capacity of the ECM in the subsequent period (the culture period from day 10) (Chapter 5, Fig 5.4 C). In contrast, GC treatment during this same period strongly enhanced the mineralization capacity of the ECM (Chapter 2, Fig. 2.2). Unfortunately, the effect of Wnt signaling on matrix composition was not studied in this way yet. Although activin A and GC treatment strongly influenced the mineralization capacity of the ECM, no major changes were found in the expression of the most abundant ECM proteins (*e.g.*, collagen I, osteocalcin and osteopontin). This proves that other ECM proteins expressed by osteoblasts control the mineralization capacity of the ECM as well.

In the microarray studies many ECM genes were found as differentially expressed, which emphasizes the importance of ECM composition for matrix mineralization. In total, 36 ECM genes were regulated during GC-induced differentiation (Chapter 4, Fig. 4.9 A), and 24 ECM genes by the activin-follistatin system (Chapter 5, Fig 5.6 A). Yet we do not know the optimal composition of the ECM and the functions of the majority of ECM proteins in bone are still unknown. Nevertheless, the observed direction of regulation of the ECM genes identified by our microarray studies coupled to the mineralization phenotype may hint at the function of these ECM genes. In other words, ECM genes that are up-regulated by activin signaling (low mineralization) may

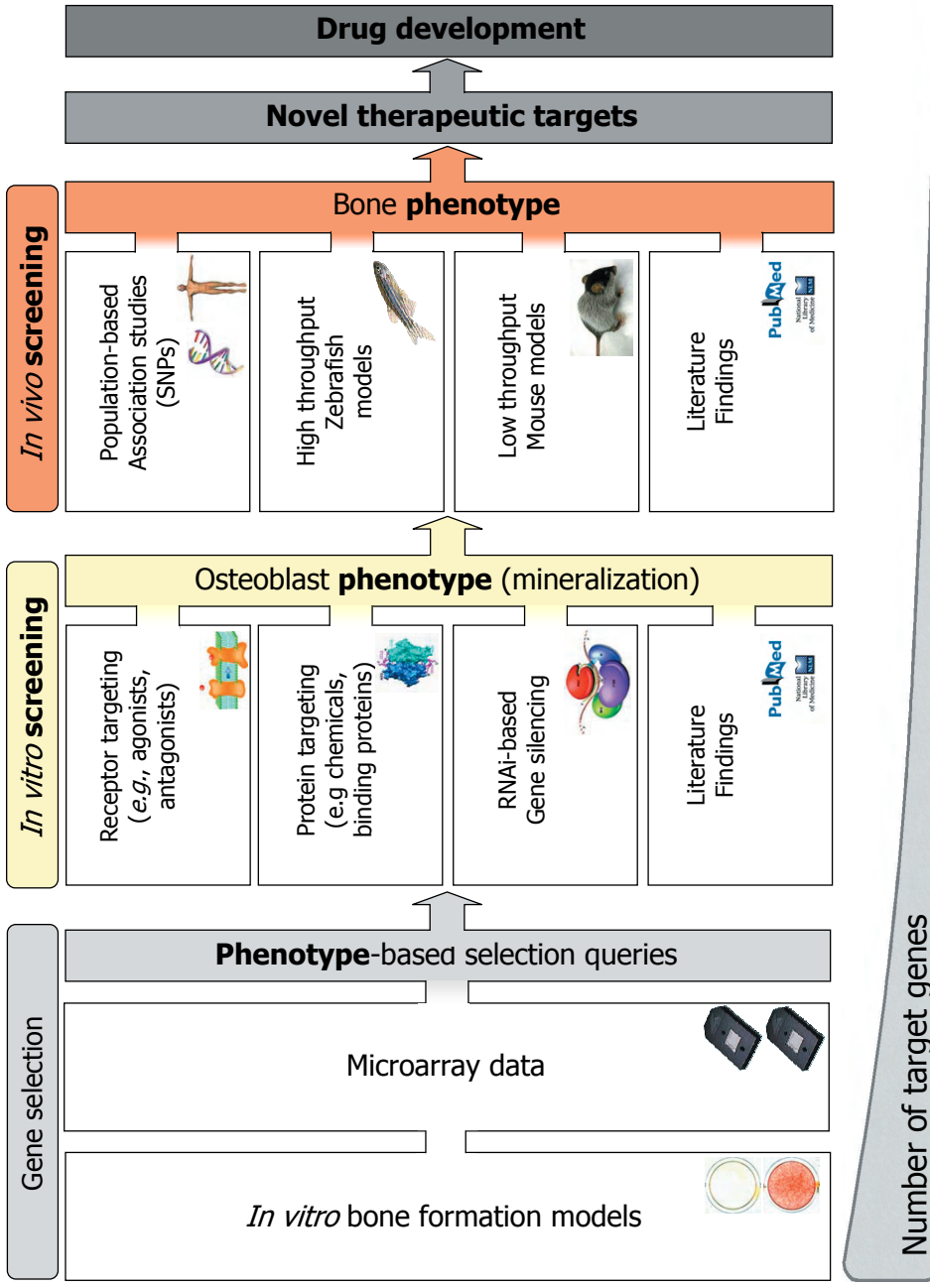

Figure 7.1: Schematic picture of generating novel therapeutic targets that stimulate bone formation. First, gene profiles are generated by microarray experiments in human *in vitro* bone formation models. Genes are selected that correlate with the phenotype of the cultures (mineralization of the ECM). Secondly, the identified genes are functionally characterized upon activation or inactivation. Genes that show an anabolic effect *in vitro* will be further characterized in several *in vivo* models. Finally, a small subset of genes will be leftover that control bone metabolism and act as novel therapeutic targets

act negatively on mineralization, whereas ECM genes that are down-regulated may act positively. In case of GC-induced differentiation, genes up-regulated by this process may act positively on mineralization, whereas the down-regulated ones may act negatively on mineralization.

