Novel insights into vitamin D enhanced mineralization

- interplay with inhibitors -

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- Interplay with inhibitors -

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Novel insights into vitamin D enhanced mineralization
- Interplay with inhibitors -

Nieuwe inzichten in vitamine D gestimuleerde mineralisatie
- Samenspel met remmers –

Neue Einblicke in die Vitamin D verstärkte Mineralisierung
- Zusammenspiel mit Hemmern -

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Gehe nicht, wohin der Weg führen mag, sondern dorthin, wo kein Weg ist, und hinterlasse eine Spur. (Jean Paul 1763-1825)

Preface

Viola und Holland – diese Begriffe gehören mittlerweile fest zusammen. Wenn man jedoch die Vorgeschichte beschaut ist es verwunderlich, dass und wie diese Kombination entstanden ist…

In meiner Kindheit waren wir einige Male in Holland im Urlaub und dann meistens in Ferienparks mit Schwimmbädern, die kaum etwas vom holländischen Leben ausstrahlen. Meine Eltern haben nach diesen Urlauben aber relativ schnell beschlossen das nächste Mal nach Spanien in den Urlaub zu fliegen – auf das Wetter ist dort mehr Verlass!

Die einzige Berührung mit Holland in den Jahren bis zu meiner Doktorarbeit im „Knochenlabor" war dann der holländische Käse, den ich en masse gegessen habe.

Ironischerweise wurde während meines Studiums – in Genetik – ein Kurs „Knochen- und Knorpelbildung" angeboten. Als ich diesen Kurs auf dem Semesterplan sah, dachte ich, „Wer sollte sich für Knochen interessieren?" und habe mich doch lieber auf das Paarungsverhalten von Hefen gestürzt.

Als mein Studium dann langsam ein Ende nahm und ich Ausschau nach einem Platz für die Doktorarbeit suchte, habe ich eigentlich mehr in Richtung Hannover, Hamburg oder Berlin gesucht. Schlussendlich landete ich auf der Homepage vom Europäischen Training Netzwerk NucSys. Es waren noch einige Plätze frei – doch wo? Hierzu gab es keine Angaben. Mir wurde nur mitgeteilt, dass meine Bewerbung nach Bedfort (England) und Rotterdam geschickt wurde. Ich betete für ein Vorstellungsgespräch in Bedfort und bekam Antwort aus Rotterdam.

Die Reise nach Rotterdam erwies sich dann als reinstes Abenteuer. Nach rund 6 Stunden im Zug hatte ich 2 Stunden bis zu meinem Vorstellungsgespräch. Diese 2 Stunden habe ich auch gebraucht, um von Centraal bis zum Labor zu kommen. Ich wurde von der RET in Tram 7 zur Erasmus Universität gesetzt doch nach etwa 40 Minuten in der Tram und 20 Minuten herumlaufen auf dem Campus musste ich feststellen, dass ich am falschen Ende der Stadt stand. Also, dieses Mal mit der Metro, fragen, welche Haltestelle das Erasmus MC ist und schon stand ich in Dijkzigt. Alles war schön und gut. Ich stand vor dem Eingang der Fakultät, doch die Eingangsschilder führten mich 15 Minuten lang um den ganzen Komplex herum.

Am „Eingang" erfuhr ich dann, dass ich wieder zurück zur Fakultät musste… schlussendlich kam ich gerade noch rechtzeitig im Labor an. Hier kam mir ein Mann aus dem Labor entgegen und fragte, wen ich suche, „Hans van Leeuwen" sagte ich. „That's me" antwortete der Mann und das einzige was ich darauf entgegnen konnte war: „Wow – you`re tall!".

Nach 5 Jahren bin ich nun immer noch in Rotterdam und meine Zeit hier ist noch lange nicht abgelaufen. Ich möchte mich bei einigen Personen bedanken, die mich während meiner Doktorarbeit maßgeblich unterstützt haben. Zu allererst möchte ich mich bei meinen Eltern, meiner Oma, meinem Bruder und Irina bedanken, die unter anderen als Umzugshelfer 500 km nach Rotterdam gekommen sind. Dass die Tochter innerhalb von wenigen Wochen ins Ausland zieht ist sicherlich nicht einfach. Dennoch haben wir in der Folgezeit herausgefunden, dass das Telefon ein guter Freund ist und vieles ersetzen kann. (Außerdem ist die Aussicht auf Kapsalon desöfteren eine Reise wert). Des Weiteren möchte ich mich bei Martijn und seiner Familie bedanken, die mich mit offenen Armen empfangen habe, mir hier eine zweite Familie gegeben habe und damit ein Vielfaches zu meinem Wohlbefinden beitragen. Zum Schluss möchte ich mich noch bei den „Botjes" bedanken, insbesondere Bram und Hans für die Begleitung während der Doktorarbeit und Marijke für die Unterstützung in der Schlussphase der Doktorarbeit

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General Introduction

What would life be without bone? Bone (greek "osteo") is a dense connective tissue, which enables locomotion, gives shape to a body, protects vital organs and harbours hematopoietic stem cells, which continuously produce new blood cells. Additionally, bone is used as reservoir for calcium and phosphates and helps to maintain stable blood levels of those ions [1].

Bone consists of two tissues: cortical bone (80%) and trabecular bone (20%). The cortical bone is a compact tissue with a porosity of 5-30%. Within the cavity of cortical bone the bone marrow is located. Those cavities are partially supported by a spongy-like network of trabecular bone. The porosity of trabecular bone is 30-90%, depending on the location [2]. By having a strong sponge-like inner network of trabecular bone the strength of the cortical bone is maximized.

The porosity of bone allows bone to be a vivid organ with blood vessels and a variety of cell types. For bone itself, three kinds of cells are important: osteoclasts, osteoblasts and osteocytes. Additionally, hematopoietic stem cells are hosted in the bone marrow and are the driving forces of blood cell production. A tightly controlled interplay between bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts) continuously remodels bone to ensure strength [3]. A very common bone disease in elderly people is osteoporosis. This disease is based on an imbalance of bone remodeling leading to more bone being degraded than formed. The result is a weak skeleton that is easily damaged [4]. To reduce the risk for osteoporosis, vitamin D supplements are often recommended [5-6]. By regulating calcium and bone homeostasis, vitamin D tips the balance of bone remodeling towards bone formation and therefore antagonizes an osteoporotic phenotype.

This thesis is focused on vitamin D, a major hormone controlling calcium and bone homeostasis. Vitamin D has established effects on bone cells and mineralization but the knowledge is limited. Moreover, an interplay between bone and immune system is known and interactions of vitamin D with cytokines of the immune system are reported. Part of my project was to study the interplay of vitamin D with the cytokine interferon (IFN) β in human osteoblasts. This first chapter will provide background information and finally the rationale of this thesis.

Bone formation

Bone is generated during fetal development by two processes: endochondral and intramembranous ossification (for review [7]). Flat bones and skull are formed by intramembranous ossification. Hereby, mesenchymal stem cells

undergo osteoblastogenesis and connective tissue is calcifed. Long bones and most of the remaining bones are formed by endochondral ossification. This ossification is characterized by extended growth of cartilage, accompanied by increased secretion of extracellular matrix. Finally, by inducing the first ossification center in the middle of the diaphysis the periosteum is formed. This is followed by calcification of the cartilaginous tissue. The growth of long bones is controlled by the second ossification center which is located at the epiphyses of long bones.

Bone Cells

For the maintenance of healthy bone multiple cell types are of importance: mesenchymal stem cells, osteoblasts, osteocytes, hematopoietic stem cells and osteoclasts.

Osteoclasts derive from hematopoietic stem cells and have therefore the same ancestor as the blood cells [8]. They are multi-nucleated cells, which are formed from mono-nucleated osteoclast precursors upon macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) stimulation [9]. Mature osteoclasts can harbor up to 20 closely packed nuclei. They can form a sealing zone upon contact with mineralized matrix, thereby forming an isolated microenvironment: the resorption cavity. Via the ruffled border, an extended part of the osteoclast cell membrane which protrudes into the resorption cavity, hydrogen and chloride ions are released to create an acidic environment, which is necessary to dissolve the mineralized matrix. Subsequent removal of proteins from the matrix is accomplished by secretion of proteases, e.g. cathepsin K. Released protein fragments are then absorbed into small vesicles and transported to extracellular fluids via a process called transcytosis [10-11]. How transport of released calcium and phosphate from the resorption cavity takes place, is not yet clarified.

Osteoblasts originate from mesenchymal stem cells (MSC). MSCs are located in the bone marrow but also in almost all other tissues undergoing continuous tissue homeostasis. MSCs have the capability to self-renew and differentiate into a wide variety of cell types. Depending on the trigger, MSCs can differentiate into osteoblasts, chondrocytes, fibroblasts, adipocytes or myocytes [12-13]. During osteoblast differentiation various functional phases can be identified: proliferation, production and maturation of extracellular matrix (ECM) and ECM mineralization [14]. Osteoblast differentiation can start by a trigger of certain growth factors. Bone morphogenic proteins (BMP) stimulate MSCs to differentiate via osteoprogenitors into pre-osteoblasts [15]. During development of pre-osteoblasts into mature osteoblasts the ECM is produced [14]. Osteoblasts actively produce an ECM by secreting the most abundant bone matrix protein collagen type I and a broad range of other

(non-collagenous) ECM proteins and enzymes. Mineralization of mature ECM is likely induced by matrix vesicles (MVs), which derive from osteoblasts [16]. When mature osteoblasts initiate mineralization of mature ECM, their fate may vary. Osteoblasts can further differentiate into osteocytes, become a bone lining cell or undergo apoptosis [17-18] (**Figure 1**). Osteoblasts become osteocytes by being entrapped in self-produced ECM, in which they may survive for decades. Osteocytes form a star-shaped network of cytoplasmic extensions. It is thought that osteocytes function as orchestrators of bone by sensing and communicating mechanical stress (i.e. bone damage) via these extensions [19]. More recently, by applying genetic approaches and transgenic animals, it has become evident that osteocytes play a central role in the regulation of bone turn-over (resorption and formation) [20-21]. Bone lining cells are less well understood. They are covering the bone surface and prevent it from being in direct contact with the bone marrow. It has been reported that these cells "clean" resorption pits after osteoclasts have retreated [22]. Bone lining cells are considered as inactive osteoblasts. It has been suggested that these cells can be activated to become osteoblasts [23-24] but they also may represent the osteoblastic part of the stem cell niche and interact with the hematopoietic stem cells.

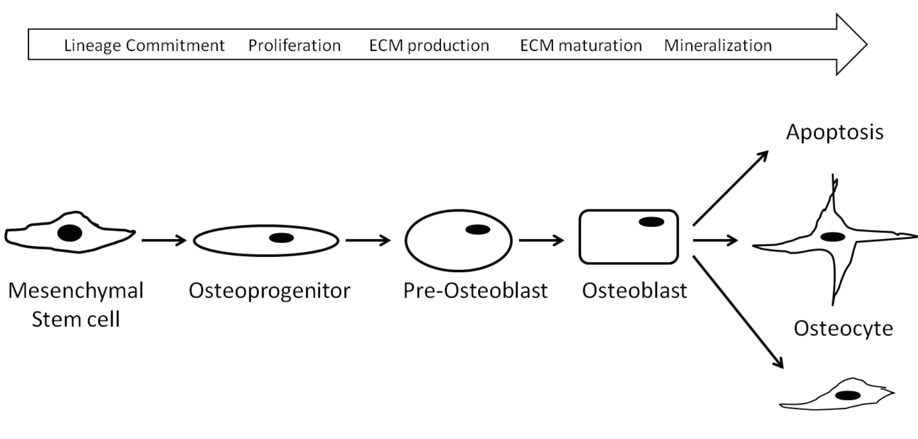

Figure 1. Osteoblastogenesis. Upon osteogenic triggers (e.g. BMPs) MSCs first differentiate into osteoprogenitors, then into ECM synthesizing pre-osteoblasts and into osteoblasts, which maturize and finally mineralize ECM. During mineralization osteoblasts either differentiate into osteocytes, bone lining cells or undergo apoptosis.

All these processes are driven by numerous factors such as BMPs, transforming growth factors (TGFs), activin A or fibroblast growth factors (FGFs) ([25], (for review see [26]). Every stage of the above mentioned differentiation is defined by specific proteins such as the transcription factors runt related transcription factor 2 (RUNX2) [27-29] and osterix (SP7) [30].

These transcription factors target osteoblastic proteins such as alkaline phosphatase (ALPL), collagen type 1 alpha 1 (COL1A1), osteopontin (SPP1), bone sialoprotein (IBSP) or osteocalcin (BGLAP) [31-32], which characterize specific differentiation stages of osteoblasts. The roles of these proteins are described in more detail later in this section.

Bone (re)modeling

Two major processes occur in bone tissues: bone modeling and bone remodeling. While bone modeling drives the growth of the skeleton, bone remodeling is responsible for the maintenance of healthy bone during adulthood [33]. Both processes are conducted by bone forming cells, osteoblasts, and bone resorbing cells, osteoclasts. The differences of the two processes are described below.

In bone modeling osteoblasts and osteoclasts act independent of each other. The physically uncoupled processes of resorption and formation result in changes of bone size or shape. Bone modeling also occurs in wound healing to repair bone damages [34]. One example for uncoupled bone formation and resorption are found in bone lining cells. These cells can differentiate into bone forming osteoblasts without resorption being the trigger [23-24]. Furthermore, a complete unloading results in bone resorption without subsequent bone formation [35].

In contrast to bone modeling which is an unbalanced process, bone remodeling is well-balanced characterized by a coupling between osteoclast and osteoblast activity. Continuous bone remodeling occurs to maintain the quality and strength of bone [3]. Remodeling occurs throughout life and always follows the same pattern within a so-called basic multicellular unit (BMU): first osteoclasts resorb bone which is then replaced by new bone matrix produced by osteoblasts. The close physical collaboration of osteoblasts and osteoclasts indicates that various coupling mechanisms exist between resorption and formation [36]. For example, structure-wise, on top of the BMU a canopy of cells with osteoblastic markers and capillaries is coupling bone resorption and formation [37-38]. Another example is the tightly controlled molecular balance between resorption and formation. Activated osteoblasts and osteocytes produce the soluble osteoclast stimulating factors RANKL and M-CSF, which upon binding to its receptors (RANK and c-Fms, respectively) drive differentitation of osteoclast progenitors as well as activation of osteoclasts [9]. Besides RANKL, osteoblasts also produce a soluble decoy-receptor: osteoprotegerin (OPG). OPG binds RANKL with high affinity leading to inhibition of osteoclast stimulation and thus leading to less bone resorption [39-40] (**Figure 2**).

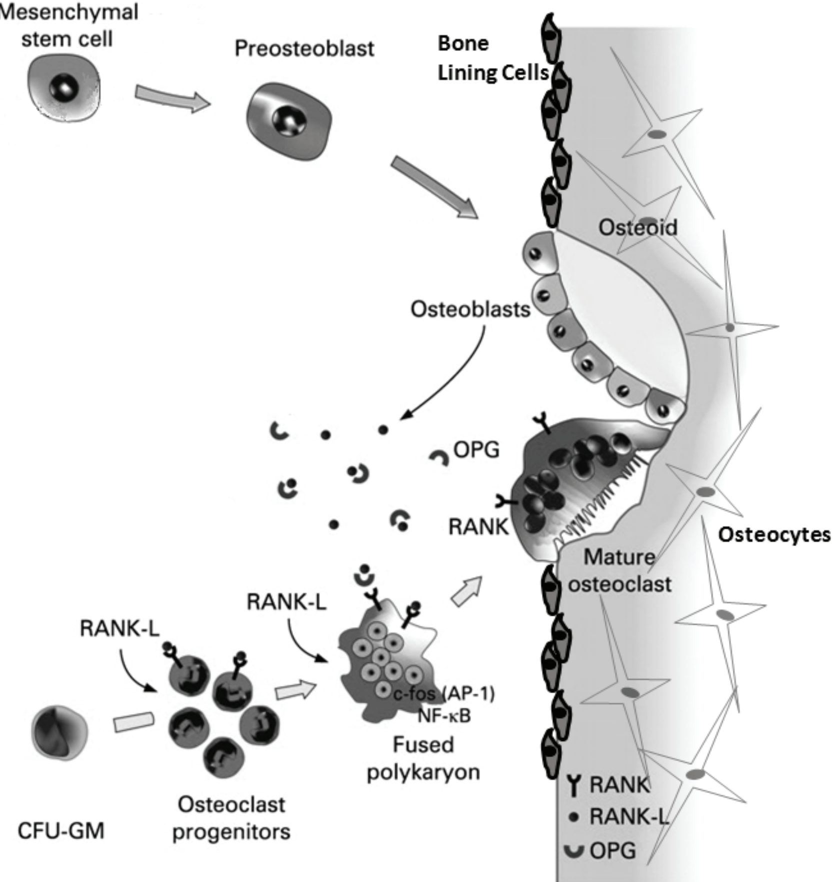

Figure 2. Bone remodeling (Adapted with permission from BMJ Publishing Group Limited (Gut, Inflammation and osteoporosis: basic and clinical concepts, Tilg et al., 57, 684-694, 2008)).