If we focus on the genes that are regulated by GC-induced differentiation and by the activin-follistatin system, we find seven ECM genes that are controlled by both processes (Table 7.1). Interestingly, six out of these seven ECM genes were regulated in opposing ways by the two conditions, which make them of great interest. COL5A3, CLEC3B, NID2

Table 7.1: ECM genes regulated by GC-induced differentiation and by the activin-follistatin system is human osteoblasts.

		Regulated by	
Gene Symbol	**Gene name**	**GC-induced differentiation**	**Activin signaling**
COL5A3	Collagen, type V, alpha 3	↑	↓
CLEC3B	C-type lectin domain family 3, member B (tetranectin)	↑	↓
NID23	Nidogen 2 (osteonidogen)	↑	↓
TIMP4	Metallopeptidase inhibitor 4	↑	↓
MMP2	Matrix metallopeptidase 2	↓	↑
SPOCK	Sparc/osteonectin (testican)	↓	↑
THBS1	Thrombospondin 1	↑	↑

and TIMP4 were up-regulated by GC-induced differentiation and down-regulated by activin signaling, which makes them potential stimulators of matrix mineralization. MMP2 and SPOCK are down-regulated by GC-induced osteoblast differentiation and up-regulated by activin signaling, which make them potential inhibitors of matrix mineralization.

Nevertheless, we should take into account that a function of a gene is not always correlated with the phenotype of the osteoblasts. For example, $1,25\alpha(OH)_2D_3$ enhances matrix mineralization [1] but at the same moment it induces proteins like osteocalcin and osteopontin, which have been negatively implicated in mineralization [46-51]. Moreover, it should be considered that not only one single ECM protein determines the mineralization capacity of the ECM. Changing the expression of one single ECM gene will probably not result in a major change in mineralization. The total ECM composition might be of greater importance in the control of matrix mineralization and eventually the quality of bone.

7.8 Control of extracellular matrix mineralization

We hypothesize that there are two distinct phases in the process of bone formation where the extent of mineralization can be influenced (Fig. 7.2). Phase 1 covers the period in which the ECM is produced and matured. At this phase regulatory factors (*e.g.*, hormones and growth factors) can directly or indirectly change the composition of the ECM. Altering ECM composition in phase 1 will eventually influence the extent of mineralization later on. After the ECM is completely synthesized and matured,

hydroxyapatite mineral is deposited to the ECM (= Phase 2). At this phase the mineralization can be controlled by factors that directly control mineral formation and deposition, *e.g.*, pH [52], (pyro)phosphate, calcium ions and alkaline phosphatase [53-56]. At the same moment osteoblast activity can still control the rate of mineralization. Osteoblasts secrete proteins like osteocalcin and osteopontin that are suggested to directly inhibit crystal formation [46-48, 50, 51]. Expression of other ECM proteins, ion channels or enzymes at this phase may also directly influence the mineralization rate.

Other compounds such as inorganic phosphate (PO_4^{3-}) act in both phase 1 and phase 2. Inorganic phosphate is a necessary compound of the mineral in bone, but several studies also demonstrate its effect on osteoblast differentiation, activity and apoptosis [54, 57].

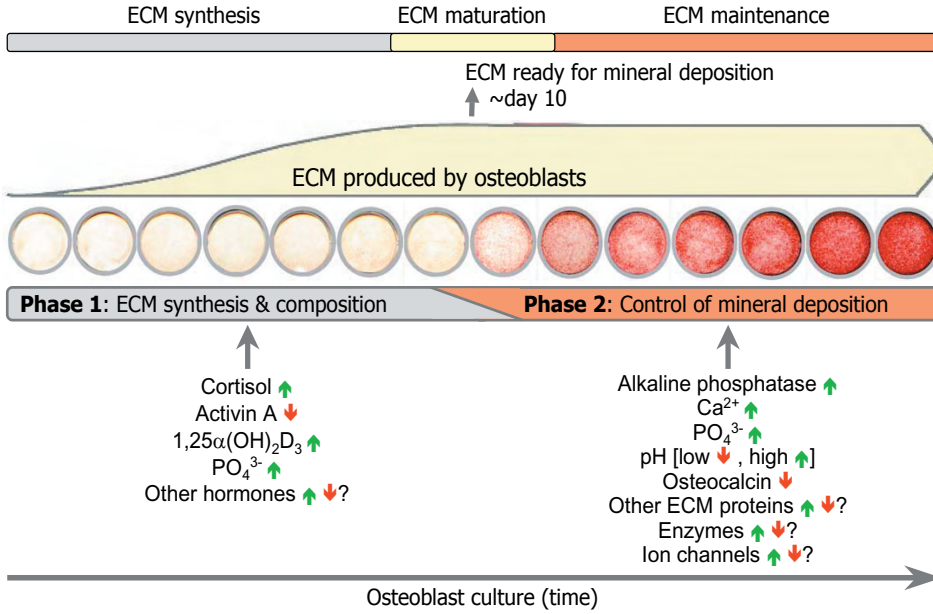

Figure 7.2: The control of extracellular matrix (ECM) mineralization. During the formation, maturation and mineralization of the ECM two phases exist where the extent of mineralization can be influenced. Within 10-days of osteoblast culture an ECM is produced that is ready for mineral deposition (Phase 1). In this phase several factors can influence the mineralization capacity of the ECM by influencing ECM composition. During mineral deposition (Phase 2), factors that directly regulate mineral deposition can regulate the extent of ECM mineralization. In Figure 7.2 mineralization of osteoblast cultures is indicated by the red color (Alizarin red staining) of the culture dishes.