Remodeling occurs simultaneously at multiple sites throughout the whole skeleton to repair micro-damages and to maintain calcium homeostasis [41-42]. Normally, the flow of calcium to and from bone due to remodeling is neutral, highlighting the balance of resorption and formation. A disturbance of serum calcium levels results in pathological situations for bone. For example, *in vitro* hypocalcemic conditions stimulate osteoclasts to release calcium from bone. Coupled to calcium homeostasis is the level of vitamin D which is an important stimulator of calcium intake via the intestine. Vitamin D affects bone remodeling by controlling serum calcium level [4, 43]. *In vitro* it has been shown that vitamin D stimulates osteoclast differentiation and activity [44-46], but also stimulates osteoblasts and enhances ECM mineralization [47]. Therefore, it appears that vitamin D can both stimulate bone formation and bone resorption. Vitamin D is an anti-rickets agent [48-50], which improves bone mineralization and is often prescribed in

combination with other osteoporosis drugs to secure a positive calcium balance. Therefore vitamin D can be considered as a stimulator of bone formation either in an indirect or direct way. The direct effects will be discussed in more detail below. Various other stimuli are able to influence remodeling and disturb the balance of bone formation and bone resorption. A good example is estrogen. Loss of estrogen after menopause results in elevated osteoclast activity and thus tipping the balance towards bone resorption, which results in high risks of osteoporosis [51]. Additionally, mechanical loading, cytokines and other hormones such as parathyroid hormone (PTH) trigger increased and unbalanced bone remodeling [52-56].

Extracellular Matrix (ECM)

Bone needs to be a flexible but stable organ at the same time. This is achieved by a defined distribution between collagens and mineral. To obtain structural integrity, bone consists of mineralized ECM and bone cells. The ECM is formed by long fibrous proteins of which the most abundant is COL1A1. In between the fibers smaller proteins are incorporated to guarantee a stable but flexible ECM. Besides collagens, other non-collagenous proteins such as proteoglycans, growth-factors, calcium-binding proteins and fibronectins are necessary to stabilize the ECM [57]. Two extensively studied proteins are BGLAP and SPP1. These two proteins are incorporated in the ECM and are often used as markers of osteoblast activity. The ECM is strengthened by the deposition of calcium phosphate crystals (hydroxyapatite) [57].

Disturbances in ECM either in mineral or in the ECM proteins such collagen can lead to pathologies. A good example of too brittle bones is osteogenesis imperfecta. In this disease, mutations in the COL1A1 gene cause malformation within collagen fibers resulting in enhanced risk of bone fractures [58-60]. Another disease, which is characterized by extreme bone density and brittle bones is osteopetrosis. This disease is not caused by disturbances in the ECM but by osteoclastic malfunction [61]. Examples of too flexible bones are rickets and osteomalacia. In these diseases, characterized by defective vitamin D signaling (for review [62]), serum calcium levels are too low, which limits the availability of calcium being deposited in the ECM. Subsequently, the bones are hypomineralized and undergo deformation. These diseases highlight the importance of both ECM composition and mineralization grade of bone for optimal quality and strength. However, the processes underlying and controlling mineralization are yet poorly understood.

Mineralization occurs after ECM maturation is completed. The trigger for completion remains unknown and the process is dependent on a number of variables. ECM composition, production of so-called matrix vesicles (MVs)

[63-65], enzyme activities and concentrations of calcium and phosphate ions all have to be orchestrated. The main mechanism of initiating mineralization is supposed to constitute the formation of hydroxyapatite arising from crystallization of calcium ions [65] and inorganic phosphate (Pi) groups, which are provided by ALPL [66]. The process of hydroxyapatite deposition may be driven by MVs. MVs bud off at specific regions on the outer membrane of osteoblasts and have enriched amounts of calcium- and phosphate transporters on their membranes. These transporters allow for high local concentrations of calcium and phosphates [67-68]. Additionally, MVs are highly loaded with ALPL, which converts phosphates to Pi [16, 63, 69]. With calcium ions, phosphate ions and ALPL, MVs have all pre-requisites to initiate the formation of mineral crystals. Besides in MVs, there are several indications that ALPL is also present in the ECM [70-72]. This suggests that other mechanisms underlying initiation and progression of hydroxyapatite deposition are possible.

Vitamin D

Intestine, kidney and bone are the main organs responsible for maintaining calcium homeostasis. Together with PTH and calcitonin, vitamin D plays a major role in regulating calcium homeostasis and is a well-known enhancer of bone formation *in vitro*. Vitamin D can be obtained from the diet such as fatty fish, fish oil or milk. However, the concentrations of vitamin D in food are not sufficient to fully cover the requirements. The other and most important source is production by the organism itself. Vitamin D production is initiated in the skin through ultraviolet B photons. Thus sun exposure is an important determinant of vitamin D formation. During seasons sun exposure and therefore also vitamin D levels vary. To compensate for this and ensure sufficient vitamin D levels, respective food supplements can easily be purchased. In some countries (Northern America, Scandinavia) vitamin D and calcium are standard additives in milk products.

In more detail, upon ultraviolet B photon exposure 7-dehydrocholesterol (pro-vitamin D_3) is transformed into (pre)vitamin D_3 (cholecalciferol) in the skin. Subsequent hydroxylation at the C25 (by CYP2R1) and 1α position (by CYP27B1) in liver and kidney, respectively, produce the most active metabolite 1α,25-dihydroxyvitamin D_3 (calcitriol or $1,25(OH)_2D_3$ (1,25D3)) (for review [73]). While being transported in plasma, the majority (>85%) of 1,25D3 is bound to vitamin D binding protein (DBP) [74-75]. 1,25D3 exerts its effects by binding to the intracellular vitamin D Receptor (VDR), which upon 1,25D3-mediated phosphorylation interacts with retinoid X receptor (RXR) to form a heterodimeric transcription factor [76-77]. The VDR-RXR heterodimer rapidly translocates into the nucleus, where it binds to the VDR response elements (VDRE) and regulates gene expression [78-79]. 1,25D3

induces gene transcription but can also switch off gene transcription such as for the PTH gene. To protect the organism against toxic levels of 1,25D3, the enzyme initiating the degradation process of 1,25D3, 24-hydroxylase (CYP24A1), is strongly activated. In fact, CYP24A1 is one of the strongest regulated genes upon 1,25D3 stimulation [80]. Hydroxylation on the C24 position leads to decreased bioactivity [81]. Further hydroxylation processes eventually lead to inactive products (**Figure 3**).

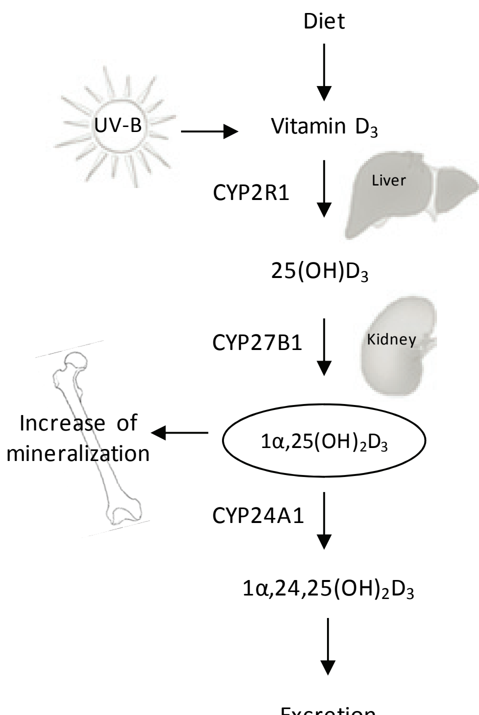

Figure 3. Vitamin D metabolism. Vitamin D3 is taken in by either diet or generated upon sun exposure. Subsequent hydroxylations in liver and kidney result in the most active metabolite $1\alpha,25(OH)_2D_3$. By further hydroxylations the activity of metabolite is reduced and finally inactivated, which then can be excreted.

Vitamin D and osteoclasts

In an early stage of osteoclast differentiation, 1,25D3 induces interferon (IFN) β expression to inhibit osteoclast differentiation and activity [82] but expression of VDR by osteoclasts and direct effects of 1,25D3 on human osteoclast formation are controversial [83-84]. However, 1,25D3 targets osteoclasts indirectly via osteoblasts in which 1,25D3 stimulates the production of specific osteoclast activating factors such as RANKL [85-87] or SPP1.

Vitamin D and bone formation

To date, it is still debatable whether 1,25D3 effects on bone formation are solely indirect via intestinal and renal regulation of calcium levels or also direct. It has been demonstrated that mice lacking the VDR gene display retarded growth, severe bone impairment, immune abnormalities and premature death at only 15 weeks of age due to hypocalcemia [49, 88-89]. A rescue diet restored all pathological effects suggesting that as long as calcium homeostasis is under control, bone itself does not seem to be affected by impaired VDR signaling. Nevertheless, the importance of physiological 1,25D3 levels for bone is demonstrated by the mutation of the CYP27B1 gene (encodes the enzyme 1α-hydroxylase, responsible for the synthesis of 1,25D3). Subjects with a mutation in this gene develop vitamin-D-dependent rickets [48]. In a mouse model for rickets the greater extensibility and lower stiffness of fibrils resulted from a decreased grade of mineral deposition [90]. This further supports the importance of an optimal grade of mineralization for healthy bones [48] and points to a role for 1,25D3 herein.

Next to the indirect effects, which are reported in *in vivo* studies, *in vitro* studies also demonstrated direct effects. We have shown that osteoblasts are able to convert 25(OH)D_3 into the biologically most active form 1,25D3, suggesting a direct relationship between 1,25D3 synthesis and bone [91]. Additionally, the expression of VDR allows 1,25D3 to directly affect osteoblast growth and differentiation. 1,25D3 regulates the osteoblast differentiation marker ALPL and various bone ECM proteins such as COL1A1. In addition, 1,25D3 regulates various cytokines and growth factors (TGFβ, insulin-like growth factor 1) [92-93]. 1,25D3 has been shown to stimulate mineralization in human *in vitro* osteoblast bone formation models [47]. The exact mechanism of 1,25D3-mediated stimulation of mineralization is not yet understood. However, *in vitro* 1,25D3 has certain protective control mechanisms in place to avoid pathological over-mineralization. The most abundant one is the induction of the above described CYP24A1, which degrades active 1,25D3 [94]. Furthermore, 1,25D3 induces BGLAP and SPP1, both established inhibitors of mineralization [95-96] and a stimulator of mineralization, bone sialoprotein (IBSP), is inhibited by 1,25D3 [97] (Overview of 1,25D3 actions on accelators and inhibitors of mineralization are depicted in **Figure 4**). These examples do not only show how important 1,25D3 for bone formation is but also highlight the importance of a well controlled system to gain optimal bone strength. The roles of the mentioned inhibitors and stimulator, as well as other abundant osteoblastic proteins, are described in more detail below.

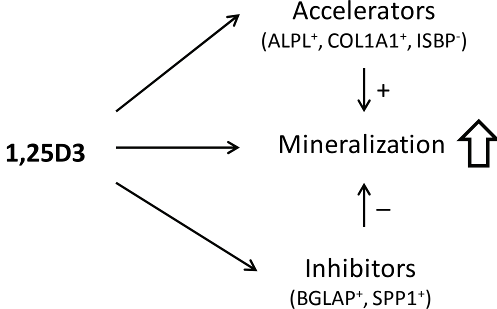

Figure 4. Overview of established 1,25D3 actions on known accelerators and inhibitors of mineralization. The accelators ALPL and COL1A1 and inhibitors BGLAP and SPP1 are stimulated by 1,25D3, whereas the accelerator ISBP is inhibited (see text for references).

Runt-related transcription factor 2 (RUNX2)

RUNX2 is the key transcription factor in osteoblast differentiation. RUNX2 binding sites are located in the promoters of BGLAP, SPP1, IBSP and COL1A1 and RUNX2 regulates their transcription [98-102]. Homozygous mice with a mutation within the RUNX2 gene died after birth due to respiratory failure. The phenotype also revealed non-mineralized bones and a complete lack of osteoblasts as well as BGLAP and SPP1 expression [27, 29]. At the BGLAP promoter, RUNX2 co-precipitated with VDR indicating a role for RUNX2 in stabilization of VDR to the VDRE [103]. This suggests that 1,25D3 influences RUNX2-mediated inductions of osteoblastic target genes.

Alkaline phosphatase (ALPL)

ALPL is a membrane-bound phosphatase, which converts pyrophosphate into inorganic phosphates (Pi) [104]. Pyrophosphates are inhibitors of mineralization and by converting pyrophosphates, ALPL reduces their negative effects [105-106]. At the same time, the product of the conversion, Pi, is crucial for hydroxyapatite formation. ALPL is present in ECM and MVs, which are both involved in mineralization. ALPL deficient mice display growth impairment and abnormal bone mineralization [107-108]. During the ECM maturation phase 1,25D3 increases ALPL activity of osteoblasts [47], indicating elevated osteoblast differentiation.

Osteocalcin (BGLAP)

BGLAP is a small non-collagenous protein with three gamma-carboxyglutamic acids which are crucial for the interaction of BGLAP with hydroxyapatite. The negatively charged protein surface of BGLAP coordinates the binding of five calcium ions which are complementary to those in hydroxyapatite [109]. BGLAP inhibits *de novo* mineralization by delaying nucleation of hydroxyapatite [110-111] and a mineral maturation defect has been reported in BGLAP deficient mice [112]. During osteoblast proliferation the BGLAP gene is repressed and only becomes up-regulated post-proliferationally or upon stimulation with 1,25D3. BGLAP has highly functional VDRE element in

its promoter leading to immediate 1,25D3-mediated stimulation of BGLAP expression.

Osteopontin (SPP1)

SPP1 is an extracellular structural protein produced by osteoblasts and only expressed during mineralization [113-114]. SPP1 is highly negatively charged and inhibits the formation of hydroxyapatite [115]. SPP1 is thought to be an anchor protein for osteoblasts and osteoclasts to the ECM [116-117]. Similar to BGLAP, SPP1 mRNA expression is also induced by 1,25D3 [96, 114-116, 118-119].

Bone sialoprotein (IBSP)

IBSP is an acidic glycoprotein bound to type I collagen [120]. It has been shown that IBSP owns negatively charged regions, which allow IBSP to be a potent nucleator of hydroxyapatite formation [121]. IBSP mRNA expression is reduced by 1,25D3, suggesting a decreased IBSP-mediated formation of hydroxyapatite [122-123].

This list of ECM proteins and transcription factors being crucial for osteoblast differentiation and mineralization is far from complete but also highlights that the mechanism of 1,25D3-stimulated mineralization is not yet clarified. We do know that 1,25D3 induces several mechanisms to avoid pathological over-mineralization (i.e. stimulation of BGLAP and SPP1, repression of IBSP) but the mechanisms by which mineralization is actually stimulated remains unresolved.

Vitamin D and the immune system

The immune system is devided in the innate (non-specific) and the adaptive (specific) immune system. Their role is to protect and defend the organism against disease (i.e. bacterial or viral infections). To successfully defend against intruders various processes for detection and destruction are in place. These processes include various cell types, such as monocytes and macrophages, and small signaling molecules called cytokines (for review [124]). Interestingly, in monocyte-macrophages 1,25D3 can activate the toll-like receptor (TLR) pathway [110]. Additionally, deficient amounts of 1,25D3 limit the activation of antimicrobial genes [125-127]. Consequently, 1,25D3 participates in host defense.

One of the major groups of cytokines within the innate immune system is the interferon (IFN) system. Several IFNs are described but the best studied are IFNα, IFNβ and IFNγ. In response to pathogens IFNs are secreted from host cells to trigger autocrine/paracrine immune responses (for review [124]). Upon binding to their receptors, Janus kinase – signal transducers and activators of transcription (STAT) signaling pathway is induced [128]. Interestingly, independent of its role in immune response STAT1 has been

associated with bone formation. STAT1-deficient mice display a strong increase in bone mass and it has been demonstrated that STAT1 is able to attenuate osteoblast differentiation via interactions with RUNX2 [129-130]. Interestingly, 1,25D3 is able to modify STAT1 protein levels and VDR can interact with STAT1 resulting in modifications of transcriptional activities [131]. Not only STAT1 acts independently on bone. Recently, various studies reported IFNβ actions on bone, which are also independent of the immune system [82, 129, 132-135]. Thereby, IFNβ plays a crucial role in osteoclast development since RANKL-induced IFNβ production inhibits osteoclastogenesis [135]. Interestingly, this process can be induced by 1,25D3 [82].

The above described processes and interactions are all within the innate immune system. Also within the adaptive immune system 1,25D3-mediated actions on several cell types are described. In lymphocytic cells, 1,25D3 inhibits proliferation and activation, as well as IFNγ production [136]. 1,25D3 also disturbs maturation and differentiation in dendritic cells. Additionally, several interleukins (IL; IL2, IL10, IL12 and IL17) are targeted by 1,25D3 in various immune cell types [137-139].

Clinical Applications of vitamin D

Clinically the role of vitamin D is most clear from conditions of severe vitamin D deficiency and mutations in the VDR and the enzyme controlling the formation of 1,25D3, CYP27B1 [48-49, 62, 89, 140-141]. These conditions leading to rickets revealed the crucial role of 1,25D3 in the control of calcium homeostasis and the consequences for bone metabolism.

The presence of the VDR in almost all tissues, including the immune system and a broad range of cancer cells, together with epidemiological studies on sun exposure and diseases have implicated a role for 1,25D3 in a broad range of physiological processes and pathological conditions including immune-related diseases and cancer [139, 142-150].

For example, vitamin D has been associated with various autoimmune diseases such as multiple sclerosis (MS) [150], rheumatoid arthritis [149] and type 1 diabetes [141, 151]. Actually, 1,25D3 deficiency is put forward as a risk factor for MS [150, 152-153]. IFNβ reduces various symptoms and disease activity and is therefore used as standard treatment [154]. Interestingly, MS has been associated with low bone mass and a higher frequency of osteoporosis [155].