7.9 Activin signaling as a novel therapeutic target to modulate mineralization of the ECM

In Chapter 5 we demonstrated that osteoblasts auto-inhibit mineralization of the ECM by endogenous expression of activin A. This auto-inhibition opens the way to use activin signaling in a therapeutic manner. Recently, Pearsall and colleagues demonstrated that treatment with an activin antagonist stimulated bone formation in mice [58], which

Table 7.2. Functional consequence on matrix mineralization after manipulating components of the activin-follistatin system. * Modulation of activin signaling can be achieved by modulation of type I or type II activin receptors or Smad signaling molecules.

	Activin signaling		Activin A		Follistatin		Activin target genes	
	Stimulate	Inhibit	Stimulate	Inhibit	Stimulate	Inhibit	Stimulate	Inhibit
Mineralization	↓	↑	↓	↑	↑	↓	↓↑	↓↑
Application								
Bone Formation		✓		✓	✓		✓	✓
Pathological calcification	✓		✓			✓	✓	✓

substantiates the therapeutic potential of activin signaling. The use of activin signaling to control mineralization is of particular interest since it can control mineralization in two directions. Inhibition of endogenous activin signaling will enhance mineralization, whereas stimulation of activin signaling will suppress mineralization. The latter gives activin signaling also potential in the control of pathological calcifications. *In vitro*, we already showed that activin inhibits the mineralization process in a vascular smooth muscle cell (VSMC)-based model for arteriosclerosis (Chapter 5, Fig 5.1 D).

To modulate activin signaling *in vivo* there are multiple potential targets for therapeutic intervention. At the cell surface level, activin receptors can be activated by activins or derivatives/analogues. Local inhibition or neutralization of follistatin will enhance free levels of activin, which would be an elegant method to enhance activin signaling locally. To inhibit activin signaling similar methods could be used, addition of follistatin or other activin binding proteins can be used to neutralize activin signaling. Activin receptors or other signaling molecules such as Smad2 and Smad3 can directly be targeted to inhibit activin activity. In addition, modulation of specific activin target genes, like the identified ECM genes, is a good alternative for therapeutic intervention. For the latter application the function of the target genes should be characterize first. Both up-regulated and down-regulated activin target genes are of interest since they potentially inhibit or stimulate the mineralization, respectively. The functional consequences of stimulating or inhibiting the actions of activins and activin signaling as described above are summarized in Table 7.2.

Since activin and TGFβ signaling show a large overlap in their signaling it might be difficult to modulate activin signaling without influencing TGFβ signaling. The challenge will be to characterize all the players in the activin and TGFβ signaling pathways and to assess the overlap, but importantly, also the differences between these signaling pathways. Another challenge will be to specifically target mineralization in bone or pathological calcifications.

As an alternative for *in vivo* applications, activin signaling can be employed in tissue engineering. Bone explants can be produced *ex vivo* in which follistatin or derivatives are added to enhance the bone formation process. In contrast, for other applications extracellular matrices can be produced *ex vivo* that do not mineralize *in vivo* upon activin treatment.

7.10 Wnt signaling

The described loss-of-function and gain-of-function mutations in the Wnt co-receptor LRP5 leading to a very high or very low bone mass in humans indicate an important role of Wnt signaling in bone metabolism [59-61]. After these findings several *in vivo* and *in vitro* studies were published that indeed showed a stimulatory role of Wnt signaling on bone formation caused by enhanced osteoblastic activity [62-64]. These findings, however, might be publication biased since the mutation in LRP5 strongly suggested a positive role of Wnt signaling in osteoblasts. Remarkably, these publications were followed by studies showing a contradicting effect in terms of inhibition of osteoblast differentiation and mineralization by Wnt signaling [65-67]. The conflicting data on whether Wnt signaling acts stimulatory or inhibitory on osteoblast differentiation is likely to reflect diverse function of Wnt signaling at different stages of osteoblast development.

In Chapter 6 we showed that within one cell model the Wnt signaling pathway stimulates early differentiation but strongly inhibits matrix mineralization later on during differentiation. This knowledge is crucial for the interpretation and comprehensibility of the complex Wnt signaling pathway in relation to bone formation. The therapeutic use of such a pathway to control bone formation will be difficult since it is almost impossible to target a specific stage of osteoblast differentiation *in vivo*. The complexity of the Wnt signaling [68], however, could open the way to target Wnt signaling in a differentiation dependent manner. It is likely that multiple ligands and receptors control Wnt signaling in the osteoblast. We showed that several Wnt signaling components are differentially expressed during osteoblast differentiation and are therefore interesting targets to control in a differentiation dependent manner.