Osteoporosis is a disease of bone characterized by low bone mineral density, subsequently resulting in an increased risk for fractures. The underlying mechanism is an imbalance of bone resorption and bone formation whereby more bone is resorbed than is formed leading to a reduction in bone density and strength (for review [4]).

Osteoporosis affects 50% of women and 20% of men being older than 50 years of age. In Europe the yearly costs for osteoporosis treatment exceed 30 billion Euros [156]. Osteoporosis is primarily an age-related disease but can also be secondary related to other diseases and therapies. There are several risk factors for osteoporosis: advanced age, estrogen [51] or testosterone deficiency [157], low calcium diet or a 1,25D3 deficiency combined with modifications of PTH levels [158]. Additionally, an individual's lifestyle can include risk factors such as excessive consumption of alcohol [159] or tobacco smoking [160]. It is also possible that drugs induce osteoporosis, of which long-term glucocorticoid or steroid treatment are examples [161-162].

Many diseases and disorders have been associated with secondary osteoporosis, for example immobilization, hypogonadal disorders (i.e. Turner Syndrome), endocrine disorders (i.e. Cushing's Disease), hematological disorders (i.e. leukemia) or neurological disorders (i.e. MS). Interestingly, in MS and hepatitis C treatment with IFNβ is under debate since it may increase the risk of osteoporosis [163-165]. Not only IFNβ is under debate but also several other medications have been associated with low bone density and increased osteoporosis, among others rosiglitazone and warfarin [166-170].

To prevent osteoporosis, various approaches are in place and include diet, exercising, fall prevention and additional supplements such as 1,25D3, calcium and biphosphonates [5-6]. Current treatments for osteoporosis include bisphosphonates and estrogen replacement therapies, which are directed to inhibit bone resorption and further bone loss [171-172]. A major aim is to develop bone anabolic agents that stimulate bone formation to restore bone mass that has been lost [173-175]. Potentially, vitamin D may play a role in this, albeit the results of vitamin D treatment in fracture prevention are yet controversial [176-180]. Interestingly, in various clinical trials on osteoporosis performed with e.g. bisphosphonates, vitamin D treatment was included as basis therapy ([181] and www.clinicaltrials.gov). The obtained results subsequently led to a FDA approved medication containing a bisphosphonate and vitamin D (fosavance®) [182]. Following the manufacturers information on osteoporosis medications (i.e. Actonel®, Evista®, Aclasta®), additional treatment with calcium and vitamin D is strongly recommended.

Aim of this thesis

During the last two years a renewed interest in vitamin D was observed. This was primarily based on two observations: 1) a trend towards a global nutritional vitamin D insufficiency [183-184] and 2) new actions of 1,25D3 within the innate and adaptive immune systems [185].

This thesis scrutinized the effects of 1,25D3 on osteoblasts and the impact on bone formation. Since mineralization has to be tightly controlled to avoid pathological over-mineralization, our aim was to investigate the interplay of 1,25D3 with inhibitors of mineralization (IFNβ, activin A, rosiglitazone).

This thesis is based on a human *in vitro* cell environment, which allowed us to translate the results easier into *in vivo* conditions. This is of importance since in bone, mechanistic discrepancies exist between species. For example, vitamin D stimulates osteocalcin in human osteoblasts while inhibiting it in murine osteoblasts. We analyzed gene chip profiles for processes involved in 1,25D3-enhanced mineralization which formed a basis for further analyses in the various chapters. In chapter 1 we focused on the process of mineralization itself. By using devitalization assays we gained more insight on ECM produced by 1,25D3-treated osteoblasts. Furthermore, we investigated the role of ECM genes and MVs in 1,25D3-stimulated mineralization. These analyses extend the knowledge of 1,25D3-stimulated mineralization and were the foundation of the subsequent chapters.

By gene chip analyses we aimed to identify new inhibitors in 1,25D3-stimulated mineralization. In chapter 2 we first characterized a potential candidate, IFNβ. We investigated the effects of IFNβ on osteoblast growth, differentiation and mineralization. To understand the role of this candidate we analysed the expression of genes associated with ECM. We finally identified IFNβ as novel inhibitor of human osteoblast mineralization.

Since 1,25D3 inhibits IFNβ expression we analysed in chapter 3 the interplay of IFNβ and 1,25D3 in osteoblasts and the regulation of mineralization. To investigate this, we analysed the effects of IFNβ on 1,25D3-stimulated osteoblast differentiation and mineralization. We extensively studied gene expression and performed gene chip profiling to understand the role of IFNβ in 1,25D3-stimulated mineralization.

In addition to IFNβ, we identified that 1,25D3 induced activin A expression in mineralizing osteoblasts. In chapter 4 we scrutinized the interaction between 1,25D3 and activin A and the role of its natural inhibitor follistatin in this interaction. We investigated the capacity of increased activin A protein levels to influence 1,25D3-stimulated mineralization by mineralization assays, reporter-gene-assays and target gene expression. Finally, we tested a possible interaction with another 1,25D3-induced inhibitor of mineralization: BGLAP.

This thesis was part of the European Union PhD traning network NucSys. Chapter 5 is based on a collaboration within this network and aimed to investigate the relationship between 1,25D3 and rosiglitazone (PPARγ ligand) on human osteoblast differentiation and mineralization. To reveal possible interactions, we assessed target gene expression, vitamin D levels in culture medium and performed osteoblast mineralization as well as differentiation assays.

Together, these approaches increased our knowledge on the mechanism by which 1,25D3 regulates mineralization. It sheds light on the interaction of 1,25D3 with mineralization inhibitors (IFNβ, activin A, rosiglitazone) to control mineralization, thereby underlining the presence of regulatory processes to prevent over-mineralization and achieving optimal mineralization.

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Chapter 1

1α,25-$(OH)_2D_3$ acts in the early phase of osteoblast differentiation to enhance mineralization via accelerated production of mature matrix vesicles

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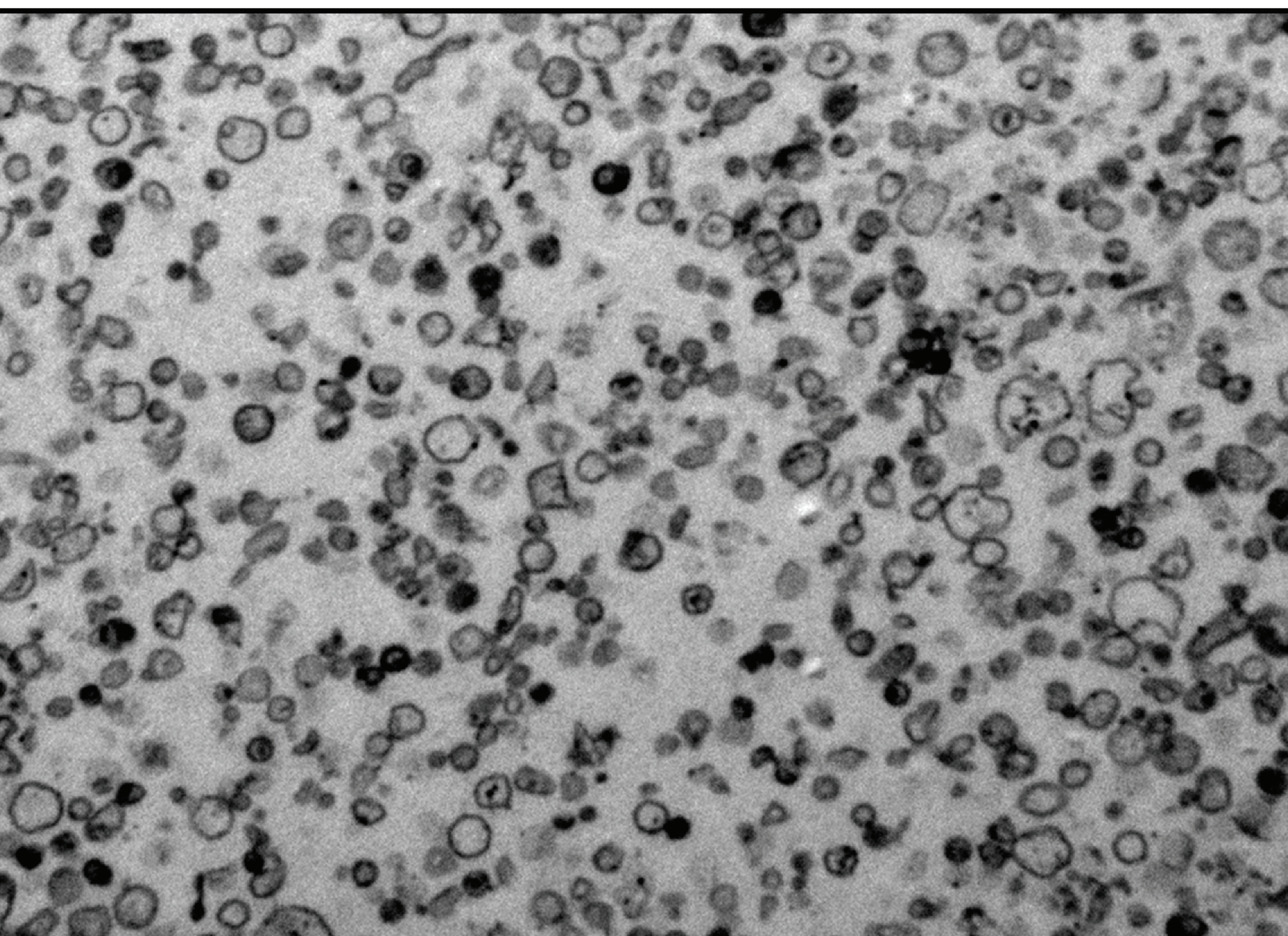

Abstract

1α,25-dihydroxyitamin D_3 (1,25D3) deficiency leads to impaired bone mineralization. We used the human pre-osteoblastic cell line SV-HFO, which forms within 19 days of culture an extracellular matrix that starts to mineralize around day 12, to examine the mechanism by which 1,25D3 regulates osteoblasts and directly stimulates mineralization. Time phase studies showed that 1,25D3 treatment prior to the onset of mineralization, rather than during mineralization led to accelerated and enhanced mineralization. This is supported by the observation of unaltered stimulation by 1,25D3 even when osteoblasts were devitalized just prior to onset of mineralization and after 1,25D3 treatment. Gene Chip expression profiling identified the pre-mineralization and mineralization phase as two strongly distinctive transcriptional periods with only 0.6% overlap of genes regulated by 1,25D3. In neither phase 1,25D3 significantly altered expression of extracellular matrix genes. 1,25D3 significantly accelerated the production of mature matrix vesicles (MVs) in the pre-mineralization. Duration rather than timing determined the extent of the 1,25D3 effect. We propose the concept that besides indirect effects via intestinal calcium uptake 1,25D3 directly accelerates osteoblast-mediated mineralization via increased production of mature MVs in the period prior to mineralization. The accelerated deposition of mature MVs leads to an earlier onset and higher rate of mineralization. These effects are independent of changes in extracellular matrix protein composition. These data on 1,25D3, mineralization, and MV biology add new insights into the role of 1,25D3 in bone metabolism and emphasize the importance of MVs in bone and maintaining bone health and strength by optimal mineralization status.

Introduction

Vitamin D is one of the major factors involved in calcium homeostasis through actions on the intestine, kidney, parathyroid gland, and bone [1]. The biologically most active form of vitamin D is 1α,25-dihydroxyvitamin D_3 (1,25D3). 1,25D3 is synthesized from the parental vitamin D molecule by sequential hydroxylations in the liver (25-hydroxylation) and kidney (1α-hydroxylation) [2]. Most of the biological activities of 1,25D3 are exerted through binding to the vitamin D receptor (VDR) followed by hetero-dimerization with Retinoid-X-Receptor (RXR) [3]. The heterodimer directly binds to the promoter of target genes and controls their expression [4].

1,25D3 is involved in bone formation and mineralization and it is used to prevent and treat osteoporosis. These influences on bone can be indirectly established through the control of calcium uptake in the intestine or reabsorption in the kidney. However, direct effects are also likely as the VDR is present in osteoblasts. In addition, 1,25D3 can directly affect osteoblasts

as shown by stimulating *in vitro* mineralization in osteoblast cultures [5-6] and by altering gene regulation [2, 6-8]. Moreover, we were able to show that osteoblasts themselves can synthesize 1,25D3 [9], emphasizing the importance of local 1,25D3 for osteoblasts.

Composition of the extracellular matrix (ECM) is an important determinant of mineralization. The ECM contains numerous proteins which can be regulated by 1,25D3 and influence mineralization. Examples are osteocalcin [10-11], osteopontin [12], bone sialoprotein [13] and collagens [14]. In addition, we recently demonstrated that follistatin and activin cause major changes in the ECM gene expression profile preceding their stimulatory and inhibitory effect on mineralization, respectively [15].

Another crucial prerequisite for mineralization are matrix vesicles (MVs). MVs are budding from the basolateral side of the osteoblast plasma membrane and are released into the ECM [16]. Various channel proteins (annexin II, V and VI) allow transportation of calcium and phosphate ions into the vesicles [17]. Furthermore, MVs contain a large amount of alkaline phosphatase (ALPL), which is an important enzyme involved in osteoblast mineralization and is generally accepted as a marker for MVs [18-19]. Mature MVs, reflected by outgrowth of hydroxyapatite, and high ALPL activity are believed to initiate mineralization [20]. It is widely believed that following MV maturation, the membrane ruptures and hydroxyapatite (calcium-phosphate) is released into the matrix.

The aim of this study was to identify the processes involved in 1,25D3 stimulation of mineralization. This was assessed by a series of experiments, including *in vitro* timing experiments with differentiating human osteoblasts, transcriptional profiling and isolation and analyses of MV production.

Material and Methods

Cell culture

SV-HFO cells [21] were cultured as described previously [15]. To induce osteoblast differentiation, medium (αMEM; Gibco BRL, Invitrogen Ltd.) was supplemented with freshly added 10 mM β-glycerophosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich), with or without 10 nM 1,25D3, and replaced every 2 or 3 days. Hence, one treatment with 1,25D3 is equivalent to a duration of 2-3 days, two treatments with 4-5 days etc.. 1,25D3 was a generous gift from Leo Pharma, Denmark. Cells were harvested at different time points during culture.

DNA, mineralization and protein assays

DNA and calcium measurements were performed as described previously [22]. Briefly, for DNA measurements cell lysates were incubated with heparin (8 IU/ml in PBS) and Ribonuclease A (50 µg/ml in PBS) for 30 minutes at

37°C. DNA was stained by adding ethidium bromide (25 μg/ml in PBS). Analyses were performed by using a Victor2 plate reader (PerkinElmer Life and Analytical Science) with an extinction filter of 340 nm and an emission filter of 590 nm. For calcium measurements, cell lysates were incubated overnight with 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 cell lysates. 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). For protein measurement 200 μl of working reagent (50 volumes BCA™ reagent A, 1 volume BCA™ reagent B; Pierce) was added to 10 μl of sonicated cell lysate. The mixture was incubated for 30 minutes at 37°C, cooled down to room temperature and absorbance was measured, using a Victor2 plate reader at 595 nm.

Affymetrix Gene Chip-based gene expression

Gene expression arrays were performed as described previously [15]. Briefly, cultures, continuously treated with or without 10 nM 1,25D3, were harvested at days 3, 7, 12 and 19 of culture and total RNA was isolated. Quality assessments of arrays were performed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). For further procedures several kits were used: cDNA synthesis (Invitrogen), purification, labelling and hybridization (Affymetrix). As platform we used the Affymetrix "GeneChip Human Genome U133 Plus 2.0 oligonucleotide Genechips" . For gene ontology (GO) analysis, selected Affymetrix IDs were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 hosted by the National Institute of Allergy and Infectious Diseases (NIAID), NIH [23].

Quantification of mRNA expression

RNA isolation, cDNA synthesis and PCR reactions were performed as described previously [24]. Reactions were downscaled to a 384-well-plate system and reactions were performed in 12.5 μl volume. Q-PCR was carried out, using an ABI PRISM 7900 HT system (Applied Biosystems). Primer pairs, all being either on exon boundaries or spanning at least one exon, were purchased from Sigma-Aldrich. Primers: decorin (DCN): TAACCGAAATCAAAGATGGAGACTT (forward primer; 2.5 pmol/reaction); TCCAGGACTAACTTTGCTAATTTTATTG (reverse primer; 2.5 pmol/reaction); fibulin-1 (FBLN1): GGAGACCGGAGATTTGGATGT (forward primer; 5 pmol/ reaction); TCAGATATGGGTCCTCTTGTTCCT (reverse primer; 5 pmol/ reaction). GAPDH: ATGGGGAAGGTGAAGGTCG (forward primer; 3.75 pmol/ reaction); TAAAAGCAGCCCTGGTGACC (reverse primer; 3.75 pmol/reaction); CGCCCAATACGACCAAATCCGTTGAC (FAM probe, 3.75 pmol/reaction).

Devitalization of osteoblast cultures
Cells were devitalized according to the protocol by Eijken *et al*. [15]. In short, on day 10 of SV-HFO cultures, medium was removed from the cells and cultures were washed once with PBS (Gibco BRL, Invitrogen Ltd.). Cells were air-dried and stored at -20°C. After thawing, cells were washed once with PBS. Devitalized cultures were incubated under standard conditions as described above.