7.11 Final remarks & Conclusion

In conclusion, many aspects of human osteoblast differentiation were studied in this thesis. The studies regarding GCs, Wnt signaling and the activin A-follistatin system resulted in a significant increase in knowledge about the role of these signaling pathways in the control of osteoblast differentiation and bone formation. We demonstrated that the actions of these signaling pathways are dependent on the stage of osteoblast differentiation, demonstrating that proper timing is essential to control osteoblast differentiation and function *in vitro* and *in vivo*. Regarding the role of GCs in bone, we showed that in normal osteoblast physiology GCs are of great importance and do not mediate negative effects directly on osteoblasts. The negative side effects of GCs on bone formation are probably mediated by other processes that indirectly affect osteoblasts and bone formation.

The novel findings concerning the potent effects of the activin A-follistatin system on matrix mineralization are of particular interest since we identified a novel therapeutic target to control bone matrix formation and subsequent mineralization. Follow-up research is needed to precisely pinpoint the mechanism of action of action and to clarify the full therapeutic potential of regulating activin in skeletal diseases or diseases of

pathological calcification. The use of activin signaling to control matrix formation and mineralization in an *ex vivo* manner (*e.g.*, tissue engineering) seems already applicable and this will be tested in the near future.

We believe that the microarray data, generated by unique phenotype-based selection queries, has great potential to discover other novel therapeutic targets that control bone formation. The generated gene expression profiles will lead researchers within the bone field to better understand the complex processes of osteoblast differentiation and bone formation. The complete microarray dataset, however, is not fully exploited yet and might be strengthened by the addition and integration of other data sets and further data mining. A good start was made by the experiments described in this thesis. It is anticipated that these genes encompass important novel regulators of osteoblast differentiation and bone formation. The functional characterization of the identified and phenotype-based selected genes is a major challenge and unfortunately beyond the time frame of this thesis. Currently, these functional analyses are in progress among others by lentiviral siRNA studies and zebrafish-based screens.

7.12 References

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Chapter 8

Samenvatting
Summary
Abbreviations
Dankwoord
Curriculum Vitae
List of Publications

8.1 Samenvatting

Osteoporose is de meest voorkomende botziekte die wordt gekenmerkt door lage botmassa en een verhoogd risico op fracturen. In het lichaam zijn osteoblasten verantwoordelijk voor de productie van nieuw bot en hebben daarom een belangrijke taak in het reguleren van de botmassa. Osteoblasten zijn afkomstig van mesenchymale stamcellen die zich onder andere in het beenmerg bevinden. Het ontwikkelingsproces van mesenchymale stamcel naar volwassen osteoblast is zeer complex en wordt gecontroleerd door verscheidene endocriene en autocriene factoren (beschreven in **Hoofdstuk 1**). Het doel van het in dit proefschrift beschreven onderzoek was om meer kennis te verkrijgen van het ontwikkelingsproces van humane osteoblasten, zodat deze als basis gebruikt kan worden om nieuwe therapieën te ontwikkelen die de botvorming kunnen stimuleren. Binnen dit onderzoek is gebruik gemaakt van humane osteoblast modellen die het botvormingsproces *in vitro* nabootsen, waarbij er voornamelijk geconcentreerd is op het bestuderen van glucocorticoïden, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), Wnt signaaltransductie en activine signaaltransductie in relatie tot osteoblast ontwikkeling en botvorming.

Ondanks het feit dat glucocorticoïden de botvorming *in vitro* stimuleren, staan glucocorticoïden vooral bekend als factoren die de botvorming negatief beïnvloeden. Deze aanname is voornamelijk gebaseerd op de negatieve bijeffecten tijdens glucocorticoïden gebruik, zoals verlies van botmassa en verhoogd risico op fracturen. In **Hoofdstuk 2** wordt juist de essentiële rol van glucocorticoïd hormonen zoals cortisol voor de correcte differentiatie van humane osteoblasten benadrukt. Er werd aangetoond dat glucocorticoïden tijdens het vroege levensstadium van osteoblasten verdere differentiatie en mineralisatie van de extra-cellulaire matrix (ECM) initiëren. Tevens is aangetoond dat glucocorticoïden de expressie van meerdere genen die positief betrokken zijn tijdens osteoblast differentiatie verhogen. Van andere genen zoals osteopontine en osteocalcine, die negatief betrokken zijn bij de mineralisatie, werd aangetoond dat de expressie juist onderdrukt wordt door glucocorticoïden. Tenslotte toonden we aan dat glucocorticoïden de proliferatie van osteoblasten verhogen terwijl de mate van celdood gelijk bleef. Concluderend laat hoofdstuk 2 zien dat de negatieve bijeffecten van glucocorticoïden niet verklaard worden door directe effecten op osteoblasten maar door andere mechanismen die waarschijnlijk indirect de botvorming onderdrukken.

Op cellulair en weefsel niveau wordt de activiteit van glucocorticoïden verhoogd door de aanwezigheid van het enzym 11β-HSD1. Dit enzym zet het inactieve glucocorticoid cortison om in het biologisch actieve cortisol. In **Hoofdstuk 3** is de regulatie van 11β-HSD1 expressie tijdens het differentiatieproces van humane osteoblasten bestudeerd. Er is aangetoond dat de activiteit van 11β-HSD1 omgekeerd gerelateerd is aan de differentiatie stadia van humane osteoblasten. Met andere woorden, wanneer osteoblasten in de aanwezigheid van glucocorticoiden differentiëren naar volwassen osteoblasten, wordt er weinig cortisol geproduceerd. Daarentegen, in osteoblasten die niet door glucocorticoïden gestimuleerd worden en dus niet verder differentiëren, is de cortisol productie hoog. Om het moleculaire mechanisme van deze differentiatie

afhankelijke expressie te achterhalen is de promotor van het 11β-HSD1 gen bestudeerd. Dit resulteerde in de identificatie van specifieke regio's binnen de 11β-HSD1 promotor die betrokken zijn bij de differentiatie afhankelijke expressie van het 11β-HSD1 gen. Tot slot is de functionaliteit van 11β-HSD1 in osteoblasten aangetoond door osteoblasten te laten differentiëren in de aanwezigheid van het nog inactieve cortison. Dit liet zien dat osteoblasten hun eigen differentiatie kunnen initiëren door cortison om te zetten in het actieve glucocorticoïd cortisol.