Isolation of matrix vesicles
MV isolation was performed as described previously [25]. Briefly, for isolation of MVs, SV-HFO were cultured with or without 1,25D3 in 175 cm² or 75 cm² flasks. For the collection of ECM associated MVs, cells were washed twice with PBS and treated with 1.5 ml (75 cm² flasks) of collagenase/dispase (1mg/ml; Roche) for 90 minutes at 37°C. After incubation, 9 ml PBS was added and the suspension was centrifuged for 10 minutes at 500g, followed by centrifugation at 20,000g for 30 minutes at 4°C and the resulting supernatant was then subjected to a second centrifugation at 100,000g for 60 minutes at 4°C. The pellet was dissolved in 100 µl PBS. Centrifuges used were the Heraeus Multifuge 1S (DJB Labcare Ltd) and the Ultracentrifuge L-70 (Beckmann Coulter).

Electron Microscopy of MVs
For electron microscopic samples, SV-HFO cells were cultured according to protocol and MVs were harvested on day 14 of culture. To achieve sufficient amounts of MVs, cultures of two flasks (175 cm²) were pooled. Isolation of MVs was performed as described; instead of dissolving the pellet in PBS we dissolved it in 200 µl fixative (4% formaline, 1% gluteraldehyde; postfixation 1% osmiumtetroxide). Samples were forwarded to the department of Pathology, Erasmus Medical Center, Rotterdam, for further processing and analyses.

Flow Cytometry Analyses of MVs
A Becton Dickinson FACS-Canto and DIVA Flow Cytometry System (BD bioscience) were used to measure and analyze MVs, respectively. 25 µl of freshly isolated MVs were incubated for 15 minutes with 25µl ELF-97 staining solution (0.2 M ELF-97 (Invitrogen), 1.1 M acetic acid, 0.011 M $NaNO_2$, pH 8.0). 250 µl PBS was added and stained vesicles were measured in AmCyan-A channel (488nm). ELF-97 is a phosphatase substrate, which at pH 8 detects alkaline phosphatase. Reference beads (BD bioscience) were added to the samples for size estimation of the isolated vesicles.

Gene nomenclature
Gene names and symbols were used as provided by the HUGO Gene Nomenclature Committee [26].

Statistics

The data provided are based on multiple independent experiments derived from independent cultures. Experiments were performed at least in triplicate; values are means ± SEM. Significance was calculated using the Student's *t*-test and p-values < 0.05 were considered significant.

Results

Human osteoblasts continuously treated with 1,25D3 showed enhanced and accelerated mineralization as the onset of mineralization is already observed on day 10 of culture (**Figure 1**).

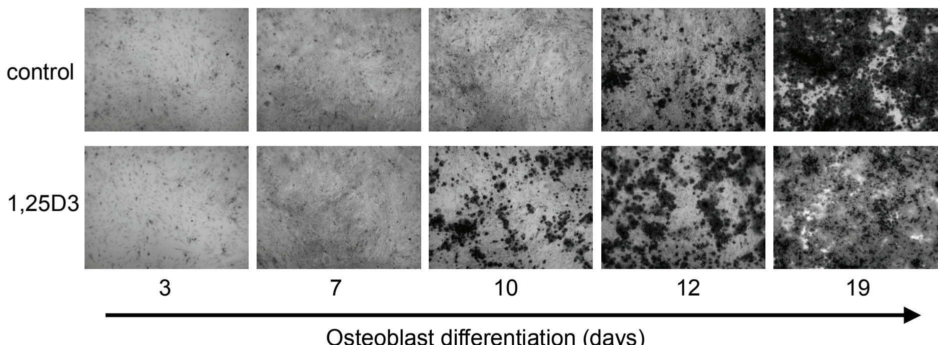

Figure 1. Alizarin Red Staining of 1,25D3 enhanced mineralization in SV-HFO cells. Cells were treated with or without 1,25D3. Cells were harvested at days 3, 7, 10, 12, 14 and 19, fixed with 70% ethanol and stained for calcium.

Next, we examined the timing of the 1,25D3 effect on mineralization. Treatment prior to the onset of mineralization (i.e. day 12 of control cultures) was crucial and sufficient to observe enhanced mineralization after 19 days of culture (**Figure 2**). Therefore, the major effects of 1,25D3 on osteoblasts to stimulate mineralization occur prior to the onset of mineralization.

We further examined the timing of the 1,25D3 effect by zooming in on the pre-mineralization period. We reasoned that when 1,25D3 would stimulate a specific step in the differentiation process the precise timing of treatment is very important. No specific time-point of treatment turned out to be crucial. There was no difference in effect whether treatment started at day 0, 3 or 5 of culture (**Supplementary Figure S1**). However, it was obvious that the number of treatments, *i.e.* duration of treatment, determined the magnitude of the 1,25D3 effect on mineralization (**Figure 3**). Taken together, the longer the cells were treated with 1,25D3 prior to the onset of mineralization, the stronger the stimulation.

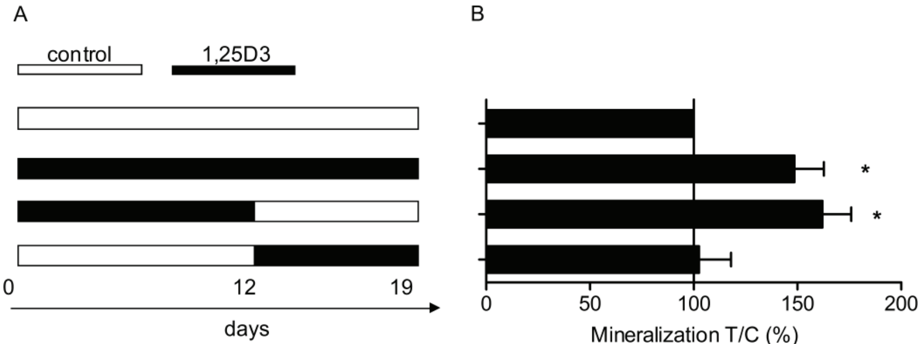

Figure 2. Timing of 1,25D3 effect on mineralization. A) Cells were treated with or without 1,25D3 for 19 days. Additionally, the treatment was shortened to only the first 12 days or only the last 7 days of culture. B) The cells were harvested at day 19 and the calcium content was measured. Control condition was set to 100%. * p-value < 0.05 versus control condition.

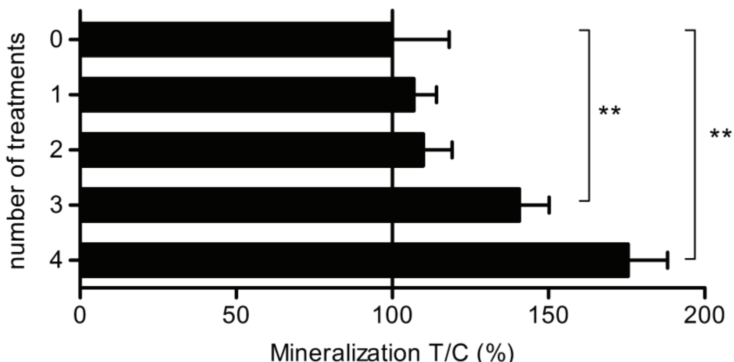

Figure 3. Impact of duration of 1,25D3 treatment on mineralization. In the pre-mineralization period (days 0-12) SV-HFO cells were treated with 1,25D3 for time periods up to 3, 5, 7 and 10 days representing 1 - 4 times 1,25D3 treatment, respectively. Cells were harvested at day 19 and calcium content was measured. Control condition was set to 100%. ** p-value < 0.01 versus no 1,25D3 treatment condition.

On basis of the findings that 1,25D3 stimulated mineralization by effects in the pre-mineralization period, we performed Gene Chip analyses to identify genes regulated by 1,25D3 at this stage in an unbiased manner. Gene chip profiling was performed at four different time points (days 3, 7, 12 and 19) during the course of osteoblast differentiation, in the presence or absence 1,25D3.

We identified genes which were at least 1.5 fold regulated by 1,25D3 in the pre-mineralization (days 3 and 7) and/or mineralization (days 12 and 19) period. To specifically select for 1,25D3 regulated genes in the mineralizing

condition, we excluded genes that were also regulated in the non-mineralizing condition, *i.e.* SV-HFO cultured in the absence of dexamethasone [27]. The analyses revealed that 6.4 times more genes are regulated in the mineralization period (470 genes) compared to the pre-mineralization period (74 genes). Amongst them, 13.5% and 18.7% were open reading frames or hypothetical proteins, respectively. Non-annotated transcribed sequences or clone IDs were excluded from further analyses. More genes were down- than up-regulated in both the pre-mineralization and the mineralization period (**Table 1**). Interestingly, there was an overlap of only 3 genes between the two sets of genes, indicating that the transcriptomes in these two time periods are strongly different and that 1,25D3 has a different role in early compared to late osteoblast differentiation. GO analyses by DAVID 2008 of the mineralization period identified processes of cell maintenance (*i.e.* RNA splicing (GO:0008380), cell cycle phase (GO:0022403), translation (GO:0006412) and cell death (GO:0008219) to be significantly overrepresented (**Supplementary Tables S1-S4**). The low number of regulated genes precluded significant Gene Ontology analyses of the pre-mineralization period.

Table 1: Overview of genes, which are 1.5 fold up- or down-regulated by 1,25D3 during osteoblast differentiation.

	pre-mineralization period	overlap	period of mineralization
total	74	3	470
up	21	0	154
down	53	3	316

We clearly showed that 1,25D3 stimulated mineralization when administered during the pre-mineralization period (ECM formation). We hypothesized that the 1,25D3 effect in the pre-mineralization period is mediated by an effect on ECM genes. To assess this we downloaded a list of genes being annotated with the Gene Ontology (GO) term extracellular matrix (GO:0031012; http://www.ensembl.org, Release 49; updated by AmiGO, http://www.geneontology.org, Release July 2009) and analyzed their expressions within the pre-mineralization period by merging the list with the Affymetrix Gene Chip expression data. The annotated genes, among others being procollagen I N-proteinase, connective tissue growth factor and matrix-forming genes such as collagens, were up-regulated during osteoblast differentiation (**Table 2**). However, 1,25D3 had no significant impact on the expression of any of these ECM genes (**Table 2**). Q-PCR analyses of a subset of these ECM genes confirmed this observation and validated the Gene Chip analyses (**Table 2**). Osteocalcin appeared not to be part of the GO term

extracellular matrix and is therefore not part of **Table 2**, but as expected 1,25D3 stimulated osteocalcin expression in human osteoblasts (data not shown). However, overall these analyses prove that 1,25D3 stimulates mineralization predominantly independent from changes ECM gene expression.

Table 2: Regulation of genes annotated to the GO term extracellular matrix during early osteoblast differentiation and compared with 1,25D3 enhanced osteoblast differentiation. Numeric values are fold changes, obtained by mean ratios of a) differentiating osteoblasts vs. non-differentiating osteoblasts and b) differentiating osteoblasts of 1,25D3 cultures vs. differentiating osteoblasts of control cultures.

	Osteoblast differentiation	1,25D3 enhanced osteoblast differentiation vs. osteoblast differentitation	Ratio verified by real-time PCR
DCN	4.9	0.9	1.1 ± 0.1
COL11A1	4.1	0.7	
COL5A2	3.5	0.8	
COL8A1	3.4	0.8	
NID2	3.3	0.8	0.9 ± 0.1
FBLN1	3.2	1.0	0.9 ± 0.1
COL4A1	2.9	1.0	
FBN2	2.8	1.0	
COL12A1	2.7	1.1	
FN1	2.7	0.9	1.1 ± 0.3
COL3A1	2.3	1.0	
MATN3	2.2	0.8	
CTGF	2.2	1.0	
COL4A2	2.2	1.3	
COL4A5	2.1	0.9	
COL5A1	2.0	1.1	
COL1A1	2.0	1.2	1.3 ± 0.2
COL6A3	1.9	0.9	
COL1A2	1.9	1.0	
FBN1	1.8	0.8	
LUM	1.7	0.7	

Following the bioinformatic analyses, showing no transcriptional relationship between 1,25D3 and ECM gene expression, we tested whether indeed 1,25D3 does not affect the ECM. We cultured SV-HFO with and without 1,25D3 until the onset of mineralization. Then cells were devitalized and subsequently the remaining ECM that was formed was put into culture again. This allowed for analyses of mineralization on a preformed ECM independent of additional cellular activity. Measurements at 0, 3 and 5 days after devitalization showed that ECM formed by control cultures did not mineralize yet. However, ECM of 1,25D3 treated cultures demonstrated significant mineralization thereby

showing an accelerated maturation of the ECM in pre-mineralization phase (**Figure 4**). This indicates an earlier onset of mineralization in cells treated with 1,25D3 and underlines the observation that 1,25D3-mediated effects are established prior to the onset of mineralization (**Figure 2**). Furthermore, despite the absence of an effect on expression of genes coding for ECM proteins (**Table 2**), the ECM itself is apparently affected by 1,25D3 treatment in the pre-mineralization period.

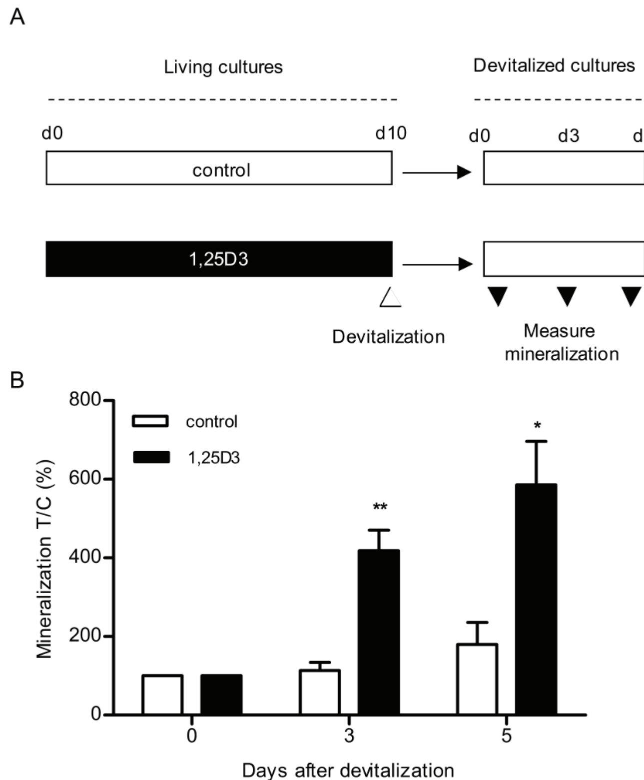

Figure 4. Effect of 1,25D3 pre-treatment on subsequent mineralization of formed ECM. A) culture and treatment scheme of SV-HFO cells. Cells were treated continuously with or without 1,25D3 and after 10 days of culture the cells were devitalized according to the protocol described in the Materials and Methods. B) Mineralization of ECM in devitalized cultures. Cultures were harvested at day 0, 3 and 5 and analyzed for calcium content. [a] p-value < 0.05; [b] p-value < 0.01 versus control condition.

A potential alternative mechanism to explain the effect of 1,25D3 on mineralization is regulation of MV production and/or MV maturation. MVs attached to the ECM were isolated at different time points after treatment of osteoblasts with vehicle or 1,25D3 in the pre-mineralization period. Electron microscopic and FACS analyses using size reference beads demonstrated that the isolates contained vesicles of the right size (**Figure 5A**, and data not shown) [19]. The TEM picture shows some heterogeneity in MVs which may reflect both ALPL-positive and MVs containing no or undetectable ALPL

(**Figure 5A**). With the progress of osteoblast differentiation the number of produced MVs increases in time. The total amount of MVs was not affected by 1,25D3 (**Figure 5A**). 1,25D3 significantly increased the number of ALPL positive MVs (**Figure 5B**) but did not change the ALPL signal per MV (**Figure 5C**). Quantitative analyses during osteoblast differentiation demonstrated that in the 1,25D3 condition at day 7 already a number of ALPL positive MVs was reached that was not yet reached at day 10 in the control condition (**Figure 5B**).

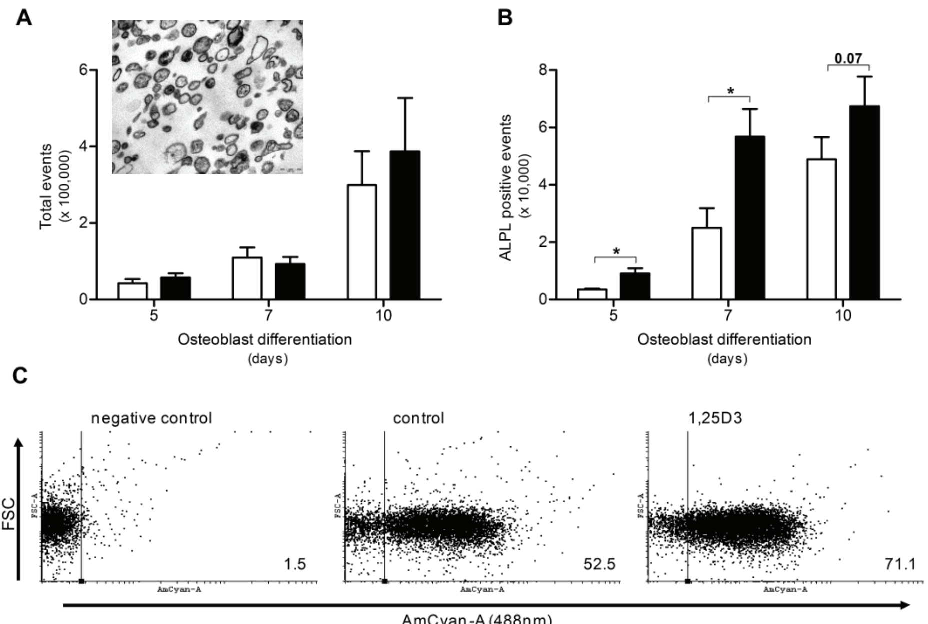

Figure 5. Effect of 1,25D3 on MVs. SV-HFO cells were treated with or without 1,25-D3. At days 5, 7 and 10 of differentiation, MV isolation and analyses were performed as described in the Materials and Methods. Open bars represent control and solid bars represent 1,25D3 condition. (A) Total number of MVs during the pre-mineralization period and an expemplified TEM picture of an MV isolate of control culture, day 5. Magnification: 44.000x. (B) Amount of ALPL positive MVs during the pre-mineralization period. Results were achieved by determining Elf-97 positive events in FACS analyses (C) An example of MVs FACS analyses of negative control (unstained MVs), control and 1,25D3 treated cultures, isolated on day 7 of culture. Numbers in the right corners of the pictures represent the percentage of ALPL positive MVs. [a] p-value < 0.05.

Discussion

In this study, we demonstrate that 1,25D3 accelerates the mineralization process through effects on human osteoblasts in the period preceding the onset of mineralization. This is the result of an accelerated maturation of the ECM and specifically via effects on MV production but not the ECM protein composition.

During osteoblast differentiation various functional phases can be identified including proliferation, ECM production and maturation and mineralization. In rodent osteoblasts, it was shown that short-term 1,25D3 treatment has different effects on the expression of various osteoblastic marker genes, depending on the period of treatment (proliferation, ECM maturation or mineralization) [28]. In the human osteoblast model that we used, the proliferation and ECM formation phases are identified as the pre-mineralization phase, which is supported by the gene profiling data showing an increase in expression of a broad range of ECM proteins (**Table 2**). It is in this pre-mineralization phase that 1,25D3 modulates osteoblast differentiation or function eventually leading to accelerated mineralization. In contrast, 1,25D3 treatment only during ongoing mineralization had no effect on mineralization. The importance of the pre-mineralization period for the 1,25D3 action is further demonstrated by the observation that living cells are not needed during the mineralization process to observe the acceleration by 1,25D3. Thereby our data are in general in line with previous data on rat osteoblasts that the effect of 1,25D3 depends on timing and phase of osteoblast differentiation [28].

The current data show the time dependency of the 1,25D3 effect on osteoblast with different effects prior to and during mineralization. This is supported by our findings that there is hardly any overlap (0.6%) between the 1,25D3 transcriptome of the pre-mineralization and mineralization period. The impact of 1,25D3 on gene transcription is about 6-fold stronger in the mineralization than in the pre-mineralization period with 470 and 74 genes being regulated, respectively. However, the current data show that the 74 genes are important for the 1,25D3 effect on mineralization as the acceleration is still observed in absence of living cells (and thus the 470 genes) during the mineralization period. Although not important for acceleration of mineralization it is interesting to understand the function of these 470 genes in osteoblast biology and bone metabolism. It is tempting to speculate that these genes and the processes they are driving are involved in the control of extent of bone formation by controlling cell survival, additional gene expression and potentially also in interaction (cross-talk) of osteoblasts with other cells in the bone micro-environment. GO analyses provided terms like cell cycle control and processes involved in transcription/translation but analyses to assess in detail these processes in osteoblast function and bone metabolism were beyond the scope of this study.

The importance of the pre-mineralization period for the regulation of mineralization is not unique for 1,25D3 as it has also been shown for activin A [15]. Moreover, it has been shown that treatment with FGF8 within the first three days of murine bone marrow cultures stimulates mineralization 14 days later [29]. In that study, treatment with FGF8 during the entire pre-mineralization period did not lead to increased bone nodule formation,

whereas continuous treatment even inhibited bone nodule formation. These observations pinpoint to the fact that timing of treatment is important for the process of osteoblast differentiation and that treatment at different time points during culture affects mineralization.

In contrast to FGF8, the effect of 1,25D3 was not dependent on a specific time-point during the pre-mineralization. It was the duration of treatment that determined the magnitude of accelerated mineralization rather than the time-point of treatment. This observation implicates that it is unlikely that 1,25D3 is stimulating a specific step in the differentiation of osteoblasts. If this would be the mechanism by which 1,25D3 accelerates mineralization, the precise timing of the effect would have been crucial. The current data about duration rather than timing suggests that 1,25D3 enhances one or more structural processes involved in the mineralization process by osteoblasts.

Two candidate processes to explain the 1,25D3 accelerated mineralization are the expression of ECM genes and/or the production of MVs. For the pre-mineralization effect of activin A on mineralization we have shown that it specifically regulates the expression of a broad range of ECM proteins [15]. However, 1,25D3 did not have any significant effects on the expression of a large list of ECM genes in the pre-mineralization period, thereby excluding a major involvement of ECM composition leading to accelerated mineralization.

Data on the effect of 1,25D3 on collagen type I are variable (van Driel et al., 2004). For example, we couldn't detect any effect of 1,25D3 on collagen type I expression in differentiating human osteoblasts while Maehata et al. have shown in MG63 osteosarcoma cells a stimulation by 1,25D3 (Maehata et al., 2006). Whether this is related to the osteosarcoma origin of MG63 is unclear but the basal collagen type I expression in these cells is already high suggesting an altered genomic promotor organization potentially making it also responsive to 1,25D3. Irrespective of this difference, the current study demonstrates that independent of changes in collagen type I expression 1,25D3 accelerates mineralization. An interesting observation by Maehata et al. was that via the collagen-mediated signaling ECM affects 1,25D3 responsiveness, which may be part of the long-term 1,25D3 effects in our osteoblast model.

Another possible explanation is the regulation of MV production by osteoblasts. The current concept is that these vesicles originate from the plasma membrane and bind to the ECM and initiate mineralization [20]. The biological importance of MVs is demonstrated in a broad range of pathological calcifications, *i.e.* pulmonary alveolar microlithiasis [30] and atherosclerosis [31]. Furthermore, patients with hypophosphatasia, a disease resulting in rickets and osteomalacia, lack the tissue non-specific isoenzyme of ALPL (TNSALP) in MVs [32]. MVs contain a large amount of ALPL (Ali et al., 1970; Majeska and Wuthier, 1975) and we have previously shown that in the pre-

mineralization period 1,25D3 enhances the expression of ALPL in osteoblastic cultures (van Driel et al., 2006). MVs can be affected in two ways, firstly, the production as assessed by the number of ALPL-positive MVs, and/or secondly, the maturation as assessed by the amount of ALPL per MV can be regulated. Our data show that the first way is the mechanism by which 1,25D3 acts. We demonstrated that in the pre-mineralization period 1,25D3 increases the number of ALPL-positive MVs. This is in line with effects of 1,25D3 on MVs from the MG63 osteosarcoma cell line [33] and on chondrocytic MVs [34-38].

In the ECM we observed an accumulation in time of both ALPL positive MVs and MVs containing low or non-detectable ALPL. Apparently osteoblasts produce both types of MVs and this heterogeneity in MVs can be seen from the TEM picture in **Figure 5A**. The function of these ALPL negative MVs is yet elusive but from our study it is clear that 1,25D3 shift the ratio towards ALPL-positive MVs and accelerates the increase in ALPL-positive MVs.

The main mechanism of initiating mineralization is supposed to constitute the formation of hydroxyapatite, arising from crystallization of inorganic phosphate (Pi) groups, which are provided by ALPL [39], and calcium ions [16]. There are several indications that ALPL is present in the ECM [40-42]. Therefore, we postulate that MVs translocate ALPL to the ECM where it is incorporated. We hypothesize that within the ECM a certain quantity threshold of incorporated ALPL has to be achieved to initiate mineralization, which is schematically depicted in **Figure 6**. Treatment with 1,25D3 reaches this threshold earlier through accelerated synthesis of ALPL positive MVs (**Figure 6A**). At the onset of mineralization, 1,25D3-treated cultures contain more ALPL within the ECM leading to a higher conversion of calcium ions and Pi into hydroxyapatite, eventually resulting in accelerated mineralization. Intermittent treatments of 1,25D3 accelerate mineralization to a lesser degree (**Figure 6B**; a = 7 days). This hypothesis is supported by the fact that a higher ratio of Pi over pyrophosphates (PPi), which is thought to be determined by ALPL levels [43], leads to increased mineralization. This new insight into MV functionality may shed light on the complex mechanism behind MV function.

In conclusion, we propose the concept that besides indirect effects via intestinal calcium uptake, 1,25D3 can directly accelerate osteoblast-mediated mineralization by stimulating the production of ALPL-positive MVs in the period prior to mineralization. More MVs lead to an earlier onset and higher rate of mineralization. These effects are independent of changes in extracellular matrix protein composition. These data on 1,25D3, mineralization, and MV biology add new insights into the role of 1,25D3 in bone metabolism and emphasize the importance of MVs in bone as well as maintaining bone health and strength by optimal mineralization status.

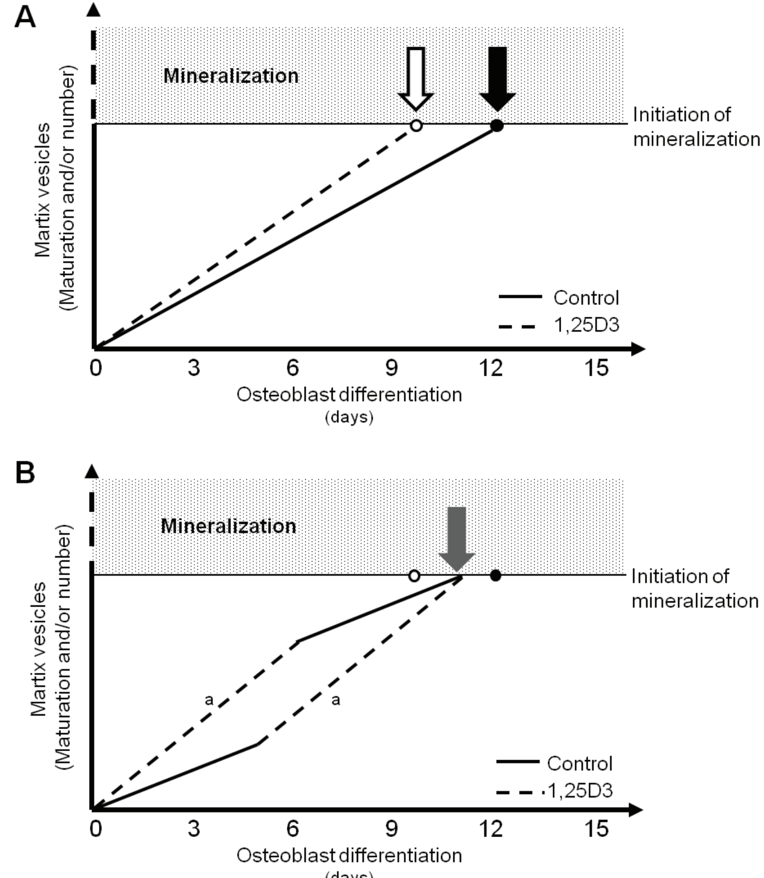

Figure 6. Diagram depicting the conclusion that length of treatment rather than starting point of treatment determines the MV-driven acceleration of mineralization by 1,25D3. A) Continuous treatment with 1,25D3 (dashed line) leads to an earlier onset of mineralization than in the control cultures (solid line). The time of mineralization in the 1,25D3 condition is indicated by the open arrow and open circle (○) and mineralization in the control condition by the solid arrow and solid circle (●). B) Illustrates the impact of two equally long (a = 7 days) intermittent treatments with 1,25D3 but starting at different days (day 0 and day 5, respectively) during the pre-mineralization period. The dashed lines indicate 1,25D3 treatment and the solid lines the control treatment. These treatments will result in the same acceleration of initiation of mineralization (indicated by patterened arrow) compared to the control condition (indicated by the solid circle (●), see also panel A) but later than in the continuous treatment with 1,25D3 (indicated by the open circle (○); see also panel A).

Acknowledgements

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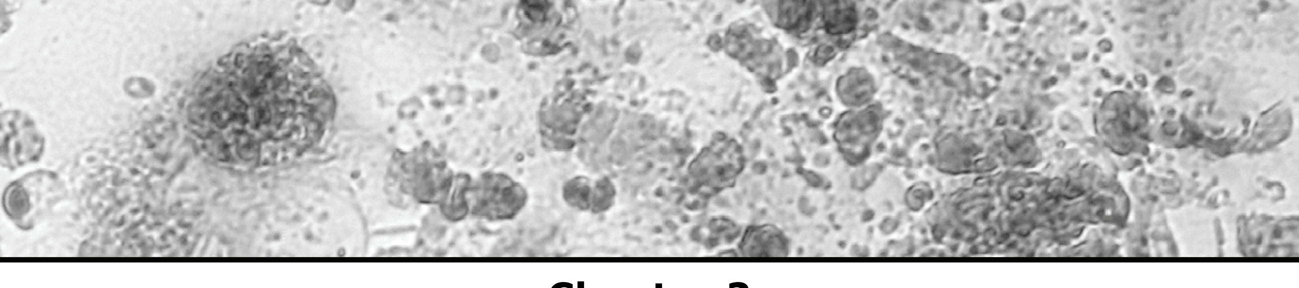

Chapter 2

IFNβ impairs extracellular matrix formation leading to inhibition of mineralization by effects in the early stage of human osteoblast differentiation

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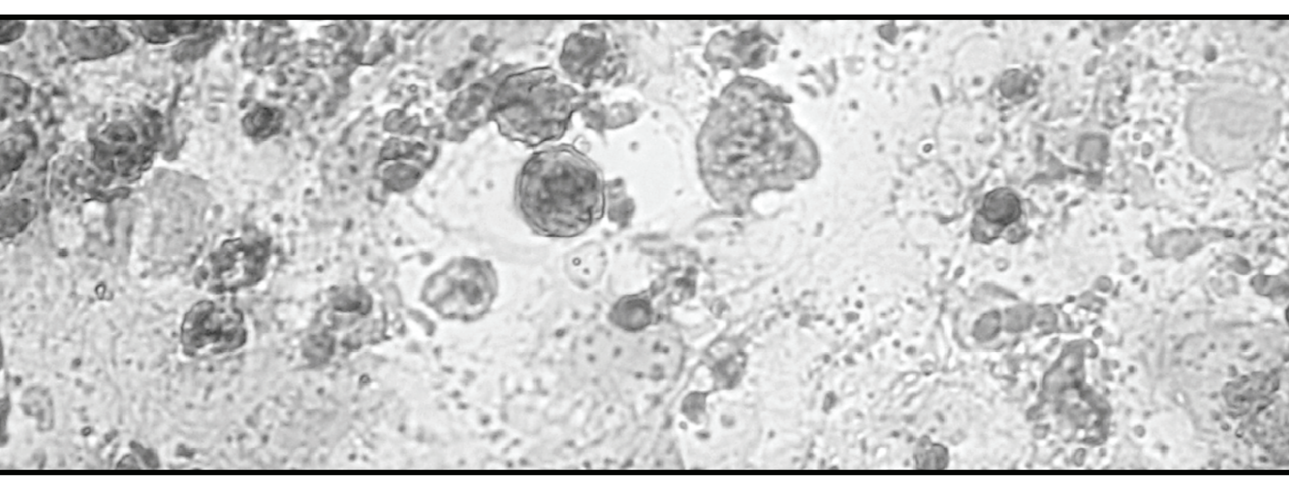

While analyzing gene chip profiles of human osteoblasts to identify mechanisms underlying the 1,25D3 stimulation of mineralization, we detected that a large set of interferon (IFN) genes was down-regulated by 1,25D3. At least 20% of all down-regulated genes could be connected to IFN signaling (Chapter 3). However, before we analyzed the effects of the IFN signaling in a 1,25D3 environment, we first focused on the effects of IFNβ on osteoblasts.

Abstract

Osteoimmunology is an emerging field of research focused on the interaction of the immune system and bone. In this study we demonstrate that human osteoblasts are sensitive to the immune cytokine interferon (IFN)β. Osteoblasts respond to IFNβ as shown by the induction of several known IFN target genes such as interferon-induced (IFI) proteins (IFIT1, IFI44L), interferon-stimulated gene factor 3 (ISGF3) complex and the induction of IFNβ itself. We demonstrated that IFNβ has severe inhibitory effects on mineralization of osteoblast-derived extracellular matrix (ECM). Analysis of the timing of the IFNβ effects revealed that committed osteoblasts in early stage of differentiation are most sensitive to IFNβ inhibition of mineralization. A single IFNβ treatment was as effective as multiple treatments. During the progress of differentiation osteoblasts become desensitized for IFNβ. This pinpoints to a complex pattern of IFNβ sensitivity in osteoblasts. Focusing on early osteoblasts, we showed that IFNβ decreased gene expression of ECM-related genes, such as type I Collagen (COL1A1), fibronectin (FN1), fibullin (FBLN1), fibrillin (FBN2) and laminin (LAMA1). Additionally, ECM produced by IFNβ-treated osteoblasts contained less collagen protein. IFNβ stimulated gene expression of osteopontin (OPN), annexin2 (ANXA2), and hyaluronan synthase 1 (HAS1), which are important factors in the adhesion of hematopoietic stem cells (HSC) in the HSC niche. In conclusion, IFNβ directly modifies human osteoblast function by inhibiting ECM synthesis eventually resulting in delayed bone formation and mineralization and induces a HSC niche supporting phenotype. These effects are highly dependent on timing of treatment in the early phase of osteoblast differentiation.