Ofschoon glucocorticoïden essentieel zijn voor de correcte ontwikkeling van osteoblasten, is de moleculaire kennis omtrent dit proces beperkt. In **Hoofdstuk 4** is het proces van glucocorticoïden- geïnduceerde osteoblast differentiatie op een moleculair niveau bestudeerd door gebruik te maken van genexpressie microarrays die het hele genoom omvatten. De genexpressie profielen van mineraliserende (behandeld met glucocorticoïden) en niet mineraliserende osteoblasten (niet behandeld met glucocorticoïden) zijn met elkaar vergeleken om regulatoire genen te identificeren die betrokken zijn bij het differentiatie en mineralisatie proces van osteoblasten. Dit leverde een variëteit aan genen, processen en gen-netwerken op die differentieel tot expressie kwamen tussen mineraliserende en niet mineraliserende osteoblasten. Verdere bewijsvoering voor dit concept werd geleverd door de identificatie van de cannabinoid receptor 1. Deze receptor kwam tijdens de mineralisatie specifiek tot expressie en activatie van deze receptor verhoogde de mineralisatie van de ECM. Tevens werden in Hoofdstuk 4 de processen van osteoblast- en vasculaire gladde spiercel-geïnduceerde mineralisatie met elkaar vergeleken. Dit toonde aan dat glucocorticoïden voor beide celtypen essentieel zijn om de mineralisatie van de ECM te induceren. Het vergelijken van genexpressie tussen de beide celtypen gaf aan dat een grote groep genen op dezelfde manier gereguleerd werden in zowel osteoblasten als vasculaire gladde spiercellen. Deze resultaten wezen dan ook op een grote overlap tussen het mineralisatie proces in bot en mineralisatie van de vaatwand tijdens het proces van arteriosclerose.

Verscheidene leden van de transforming growth factor-β (TGFβ) superfamilie staan bekend om hun effect op het botmetabolisme. Activines behoren ook tot de TGFβ superfamilie maar over hun functie in bot is nog weinig bekend. **Hoofdstuk 5** beschrijft ons werk omtrent de functie van activine A in osteoblasten. Er werd aangetoond dat activine A de mineralisatie van de ECM in osteoblasten zeer sterk remt. Daarentegen, als de endogene activine A activiteit juist onderdrukt werd door gebruik te maken van het activine A bindend eiwit follistatine, werd de mineralisatie juist verhoogd. Deze bevindingen laten zien dat door gebruik te maken van activine of follistatine, de mineralisatie in tegenovergestelde richting gestuurd kan worden. Verder wordt in hoofdstuk 5 aangetoond dat activine A in de periode van ECM synthese de samenstelling van de ECM zo verandert dat deze niet of nauwelijks meer kan mineraliseren. Dit werd bevestigd door genexpressie microarrays uitgevoerd op activine A en follistatine behandelde osteoblast-kweken. Deze studies identificeerden een bepaalde groep ECM genen die onder controle staan van de activine signaaltransductie. Als laatste lieten we zien dat activine A ook vasculaire gladde spiercellen-geïnduceerde mineralisatie van de ECM kan remmen. Deze studies geven aan dat naast osteoporose, de bevindingen omtrent activine ook belangrijk kunnen zijn voor aandoeningen zoals arteriosclerose.

Wnt signaaltransductie heeft een belangrijke functie in het controleren van celdifferentiatie van verscheidene celtypen waaronder osteoblasten. Tegenstrijdige data zijn gepubliceerd die zowel positieve als negatieve effecten van Wnt signaaltransductie op osteoblasten laten zien. In **Hoofdstuk 6** wordt aangetoond dat het effect van Wnt signaaltransductie afhankelijk is van het differentiatiestadium van osteoblasten. Wnt signaaltransductie stimuleerde vroege osteoblasten in hun capaciteit om zich verder te differentiëren, terwijl volwassen osteoblasten sterk geremd werden in hun vermogen om te mineraliseren. Tevens is in hoofdstuk 5 de endogene activiteit van de Wnt signaaltransductie bestudeerd. Dit werk gaf aan dat tijdens osteoblast differentiatie de activiteit van Wnt signaaltransductie wordt onderdrukt en de expressie van verscheidene Wnt signaaltransductie-gerelateerde genen sterk wordt gereguleerd.

Samenvattend zijn verscheidene aspecten van humane osteoblast differentiatie bestudeerd in dit proefschrift. De experimenten betreffende glucocorticoïden, Wnt signaaltransductie en het activine A-follistatine systeem heeft aanzienlijk meer kennis opgeleverd omtrent de functie van deze factoren in het osteoblast differentiatie proces (bediscussieerd in **Hoofdstuk 7**). De nieuwe bevindingen aangaande de sterke effecten van het activine A-follistatine systeem op de mineralisatie van de ECM heeft momenteel onze primaire interesse aangezien we een nieuw therapeutisch doelwit geïdentificeerd hebben met betrekking tot botvorming en arteriosclerose. Tevens vermoeden we dat binnen de microarray data, beschreven in hoofdstuk 4 en 5, nog vele nieuwe therapeutische doeleiwitten te vinden zijn die de botvorming kunnen controleren. Het functioneel testen van de geïdentificeerde genen en het bestuderen van deze genen in interactie met elkaar is een enorme uitdaging die in de nabije toekomst uitvoerig zal worden onderzocht.

8.2 Summary

Osteoporosis is the most common bone disease and is characterized by low bone mass, micro architectural deterioration and decreased bone quality resulting in increased risk of fractures. Osteoblasts, the bone forming cells, play a crucial role in the regulation of bone mass and bone quality. Osteoblasts are of mesenchymal origin and undergo a complex differentiation process regulated by many endocrine and autocrine factors (**Chapter 1**). In order to develop novel bone anabolic drugs, more knowledge concerning osteoblast biology is required. In this thesis we investigated the processes of human osteoblast differentiation and matrix mineralization. Human osteoblast-based models of bone formation were used in which the role of glucocorticoids (GCs), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), the Wnt signaling pathway and the activin A-follistatin system were studied.

GCs such as cortisol are crucial initiators of human osteoblast differentiation. Despite the clear stimulation of bone formation by GCs *in vitro*, GCs are still regarded as negative regulators of bone formation. This is mainly based on the negative side effects initiated by the clinical use of GCs, like bone loss and increased risk of fractures. **Chapter 2** emphasizes the crucial role of physiological levels of GCs for proper differentiation of human osteoblasts. We demonstrated that human osteoblast differentiation needs to be triggered by GCs in a specific time-window during the

early stages of development. GCs increased the expression of several genes positively involved in osteoblast differentiation. Other genes such as osteopontin and osteocalcin, which are negatively involved in mineralization, were suppressed by GCs. Moreover, GC treatment increased osteoblast proliferation, whereas apoptosis was unchanged. In conclusion, the data in Chapter 2 indicates that the negative side effects of GCs are not caused by direct effects on osteoblast but are probably caused by several other indirect mechanisms that affect bone formation.

At the tissue and cellular level GC activity is enhanced by local expression of 11β-HSD1. This enzyme is expressed in several GC-target cells including osteoblasts and converts the inactive GC cortisone into the biologically active cortisol. In **Chapter 3** we investigated the control and functional consequences of osteoblastic 11β-HSD1 activity. We demonstrated that the activity of 11β-HSD1 was inversely related to the differentiation stage of human osteoblasts. In other words, when pre-osteoblasts were differentiated towards mature osteoblasts, 11β-HSD1 activity was suppressed, whereas in the absence of osteoblastic differentiation (no GC treatment) 11β-HSD1 expression and cortisol production strongly increased. In order to reveal the molecular mechanism behind this differentiation dependent regulation of 11β-HSD1, the promoter of the 11β-HSD1 gene was analyzed. This provided evidence that specific regions of the 11β-HSD1 promoter were involved in the differentiation-controlled regulation of this enzyme. The functional consequence of osteoblastic 11β-HSD1 expression was demonstrated by the induction of differentiation in the presence of the inactive GC cortisone, which shows that osteoblasts auto control their differentiation by converting cortisone into cortisol.

Although GCs are crucial for osteoblast differentiation, little is known about the genes and molecular networks underlying GC-induced osteoblast differentiation and mineralization. In **Chapter 4** the process of GC-induced osteoblast differentiation was studied at the molecular level using genome-wide gene expression microarrays. GCs were used to create nonmineralizing (non GC treated) and mineralizing (GC treated) osteoblast cultures. Subsequently, the gene expression profiles of these nonmineralizing and mineralizing cultures were compared to identify novel osteoblast regulatory genes. A wide variety of genes, processes and gene networks were identified that were differentially expressed between nonmineralizing and mineralizing cultures. A proof of principle was given by the identification of the cannabinoid receptor 1. This receptor was specifically up-regulated during stages of mineralization and its activation resulted in increased matrix mineralization in human osteoblast cultures. Moreover, in Chapter 4 the processes of osteoblast- and vascular smooth muscle cell (VSMC)-induced matrix mineralization were compared. These data indicate that in both processes GC treatment was essential to induce matrix mineralization. Comparative gene expression analysis demonstrated that there was a large subset of genes similarly regulated during osteoblast- and VSMC-induced matrix mineralization, indicating strong similarities between bone and vascular mineralization.

Several members of the transforming growth factor-β (TGFβ) superfamily, including TGFβ and bone morphogenic proteins (BMPs), are well-known regulators of bone formation. Activins also belong to the TGFβ superfamily, but their precise role in bone formation is unknown. **Chapter 5** describes the studies regarding the role and impact of

activin signaling in human osteoblasts. We demonstrated that activin A strongly inhibits matrix mineralization in osteoblast cultures. In contrast, when endogenous activin A was inhibited, using the activin A binding protein follistatin, matrix mineralization was increased. These findings implicate that it is possible to control matrix mineralization in two directions using either follistatin or activin A. Moreover, we demonstrated in Chapter 5 that activin signaling changes the composition of the extracellular matrix in such way that it leads to an extracellular matrix with no or reduced mineralizing capacity. This reasoning was substantiated by gene expression microarray studies of follistatin and activin A treated osteoblasts, which identified a group of extracellular matrix genes that was under control of activin signaling. Finally, we showed that activin A inhibited VSMC-induced matrix mineralization, which indicates that, besides osteoporosis, these findings can also be of great importance for understanding the process of atherosclerosis.