Introduction

Research on bone destruction that is associated with inflammatory diseases (i.e. rheumatoid arthritis) led to an association between the skeletal and immune system. The field of osteoimmunology is rapidly expanding and reveals numerous interplays between the immune system and bone cells [1]. Interferons (IFN) are important players in the immune system. There are different types of IFNs and their classification is based on the receptors they bind to. The classical and best studied IFNs are IFNα,-β, and -γ. IFNα and IFNβ are type I IFNs and bind to IFN-α/β receptor (IFNAR) 1 and IFNAR2. IFNγ is a type II IFN and binds to IFN-γ receptor (IFNGR) 1 and IFNGR2.

It has been shown that various IFNs inhibit osteoclastogenesis [2-5]. One mechanism of action is that RANKL induces IFNβ production in osteoclast precursors resulting in interference with the RANK/RANKL system, leading to inhibition of osteoclastogenesis and thereby introducing a negative feedback loop [4, 6-8].

IFNs modify differentiation of mesenchymal stem cell (MSC) and osteoblasts [9-10]. IFNβ stimulates mineralization in murine MC-3T3 osteoblastic cells [11] whereas IFNα decreases alkaline phosphatase (ALPL) activity in human osteoprogenitors [12]. Signal transducer and activator of transcription 1 (STAT1) is the main mediator of IFN signaling. In its unphosphorylated form STAT1 has been shown to bind the key driver of osteoblast differentiation, runt-related transcription factor 2 (RUNX2), leading to attenuation of its transcriptional activity and suppression of osteoblast differentiation [13-15].

An important function of osteoblasts is the production of an extracellular matrix (ECM) that eventually mineralizes. The major constituent of ECM is collagen I but it also contains other collagens [16-18] and numerous so-called non-collagenous proteins such as osteocalcin [19], osteopontin [20] and bone sialoprotein [21] that can influence mineralization. There are indications that type I IFNs can decrease collagen production in human osteoblastic cells [22] and it has been shown that osteocalcin is increased and osteopontin decreased in plasma of IFNβ-treated multiple sclerosis patients [23-24].

The aim of the current study was to extend these observations and to examine in detail the effect of IFNβ on the differentiation of and mineralization by human pre-osteoblasts. This is important from a clinical point of view as type I IFNs are widely used to treat several diseases such as multiple sclerosis and hepatitis C. In both conditions, patients are prone to acquire pathological bone phenotypes [25-28]. Combinatory medication of IFNα and Ribavirin (in the case of Hepatitis C) is under debate as it affects bone mineral density [27-28]. There are also indications that medications including IFNβ affect the RANK/RANKL system and may modulate bone phenotypes in multiple sclerosis patients [24]. Therefore, an understanding of type I IFN-mediated effects on bone is of great interest to improve medication and circumvent potential bone side effects.

Material and Methods

Cell culture

The human pre-osteoblast cell line SV-HFO [29] was cultured as described previously [30]. To induce osteoblast differentiation, medium (αMEM; Gibco BRL, Life Technologies) was supplemented with freshly added 10 mM β-glycerophosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich), with or without 100U/ml IFNβ (Cell Signalling), and replaced every 2 or 3 days. Cells were harvested at different time points during culture. Bone marrow-derived human mesenchymal stem cells (MSCs) were purchased from Lonza Group Ltd and cultured as described previously [31]. Osteogenic differentiation of the MSCs and treatment with IFNβ was identical to that of

SV-HFO cell cultures (see above), and medium was replaced every 3 or 4 days.

DNA, alkaline phosphatase activity, protein and mineralization assays
DNA, alkaline phosphatase (ALPL) and calcium measurements were performed as described previously [32]. Briefly, for DNA measurements cell lysates were incubated with heparin (8 IU/ml in PBS) and Ribonuclease A (50 µg/ml in PBS) for 30 minutes at 37°C. DNA was stained by adding ethidium bromide (25 µg/ml in PBS). Analyses were performed by using a Victor^2 plate reader (PerkinElmer Life and Analytical Science) with an extinction filter of 340 nm and an emission filter of 590 nm. ALPL activity is determined by an enzymatic reaction by which the ALPL-mediated conversion of PNPP to PNP for 10 minutes at 37°C is measured. For calcium measurements, cell lysates were incubated overnight with 0.24 M HCl at 4 °C. Calcium content was determined colorimetrically with a calcium assay kit (Sigma) according to the manufacturer's description. Calcium and ALPL results were adjusted for DNA content of the cell lysates. For protein measurement 200 µl of working reagent (50 volumes BCA™ reagent A and 1 volume BCA™ reagent B; Pierce) was added to 10 µl of sonicated cell lysate. The mixture was incubated for 30 minutes at 37°C, cooled down to room temperature and absorbance was measured, using a Victor^2 plate reader at 595 nm.

Quantification of mRNA expression
RNA isolation, cDNA synthesis and PCR reactions were performed as described previously [33-34]. Oligonucleotide primer pairs, all being either on exon boundaries or spanning at least one intron, were purchased from Sigma-Aldrich. Primer sequences are listed in Table 1. Gene names and symbols were used as provided by the HUGO Gene Nomenclature Committee [35].

Collagen staining and quantification
Collagen staining was performed, using Sirius Red staining. SV-HFO cell cultures were terminated at several time points during differentiation by removal of medium, washing with PBS (Gibco BRL., Life technologies) and fixed with 10% formalin (Merck) for 30 minutes at room temperature. Following another wash step with PBS, cells were stored at 4°C. For the staining procedure, cells were washed twice with tap water. 500 µl Sirius Red solution (0.1% Direct Red (Sigma-Aldrich) in saturated picric acid) was added to the wells for 90 minutes under shaking conditions at room temperature, followed by three washes with 0.01 M HCl. To dissolve the staining, 0.1M NaOH was added and incubated on a shaker for 60 minutes. 100 µl of the dissolved solution was transferred to a 96-well plate and measured at 532 nm in a Victor^2 plate reader (Perkin Elmer Life and Analytical Science). For

quantification, a standard curve was created, using 10 dilutions between 0 µg and 100 µg of bovine collagen solution (Stem Cell Technologies Inc.). The staining procedure was identical to the cells, but after each incubation or wash step, collagen had to be spun down for 5 minutes at 14,000 rpm to pellet the stained collagen.

Table 1: Sequences of primer sets used for qPCR in this study. Most genes were detected using SYBR green; ALP and COLA1 PCRs were performed with a specific probe (FAM-TAMRA).

Gene Symbol	Forward Primer	Reverse Primer	pmol/ reaction
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC	3.75
	Probe FAM-TAMRA: CGCCCAATACGACCAAATCCGTTGAC		3.75
IFIT1	TCCTTGGGTTCGTCTACAAATTG	TCAAAGTCAGCAGCCAGTCTCA	1.25
IFI44L	GGCAGAAGGAGCAGGACTGT	GGTTTACGGGAATTAAACTGATATCTGT	1.25
IFN β	CTAGCACTGGCTGGAATGAGACTA	CCAGGACTGTCTTCAGATGGTTT	1.25
IRF9	GCCCTACAAGGTGTATCAGTTGCT	TCGCTTTGATGGTACTTTCTGAGT	2.5
STAT1	GTCACCAAAAGAGGTCTCAA	AAACCTCGTCCACGGAATGA	5
STAT2	CCCCATCGACCCCTCATC	GAGTCTCACCAGCAGCCTTGT	2.5
ALP	GACCCTTGACCCCCACAAT	GCTCGTACTGCATGTCCCCT	3.75
	Probe FAM-TAMRA: TGGACTACCTATTGGGTCTCTTCGAGCCA		0.625
COL1A1	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC	1.25
	Probe FAM-TAMRA: CCGGTGTGACTCGTGCAGCCATC		3.75
COL4A5	TGGTTCTCCGGGTCCAGCTCT	CCTGGAGGACCTTCTGGACCTGGTA	2.5
COL5A3	CCTGCCTCTATCCCGACAAG	GCGTCCACGTAGGAGAACTTCT	2.5
FBLN1	GGAGACCGGAGATTTGGATGT	TCAGATATGGGTCCTCTTGTTCCT	2.5
FBLN5	AACCGAAGCTGCCAAGACAT	AGCCCCCTTGTAAATTGTAGCA	2.5
FBN2	AGCAGGCTCTGAAGGCGGGT	GTTGGGCCCTCGGAGCACG	2.5
FN1	ACCTTCACGTCTGTCACTTCCA	GAAAGTACACCTGTTGTCATTCAACA	2.5
LAMA1	GGACCCCAGTAACGCTGGGC	CAGGAAGTCGGGGGCCTGGT	5
PODN	CGCCTGACTTCCCGAGGGCT	GGAAGCGGGGTGCCAAGGTC	5
HAS1	CGGTGGACTACGTGCAGGTCTGT	TACCCGGGGGTCCTCGTCCA	2.5
HAS2	TTGGAAACTGCCCGCCACCG	GGAGAGAGACTCCAAAGAGTGTGGT	2.5
HAS3	GGAGATGTCCAGCCCCCAGGGAA	GAAACGTGGCGTTGGTGAACGGA	10
ICAM1	GACCGCAGAGGACGAGGGCA	GGGCGCCGGAAAGCTGTAGATG	5
VCAM1	CGCTGACCCTGAGCCCTGTG	GGAATGAGTAGAGCTCCACCTGGA	2.5
SDF1	CAGATTGTAGCCCGGCTGAAGAACA	TCCTCAGGCGTCTGACCCTCTCA	2.5
OPN	CTCAGGCCAGTTGCAGCC	CAAAAGCAAATCACTGCAATTCTC	12.5
	Probe FAM-TAMRA: AAACGCCGACCAAGGAAAACTCACTACC		5
ANXA2	ACTTCCGCAAGCTGATGGTT	GAGCATCTTGGTCAATCAGTTCAT	2.5

Illumina gene chip-based gene expression

Illumina HumanHT-12 v3 BeadChip (Illumina, Inc.) human whole-genome expression arrays were used. RNA integrity of isolated RNA was assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). The RNA of three biological replicates for each condition (control, 10^{-8}M 1,25D3, 100U/ml IFNβ and 10^{-8}M 1,25D3/100U/ml IFNβ at 2h and 24h) was analyzed. The Illumina TotalPrep RNA Amplification Kit (Ambion) was used for RNA amplification of each sample according to manufacturer's instructions. In short, T7 oligo(dT) primer was used to generate single stranded cDNA followed by a second strand synthesis to generate double-stranded cDNA. In vitro transcription was done to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA was column purified and checked for quality by RNA 6000 Nano assay. A total of 750 ng of cRNA was hybridized for each array using standard Illumina protocol with streptavidin-Cy3 (GE healthcare) being used for detection. Slides were scanned on an iScan and analyzed using BeadStudio (both from Illumina, Inc.).

Microarray analysis

Raw data was background subtracted using GenomeStudio v2010 (gene expression module 1.6), and processed using the Bioconductor R2.10.0 lumi-package [36]. Data was variance stabilization transformed and quantile normalized. Probes that were at least 5 times present in the experiments (detection p-value <0.01), were considered to be expressed and further analyzed. Differential expressed probes were identified using Bioconductor package 'limma', linear models and empirical Bayes methods for assessing differential expression in microarray experiments [37]. Selected Illumina IDs (genes regulated with a p-value of < 0.005) were analyzed for gene ontology (GO) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 hosted by the National Institute of Allergy and Infectious Diseases (NIAID), NIH and through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

Statistics

The data provided are based on at least three independent experiments derived from three independent cultures. Values are means ± SEM. Significance was calculated using the Student's t-test and p-values < 0.05 were considered significant.

Results

Using RT-PCR we found that the receptors IFNAR1 and IFNAR2 are abundantly expressed in SV-HFO, but expression was not affected by IFNβ treatment (data not shown). The IFNARs are functional as shown by IFNβ-induced mRNA expression of IFIT1, IFI44L 24 hours after start of

differentiation, which are IFN target genes involved in type I IFN signal transduction. In addition, IFNβ induced expression of all three components of the IFN-stimulated gene factor 3 (ISGF3) (STAT1, STAT2 and IFN regulatory factor 9 (IRF9)) as well as its own expression (**Figure 1**).

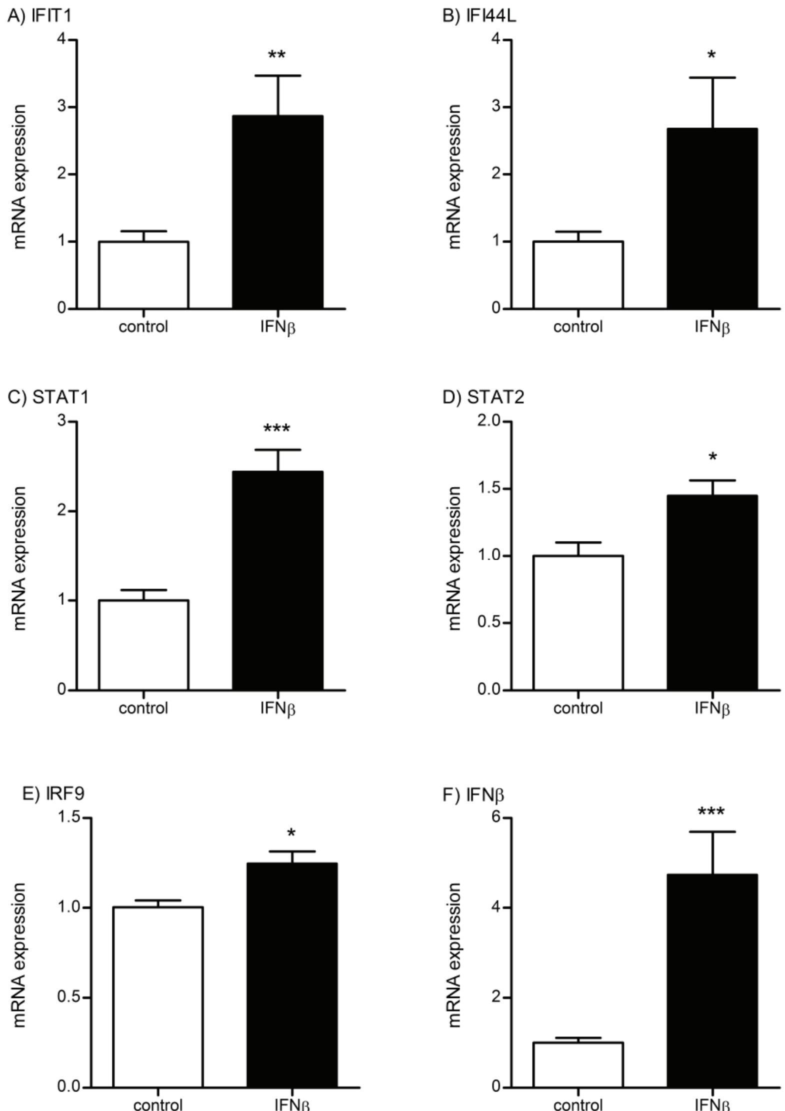

Figure 1. Effect of IFNβ on expression of IFN β target genes. SV-HFO cells were treated for 24 hours with 100 U/ml IFNβ at day 0 of culture. Expression of IFNβ target genes A) IFIT1 and B) IFI44L, C) STAT1, D) STAT2, E) IRF9 and F) IFNβ itself were determined by qPCR and expressed relative to GAPDH expression. Values are normalized to control = 1. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001 versus control.

Next we assessed whether IFN β treatment lead to phenotypic alterations in the osteoblast differentiation program and mineralization. Treatment with IFNβ up to day 14 of culture did not change DNA and protein content compared to control cultures (**Supplementary Figures 1A and 1B**). The activity of the differentiation marker ALPL was also not affected by IFNβ at several time points during differentiation (**Supplementary Figure 1C**). However, mineralization was significantly inhibited by IFNβ (**Figures 2A and 2B**). Similar effects were observed after treatment with the other type I IFN, IFNα (data not shown).

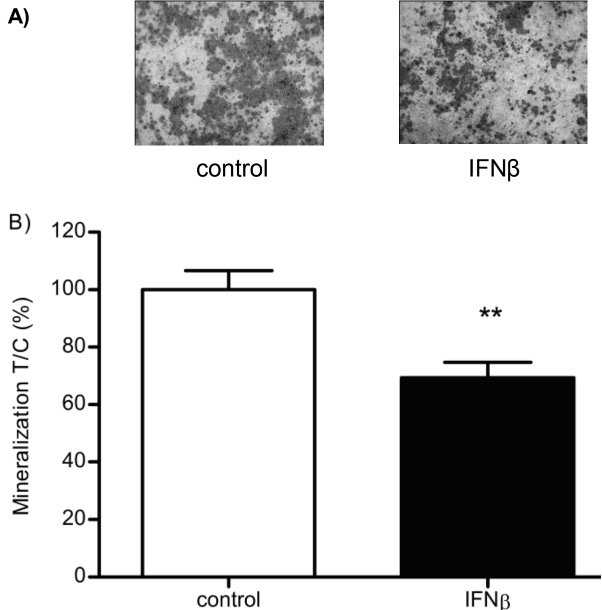

Figure 2. IFNβ effects on mineralization. A) SV-HFO cells, cultured in control or IFNβ-containing medium, stained for Calcium by Alizarin Red at day 14. B) SV-HFO cells were continuously cultured in control or IFNβ-containing medium until the onset of mineralization (day 14), harvested and mineralization was measured by determining the calcium content. Normalized to control = 100%. ** p-value < 0.01 versus control.