The Wnt signaling pathway controls cellular differentiation in a variety of cell types including osteoblasts. Conflicting data have been published demonstrating both positive and negative effects of Wnt signaling on osteoblasts. We show in **Chapter 6** that the effects of Wnt signaling are dependent on the stage of osteoblast differentiation. Wnt signaling stimulated early osteoblasts in their capacity to differentiate, whereas mature osteoblasts were strongly inhibited in their capacity to induce matrix mineralization. Moreover, we showed that endogenous Wnt signaling was under control of the GC-induced osteoblast differentiation process. Differentiating osteoblasts had suppressed Wnt signal and changed expression of various Wnt signaling related genes.

In conclusion, many aspects of human osteoblast differentiation were studied in this thesis. The studies regarding GCs, Wnt signaling and the activin A-follistatin system resulted in a significant increase in fundamental knowledge about the role and control of these signaling pathways in osteoblasts (discussed in **Chapter 7**). The novel findings concerning the potent effects of the activin A-follistatin system on matrix mineralization are of particular interest since we identified a novel therapeutic target in the control of both bone formation and vascular mineralization. In addition, we believe that the microarray data generated by unique phenotype-based selection queries described in Chapter 4 and 5 have great potential to discover other novel therapeutic targets that control bone formation. Studying these genes in interaction with each other and to perform a functional characterization is a major challenge for the near future.

8.4 Abbreviations

11β-HSD	11β-hydroxysteroid dehydrogenase
$1\alpha,25\text{-}(OH)_2D_3$	1α,25-dihydroxyvitamin D_3
ACEA	arachidonyl-2'-chloroethylamide
ACVR1B	activin A receptor, type IB
ACVR2A	activin A receptor, type IIA
ACVR2B	activin A receptor, type IIB
AHM	anti-Mullerian hormone
ALP	alkaline phosphatase
ALPL	alkaline phosphatase
αMEM	minimum essential medium, alpha
aP2	adipocyte lipid-binding protein / fatty acid binding protein 4
APC	adenomatosis polyposis coli
BMP	bone morphogenic protein
BRE	bone morphogenic protein responsive element
C/EBP	CAAT/enhancer binding protein
$Ca_{10}[PO_4]_6[OH]_2$	hydroxyapatite
CBG	cortisol binding globulin
c-Fms	macrophage colony stimulating factor receptor
CNR1	cannabinoid receptor 1
CT-FCS	charcoal-treated fetal calf serum
D	day
DAB	3'3-diaminobenzidine hydroxy chloride
DEX	dexamethasone
DKK	dickkopf
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
EC_{50}	half maximal effective concentration
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immuno sorbent assay
FCS	fetal calf serum
FSH	follicle stimulating hormone
g	gravity
GA	18β-glycyrrhetinic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GDF	growth and differentiation factor
GO	gene ontology
GR	glucocorticoid receptor
GSK3	glycogen synthase kinase 3
h	hour
HSD11B1	11β-hydroxysteroid dehydrogenase 1
IGF-I	insulin-growth factor I
INHA	inhibin, alpha
INHBA	inhibin, beta A
INHBB	inhibin, beta B
LDL	low density lipoprotein
LiCl	lithium chloride
LRP5	low density lipoprotein receptor-related protein 5

M	molar
MCSF	macrophage colony-stimulating factor
MgCl	magnesium chloride
micro CT	microfocus computed tomograhy
Min	minute
MR	mineralocorticoid receptor
mRNA	mesenger RNA
MSC	mesenchymal stem cells
MSX2	msh homeo box homolog 2
MV	matrix vesicles
NaCl	sodium chloride
NaOH	sodium hydroxide
NF-kB	nuclear factor kappa B
NHOst	normal human osteoblasts
OCN	bone gamma-carboxyglutamate (gla) protein / osteocalcin
OPG	osteoprotegerin
OPN	secreted phosphoprotein 1 / osteopontin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
qPCR	quantitative polymerase chain reaction
RANK	tumor necrosis factor receptor superfamily, member 11a / receptor activator of NF-κB
RANKL	tumor necrosis factor (ligand) superfamily, member 11 / receptor activator of NF-κB ligand
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
RU	RU 38486 / mifepristone
RUNX2	runt-related transcription factor 2
SEM	standard error of the mean
SFRP	secreted frizzled-related protein
siRNA	short interference RNA
SP7	sp7 transcription factor / osterix
SV-HFO	simian virus 40 immortalized - human fetal osteoblasts
TGFβ	transforming growth factor β
TWIST1	twist homolog 1
VDRE	vitamin D responsive element
vitD	1α,25-dihydroxyvitamin D_3
VSMC	vascular smooth muscle cell
WIF	WNT inhibitory factor 1
ZBTB16	zinc finger and BTB domain containing 16

Gene abbreviations in Chapter 4 to 7 are according to the HUGO Gene Nomenclature Committee
(Wain *et al.*, Guidelines for human gene nomenclature. Genomics. Apr 2002;79(4):464-470).

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8.6 Curriculum Vitae

Hermanus Johannes Marco Eijken was born on April 9th, 1978 in The Hague, The Netherlands. In 1995 he finished secondary school (HAVO) at the Dalton scholengemeenschap in The Hague. From 1995 to 1999 he attended technician school (Hogere Laboratorium Opleiding) at the Hogeschool Rotterdam & Omstreken in Delft. For this he conducted a study in constructing and validating a phage-display vector at the department of Biochemical Modifications of Numico Research B.V. in Wageningen. Next, he studied Biology at Leiden University, for which he performed a research project at the Institute of Molecular Plant Sciences, Center for Phytotechnology RUL/TNO in Leiden. Here he worked on a patch-clamp study of anion channels on the tonoplast membrane of Chara corallina. In 2001 he received his biology degree and started as a PhD student at the department of Internal Medicine at the Erasmus MC in Rotterdam, where he worked on the project 'Human osteoblast differentiation and bone formation' under the supervision of prof. dr. H.A.P. Pols and prof. dr. J.P.T.M. van Leeuwen. Since 2006 he is employed as a post-doc at the department of Internal Medicine. Currently he is involved in the establishment of Ricardus B.V., a spin-off company of the Erasmus MC in part based on the research described in this thesis concerning the activin A-follistatin system and matrix mineralization.

8.7 List of Publications

Eijken M, Meijer IMJ, Westbroek I, Koedam M, Pols HAP, van Leeuwen JPTM. Wnt signalin acts and is regulated in a human osteoblast differentiation dependent manner. Journal of Cellular Biochemistry, *provisionally accepted*.

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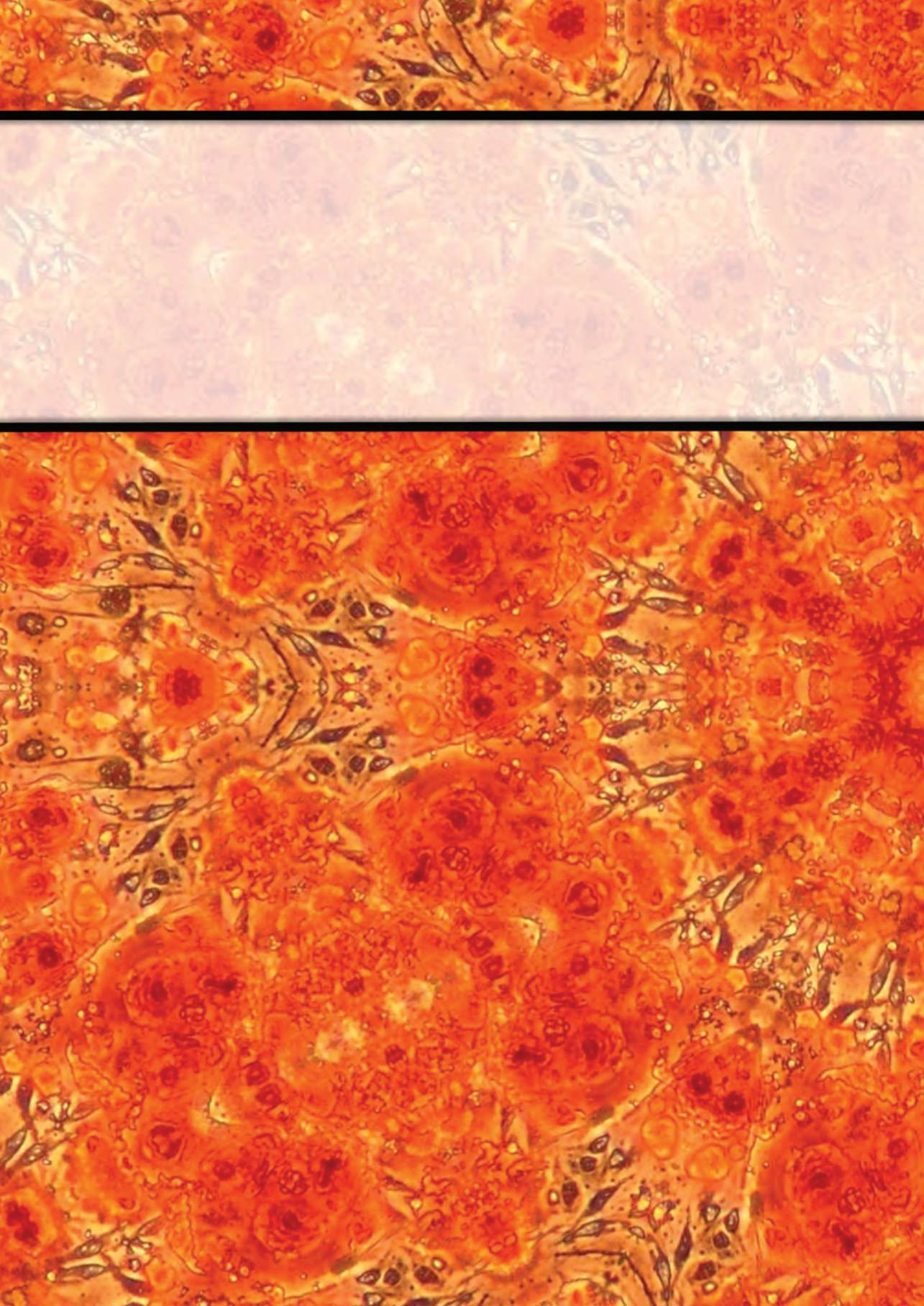

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