From earlier studies using the SV-HFO pre-osteoblasts [30, 34], we knew that timing and duration of treatment are important determinants for regulation of osteoblast differentiation. We assessed whether the effect of IFNβ on mineralization are stage and treatment-duration dependent. First, we investigated treatment periods starting at the onset of differentiation (day 0 of culture) ranging from 8 hours to 7 days treatment during the pre-mineralization period. Interestingly, 40-60% mineralization inhibition is achieved by all treatments regimens (**Figure 3A**). Treatments starting at later time points while differentiation is ongoing (for example starting at day 7 of culture) led to only 20-30% inhibition of mineralization (**Figure 3B**). These observations demonstrated that a single short-term treatment in the very early phase of pre-osteoblast differentiation is sufficient to reach maximal inhibition of mineralization.

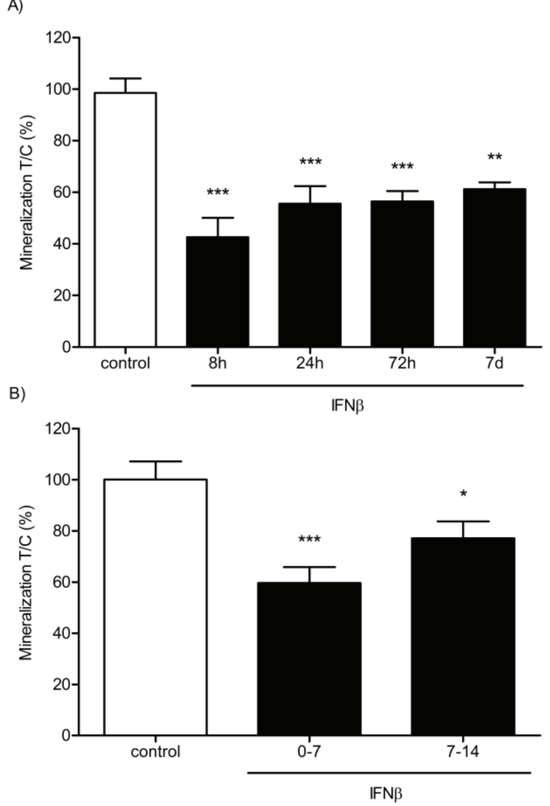

Figure 3. Time-course of IFNβ effect on mineralization. A) SV-HFO cells treated on day 0 on for 8 hours (8h), 24 hours (24h), 72 hours (72h), or 7 days (7d) with 100U/ml IFNβ. After IFNβ treatment for the indicated time periods was finished, the cells were cultured in control condition until the onset of mineralization (day 14). Data were normalized to control (= 100%). B) SV-HFO cells were cultured either in control medium, or in IFNβ containing medium from days 0-7 (0-7) or days 7-14 (7-14). At day 14 cultures were harvested and Calcium content was determined. Data were normalized to control (= 100%). * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001 versus control.

This suggests IFNβ desensitization either induced by IFNβ itself or due to a mechanism intrinsic to osteoblast differentiation. We next studied in more detail mRNA profiles of the 6 previously mentioned IFNβ target genes (IFIT1, IFI44L, STAT1, STAT2, IRF9 and IFNβ) during early pre-osteoblast differentiation. We investigated the impact of single and multiple IFNβ treatments on the expression of these genes. To this end, IFNβ was administered to the cells at days 0, 3 and 5 of culture and RNA was isolated 24 hours after each administration, i.e. at days 1 (= a single treatment), 4 (= two treatments) and 6 (=three treatments), respectively. As expected, after the first treatment with IFNβ the expression of all six genes was significantly induced (**Figures 4A-F** and **Figures 1A-F**). Interestingly, after the second IFNβ treatment none of the genes, except IFI44L, were induced (**Figure 4**). IFI44L was still induced but significantly less strong than after a single treatment (**Figure 4B**). After the third IFNβ treatment expression of IFIT1, IFI44L and IRF9 were significantly decreased (**Figures 4A, B and E**).

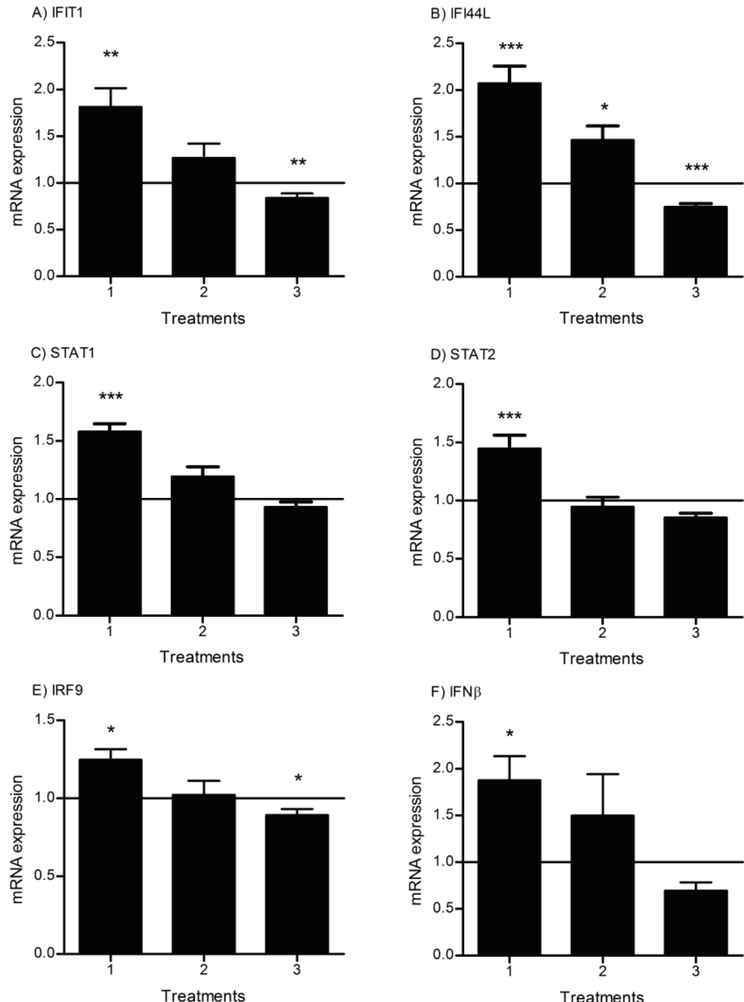

Figure 4. Osteoblast responsiveness towards multiple treatments of IFNβ. SV-HFO were treated 1x (day 0), 2x (day 3) or 3x (day 5) with IFNβ. 24h later RNA was isolated and RT-PCR is performed on A) IFIT1, B) IFI44L, C) STAT1, D) STAT2, E) IRF9 and F) IFNβ. Values are the IFNβ induced expressions (relative to GAPDH) stated in fold change. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001 versus control.

To assess an osteoblast differentiation intrinsic desensitization we investigated the response of osteoblasts to a single IFNβ treatment at various days during early differentiation. At days 0, 3 or 5, osteoblasts received a single IFNβ treatment for 24 hours. IFNβ treatment at the start of differentiation (day 0) induced IFIT1, IFI44L, STAT1, STAT2, IRF9 and IFNβ mRNA expression (**Figures 5A-F** and **Figures 1A-F**). A single IFNβ 24-hours treatment at day 3 of culture caused a similar induction of the 6 genes compared to treatment at day 0. However, with the exception of a small

increase in STAT1 mRNA expression, the osteoblasts did not respond to IFNβ when treated for 24 hours at day 5 of culture (**Figures 5A-F**). The expression of none of the STAT1 inhibitors (protein inhibitor of activated STAT1 (PIAS1), suppressor of cytokine signaling proteins 1 and 3 (SOCS1, SOCS3) and protein-tyrosine phosphatase, nonreceptor-type 2 and 11 (PTPN2, PTPN11)) did change during these first days of osteoblast differentiation (days 1, 4 and 6) or was affected by IFNβ treatment (data not shown).

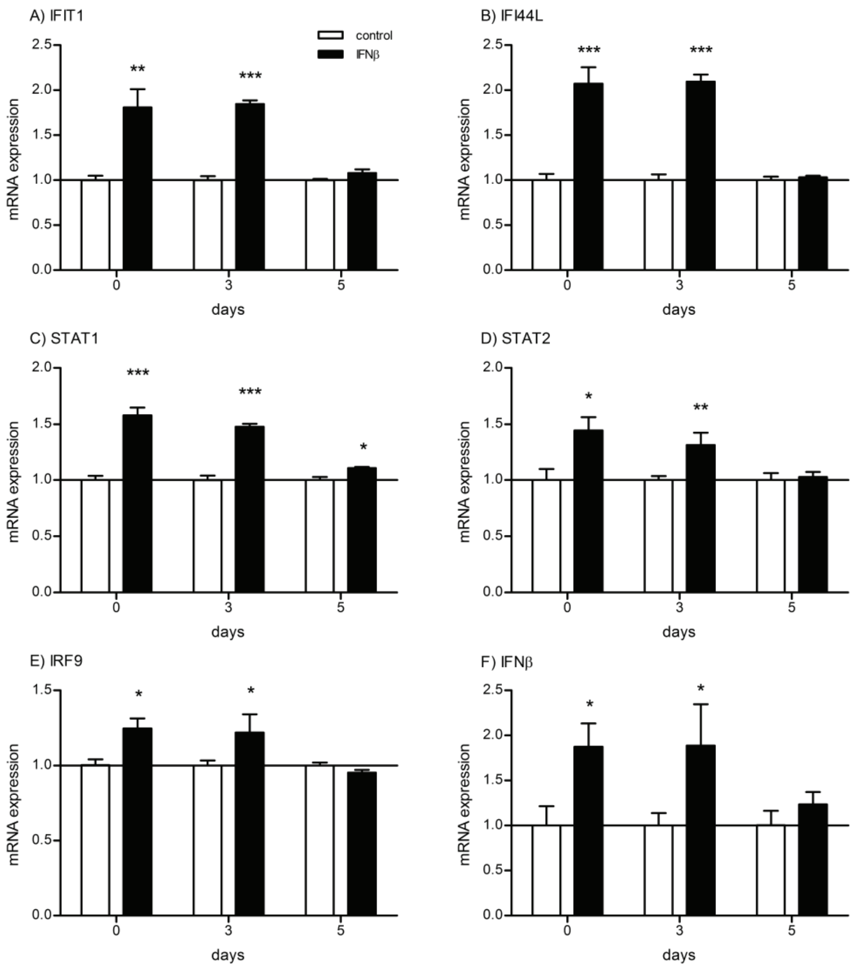

Figure 5. Osteoblast responsiveness towards a single treatment of IFNβ. SV-HFO cells were treated at days 0, 3 and 5 once with IFNβ (white bars: control; black bars: IFNβ). 24h later RNA was isolated and RT-PCR is performed on A) IFIT1, B) IFI44L, C) STAT1, D) STAT2, E) IRF9 and F) IFN β. Control condition was normalized to 1 and values are stated in fold change. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001 versus control.

To focus further on the impact of treatment window we investigated the effect of IFNβ on MSCs, the precursor of osteoblasts. Osteogenic induction of MSCs led to a 6-fold increase in the expression of the osteoblast differentiation marker ALPL within 48 hours providing evidence for osteoblastic commitment (**Figure 6A**). Treatment with IFNβ starting at day 0, prior to commitment to the osteoblast lineage, or starting after 48 hours osteogenic induction (committed MSCs) lead to different effects at the onset of mineralization. Treatment at day 0 simultaneous with the osteogenic induction had no effect on mineralization, whereas treatment starting after osteoblast commitment led to inhibition of mineralization as seen for the SV-HFO cells (**Figure 6B**). These data further emphasized the importance of IFNβ treatment timing for the subsequent consequences on mineralization.

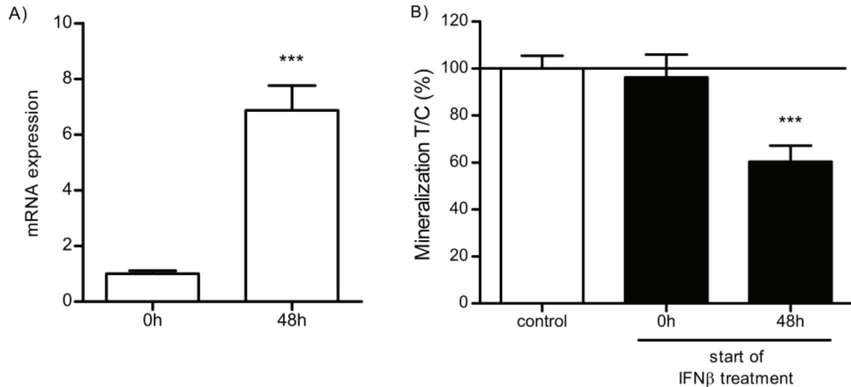

Figure 6. IFNβ inhibition of mineralization is dependent on the commitment of MSCs. A) ALPL expression of control MSCs was determined at time points 0h and 48h of the process of differentiation. B) The rate of mineralization was determined at the onset of mineralization (dependent on the donor between day 14 and 21). IFNβ treatment was started either at 0h or at 48h after differentiation was initiated. *** p-value < 0.001 versus control.

To address the mode of mineralization inhibition by IFNβ, we first focused on the production of ECM. Mineralization critically depends on the formation of ECM by osteoblasts. We assessed the effect of IFNβ on expression of various ECM genes after 24, 72 hours and 6 days treatment. No changes in gene expression were observed after 24-hours treatment with IFNβ (data not shown). However, at 72 hours the expression of various matrix genes is down-regulated (**Table 2**). This effect was temporary since after treatment for 6 days expression of these genes was not different from control anymore (**Table 2**).

Table 2: IFNβ effect on expression of ECM genes. SV-HFO cells were incubated with or without 100 U/ml IFNβ for 24h, 72h or 6 days starting at day 0 of culture. After these incubation periods cells were harvested and RNA was isolated. Expression values were corrected for GAPDH. Values are fold changes compared to control, n.d. is not determined.

Gene Symbol	Regulated by IFNβ (T/C) – 24h	p-value	Regulated by IFNβ (T/C) – 72h	p-value	Regulated by IFNβ (T/C) – 6 d	p-value
COL5A3	1.22 ± 0.14	0.441	0.59 ± 0.10	0.002	1.01 ± 0.05	0.944
FBN2	1.08 ± 0.16	0.690	0.62 ± 0.09	0.005	1.09 ± 0.03	0.210
COL1A1	1.24 ± 0.23	0.482	0.66 ± 0.12	0.049	0.98 ± 0.05	0.867
LAMA1	1.40 ± 0.15	0.087	0.66 ± 0.07	0.008	1.11 ± 0.06	0.181
FBLN1	1.05 ± 0.07	0.729	0.67 ± 0.10	0.014	n.d.	
FN1	1.15 ± 0.06	0.319	0.68 ± 0.09	0.008	n.d.	
PODN	1.33 ± 0.18	0.177	0.72 ± 0.05	0.001	1.08 ± 0.03	0.297
FBLN5	1.05 ± 0.06	0.689	0.74 ± 0.10	0.034	n.d.	
COL4A5	1.12 ± 0.15	0.536	0.75 ± 0.10	0.035	n.d.	

Finally, we assessed the impact of IFNβ treatment on total collagen production in the ECM by Sirius Red staining. After the first 3 days of culture we detected a 10% decrease in collagen staining in IFNβ-treated cultures (**Figure 7A**). This 10% deficit persisted during the entire differentiation period as the difference in collagen staining from day 3 onwards did not differ between control and IFNβ cultures (**Figure 7B**).

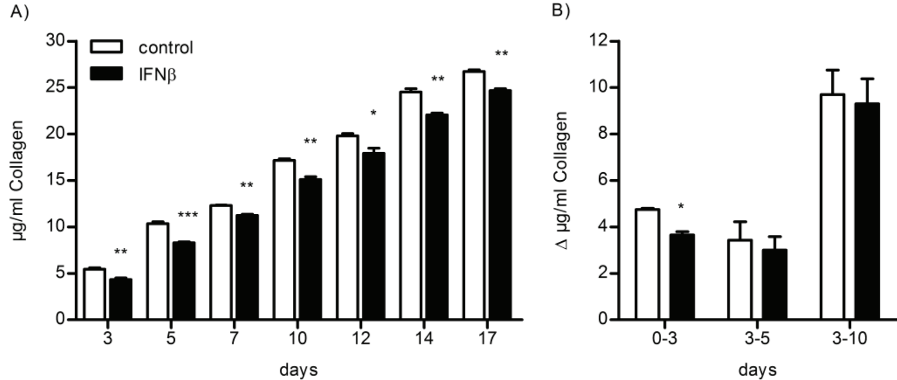

Figure 7. IFNβ inhibits Collagen production. SV-HFO cells were treated with or without 100 U/ml IFNβ for the first 72h of culture. Every 2-3 days cells were fixed and stained with Sirius Red which extraction allowed collagen quantification. White bars represent control cultures and black bars represent IFNβ treated cultures. A) Quantification of collagen at various time points during the progress of differentiation. B) Collagen produced by osteoblasts between day 0 to 3, day 3 to 5, day 3 to 10 and day 3 to 17. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001 versus control.

During the last years it has become evident that hematopoietic stem cells (HSC) attach to cells with an early osteoblastic character (N-cadherin positive, CD45 negative) [38] and that ECM-related genes, in particular OPN as suppressor of HSC activation, is playing an important role in niche formation [39-40]. At day 3 of culture, SV-HFO cells do not show gene expression of CD45 but express N-cadherin mRNA (data not shown) suggesting that early SV-HFO could possibly be involved in forming an HSC niche. Recently, it has been demonstrated that type-I-IFNs are capable to activate quiescent HSCs [41-42]. As demonstrated, IFNβ acted on early osteoblasts (**Figures 3 and 6**) and we were interested whether IFNβ influences early osteoblasts in such a way that the HSC niche may be affected. Therefore, we analyzed gene expression of various proteins shown to be involved in HSC adhesion (OPN, annexin A2 (ANXA2), hyaluronan synthase (HAS) 1, 2 and 3, Inter-Cellular-Adhesion Molecule 1 (ICAM1), Vascular-Cellular-Adhesion Molecule 1 (VCAM1) and stromal-cell-derived factor 1 (SDF1)) at day 1 of culture. IFNβ induced expression of OPN, HAS1 and ANXA2 after 24 hours. HAS3, SDF1, ICAM1 and VCAM1 were not significantly regulated (**Table 3**).

In summary, our data clearly demonstrated that IFNβ exclusively acts during early osteoblast differentiation to impair ECM production, which finally leads to decreased mineralization. Further studies have to be completed to elucidate the role of IFNβ in the osteoblast-HSC niche.

Table 3: IFNβ effect on mRNA expression of genes being involved in adhesion of HSCs to its niche. SV-HFO cells were incubated with or without 100 U/ml IFNβ for 24h starting at day 0 of culture. After these incubation periods cells were harvested and RNA was isolated. Expression values were corrected for GAPDH. Values are fold changes compared to control.

Gene Symbol	Regulated by IFNβ (T/C)	p-value
OPN	2.05 ± 0.46	0.044
HAS1	1.72 ± 0.18	0.016
HAS2	3.49 ± 1.37	0.092
HAS3	1.05 ± 0.11	0.845
ANXA2	1.23 ± 0.07	0.012
SDF1	0.93 ± 0.11	0.846
VCAM1	1.77 ± 0.48	0.142
ICAM1	1.06 ± 0.07	0.666

Discussion

In this study we clearly demonstrate that IFNβ directly influences human osteoblast function by affecting extracellular matrix synthesis eventually resulting in reduced mineralization. In the early phase of osteoblast differentiation IFNβ reduces the expression of various extracellular matrix proteins as well as total collagen protein content. Most interestingly, simultaneously IFNβ regulates the expression of genes known to be involved in the HSC stem cell niche [39-40, 43-44]. This positions IFNβ as an important regulator of the osteoblast phenotype and at the interface of osteoblasts and HSC and thereby control of development of immune cells.

In the microenvironment of osteoblasts IFNβ can be produced by immune cells or by osteoclasts [7]. Here we show that IFNβ is expressed by human osteoblasts and that expression is homologous up-regulated suggesting that autocrine/paracrine signaling occurs. Immune-related responses in osteoblasts have been described before, as they respond to various bacterial infections via toll-like receptors (TLR) and produce interleukin 6 (IL6) [45-46]. The current results show that osteoblasts typically respond by up-regulating interferon-induced-proteins (IFIT1 and IFI44L) as well as IRF9, STAT1 and STAT2 as part of the heterotrimeric signaling transducer ISGF3.

This is the first time showing type I IFN effects on osteoblast-mediated mineralization. The IFNβ concentration used did not affect cell growth or osteoblast differentiation as assessed by ALPL activity. The effect on mineralization is apparently established within the first hours after the initiation of differentiation, as an 8 hour IFNβ treatment is sufficient to reach the same degree of mineralization inhibition compared to a 14 day treatment. This immediate response is not surprising as IFNβ belongs to the innate immune system. IFNβ reacts to viral infections and its immediate signal transduction is of importance for survival of the system by triggering an immune response of uninfected neighbor cells (Reviewed by Biron et al. [47]). The mechanism by which IFNβ exerts this effect on mineralization is not fully understood yet but some aspects of it can be addressed. First, at a molecular level interaction between STAT and RUNX2 is likely. IFNβ increases the expression of classical IFN response genes including STAT1 within 24 hours. The importance of STAT1 in osteoblast differentiation has been shown by the fact that even independent of phosphorylation STAT1 can interact with RUNX2 and inhibit its nuclear translocation [13]. Secondly, at a cellular level IFNβ regulation of ECM protein expression and deposition is likely involved. We demonstrated that the expression of numerous ECM genes is down 72 hours after IFNβ treatment. This effect was not observed after 24 hours indicating that it is part of an indirect, second wave of effects, following the activation of the primary target genes and fitting the observation that only a short pulse of IFNβ is sufficient to inhibit mineralization. The effect on down-regulation of ECM genes is in line with studies showing a down-regulation of

collagens in fibroblasts and osteogenic cells by type-I and type-II IFNs [22, 48]. However, we found that the down-regulation was temporary since continuous IFNβ treatment did not affect ECM-associated genes at later time points. Quantification of collagen produced by control versus IFNβ-treated cultures supported the gene expression observations. Irrespective of SV-HFO cells being treated for the first 72 hours or continuously, IFNβ indeed only affected the collagen production of early osteoblasts since later during osteoblast differentiation no disturbances were observed.

These latter observations on temporal suppression of ECM genes and collagen production point to reduced sensitivity towards IFNβ during osteoblast differentiation. The observation that inhibition of mineralization was already maximal after a single treatment at the start of culture can also be linked to desensitization. We addressed this further by detailed analyses of timing and number of IFNβ treatments. After 5 days of differentiation, osteoblasts did not respond to a single first pulse of IFNβ anymore (**Figure 5**). Following a single pulse of IFNβ osteoblasts did not respond to a second pulse of IFNβ already after 3 days. These analyses demonstrate that IFNβ desensitization is an intrinsic process of osteoblast differentiation, which is accelerated by treatment with IFNβ. Different mechanisms have been described for desensitization to IFN. Following an IFN stimulus, dendritic cells modulate the expression of IFNARs to saturate signaling and alter sensitivity afterwards [49]. In our osteoblast model modulation of IFNAR1 and IFNAR2 mRNA is unlikely, since their gene expression was not regulated following IFNβ treatment but modifications at the protein level cannot be excluded. Desensitization can also be obtained by the ability of cells to dephosphorylate STAT1 and thus modifying the activity of the ISGF3 complex. It is postulated that desensitization of STAT1 is mediated by phosphatases [50]. In IFNβ-treated osteoblasts no alterations in gene expression of various phosphatases, PTPN2, PIAS1, SOCS1, SOCS3 and PTPN11, known to affect STAT1, were observed (data not shown). However, we cannot exclude that modifications of STAT1 phosphorylation occur. Nevertheless, it has been reported that the STAT1 inhibition of RUNX2 is independent of its phosphorylation [13] making it questionable whether changes in phosphorylation may contribute to the desensitization. Although the precise mechanism of desensitization during osteoblast differentiation remains to be elucidated, the current data demonstrate that an IFNβ pulse in the early phase of human osteoblast differentiation is sufficient to repress bone formation as assessed by ECM gene expression and collagen deposition as well as eventual decreased mineralization. The findings of negative effects of IFNβ on bone formation are of importance from a clinical point of view as multiple sclerosis (MS) and hepatitis C patients are often treated with IFNβ. Interestingly, in both diseases IFN-based medications are under debate for their effect on existing bone diseases (i.e. osteoporosis) [24, 27-28].

The results discussed so far were obtained with already committed pre-osteoblasts. As the IFNβ inhibition of mineralization is due to effects at the early stage of osteoblast differentiation we took this further and investigated human MSCs as precursors of early osteoblasts for their response to IFNβ. These analyses together with those of SV-HFO cells demonstrate that mineralization is only inhibited via effects of IFNβ on early committed osteoblasts. Treatment of MSCs before commitment to the osteoblast lineage did not result in inhibition of mineralization. Overall, these data demonstrate that human osteoblasts are only sensitive to IFNβ during a narrow time window of early differentiation.

Finally, our data suggest that IFNβ acts as a functional switch for osteoblast activity transforming it from a bone forming cell towards a cell with a stem cell niche function. It has been shown that the hematopoietic stem cell (HSC) niche is formed by cell-cell interactions of HSCs and early osteoblasts [38]. Upon type I IFN stimulation, HSCs start to proliferate and differentiate [41, 51]; changes within the niche are anticipated. Adhesion of HSCs to osteoblasts derived from annexin-2 (ANXA2)−deficient mice was significantly impaired compared to osteoblasts obtained from wild type animals [52]. Most interestingly, we found that IFNβ increased ANXA2 expression. The expression of SDF1, a key regulator of HSCs [53-54], was not regulated by IFNβ. However, it has been shown that SDF1 binds directly to ANXA2 [43]. IFNβ can therefore enhance the ANXA2-mediated presentation of SDF1 to HSCs and fortify HSC adhesion. Additionally, we showed that IFNβ increased in OPN expression and it has been shown that HSCs adhere to OPN. In OPN-deficient mice HSCs are randomly distributed within the bone marrow, suggesting that OPN is an important factor for attracting HSCs to its niche [39-40]. Another adhesion molecule is hyaluronan (HA) which is produced by HAS. In the absence of HA, 40% less HSCs were located in the endosteum suggesting an important role of HA within the lodgement of HSCs [44]. In our study, the expression of HAS1 was significantly increased by IFNβ and therefore increased HA production is likely, which will stimulate HSC lodgement. Overall, we were able to demonstrate that IFNβ modified HSC-niche related gene expression of early osteoblasts in such a way that adhesion of HSCs within the niche may be fortified [43-44]. Thus the observed gene expression suggests that IFNβ induces an osteoblast phenotype that supports HSC adhesion and lodgement. Further studies are needed to elucidate in detail type I IFN signaling as a switch in osteoblasts towards a HSC niche supporting function and thereby substantiating the link between osteoblasts and HSC.

Based on our current data and data from literature we propose the following concept to position IFNβ in the physiology of bone and bone marrow homeostasis. We put forward that IFNβ plays an important role in generating and/or maintaining the HSC stem cell niche at the bone surface. Firstly, in

osteoblasts, IFNβ delays the development of a bone forming phenotype and secondly, enhances the expression of genes enabling the osteoblasts to function as part of the stem cell niche. Thirdly, in order to prevent an extreme disturbance of bone formation/metabolism and as a consequence to a reduction in bone strength and potentially fractures, two processes are of importance. 1) To balance the reduction in bone formation, IFNβ also inhibits osteoclastogenesis [7], and 2) to prevent massive and complete block of bone formation osteoblasts are only sensitive to the bone forming inhibitory effect of IFNβ during a very short window of differentiation.

Overall, our data clearly demonstrated a role for IFNβ in osteoblast differentiation and activity involving impairment of ECM production in early stage of osteoblast differentiation eventually leading to reduced mineralization.

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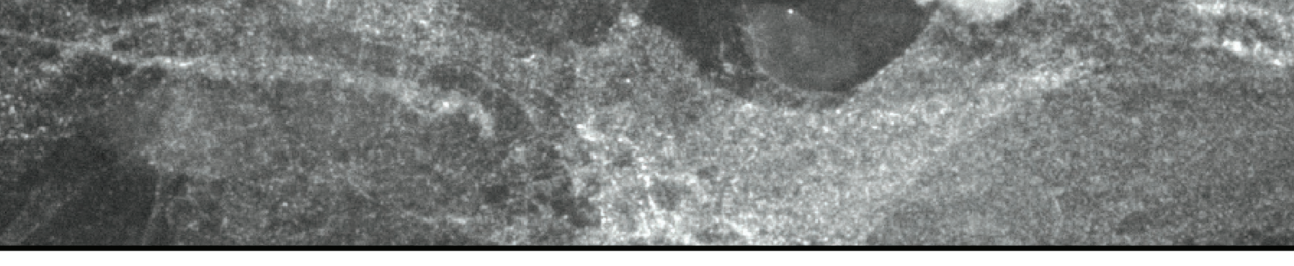

Chapter 3

Evidence of Vitamin D and interferon-β cross-talk in human osteoblasts with 1α,25-dihydroxyvitamin D_3 being dominant over interferon-β in stimulating mineralization

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Abstract

It is well established that 1α-25-dihydroxyvitamin D3 (1,25D3) regulates osteoblast function and stimulates mineralization by human osteoblasts. The aim of this study was to identify processes underlying the 1,25D3 effects on mineralization. We started with gene expression profiling analyses of differentiating human pre-osteoblast treated with 1,25D3. Bioinformatic analyses showed interferon-related and –regulated genes (ISG) to be overrepresented in the set of 1,25D3-regulated genes. 1,25D3 down-regulated ISGs predominantly during the pre-mineralization period. This pointed to an interaction between the vitamin D and IFN signaling cascades in the regulation of osteoblast function. Separately, 1,25D3 enhances while IFNβ inhibits mineralization. Treatment of human osteoblasts with 1,25D3 and IFNβ showed that 1,25D3 completely overrules the IFNβ inhibition of mineralization. This was supported by analyses of extracellular matrix gene expression, showing a dominant effect of 1,25D3 over the inhibitory effect of IFNβ. We identified processes shared by IFNβ- and 1,25D3-mediated signaling by performing gene expression profiling during early osteoblast differentiation. Bioinformatic analyses revealed that genes being correlated or anti-correlated with Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) were associated with osteoblast proliferation. In conclusion, the current study demonstrates a cross-talk between 1,25D3 and IFNβ in osteoblast differentiation and bone formation/mineralization. The interaction is complex and depends on the process but importantly, 1,25D3 stimulation of mineralization is dominant over the inhibitory effect of IFNβ. These observations are of potential clinical relevance considering the impact of the immune system on bone metabolism in conditions such as rheumatoid arthritis.

Introduction

Vitamin D is involved in calcium homeostasis, including effects on intestine, kidney, parathyroid gland and bone [1]. Its biologically most active form, 1α,25-dihydroxyvitamin D3 (1,25D3), is produced by vitamin D 25-hydroxylase (CYP2R1) and 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the liver and kidney, respectively [2].

Bone formation is indirectly influenced by 1,25D3 through the stimulation of calcium uptake in the intestine and re-absorption in the kidneys. Direct effects on bone formation have also been established: 1,25D3 stimulates mineralization of osteoblast cultures *in vitro* [3-4] and modifies gene expression of various osteoblast differentiation and mineralization-related genes such as alkaline phosphatase (ALPL), osteocalcin (BGLAP) and osteopontin (SPP1) [3, 5-6]. Recently, we have shown that 1,25D3 stimulates mineralization during the pre-mineralization period. We identified

a mechanism in which 1,25D3 directly stimulates mineralization by increasing the amount of mature matrix vesicles produced by human osteoblasts [7]. In MC3T3-E1 cells it has been shown that collagen modifying enzymes were strongly up-regulated by 1,25D3 within 72 hours [8]. Besides 1,25D3, oxygen tension and activin A also influence mineralization pre-dominantly within the pre-mineralization period [9-10]. Mineralization is a very complex process in which the pre-mineralization period is of great importance. Without tight and balanced control systems, excessive mineralization would occur. An example is marble bone disease. Patients suffer from deformation of bones, stunted bone growth and an increased risk of fractures due to reduced bone flexibility. Interestingly, 1,25D3 controls several mechanisms in osteoblasts to prevent over-activation and safe-guard excessive mineralization by 1,25D3. First, the classical process of induction of its degradation. 1,25D3 up-regulates 1α,25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1) which leads to fast inactivation and degradation of 1,25D3 [11]. Additionally, 1,25D3 is a potent regulator of osteocalcin [12] which has been reported to inhibit bone formation and mineralization [13]. However, still little is known about the mechanism of 1,25D3 regulation of human osteoblast differentiation and bone formation and mineralization. The aim of this study was to identify processes and mechanisms underlying the 1,25D3 effects on human osteoblasts and mineralization. We performed gene expression profiling analyses of 1,25D3 treated human osteoblasts against the backdrop of differentiation and mineralization in combination with functional analyses. These studies revealed a novel interaction between the seco-steroid hormone 1,25D3 and immune modulator IFNβ in osteoblasts, substantiating the emerging concept of the close interplay between bone metabolism and the immune system.

Material and Methods

Cell culture

The human pre-osteoblast cell line SV-HFO [14] and NHost cells were cultured as described previously [10]. To induce osteoblast differentiation leading to production of a mineralized extracellular matrix cells were cultured in αMEM (Gibco BRL, Life Technologies) supplemented with freshly added 10 mM β-glycerophosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich), which was, refreshed every 2 or 3 days. For Affymetix GeneChip expression analyses cells were treated with 1,25D3 from the start of culture and when medium was refreshed new 1,25D3 was added (= "continuous treatment"). Cells were treated with 250 U/ml IFNα (R&D Systems), 100 U/ml IFNβ (Cell Signalling), 5 ng/ml IFNγ (PeproTech) or 10^{-8}M 1,25D3 (LeoPharma). For combined treatments, 100 U/ml IFNβ and 10^{-8}M 1,25D3 were used.

Matrix vesicle isolation and analyses

Matrix vesicles were isolated and analyzed as described before [7]. Briefly, SV-HFO were cultured with or without 100 U/ml IFNβ for 5 days. Cultures were treated with collagenase/dispase (1mg/ml; Roche) to disrupt the extracellular collagenous matrix and next underwent a multi-step centrifugation procedure to obtain the matrix vesicles in the 100.000 g fraction. Isolated matrix vesicles were a) stained with ELF-97 (Invitrogen) and analyzed by flow cytometry (BD bioscience) and b) analysed for ALP activity.

Affymetrix Gene Chip-based gene expression

Long-term gene expression profiling was performed using GeneChip Human Genome U133 Plus 2.0 and as described previously [10]. Briefly, osteoblasts were treated with or without 10 nM 1,25D3 (added every medium refreshment) and harvested at multiple days during differentiation and mineralization. Next total RNA was isolated. Quality assessments of the arrays were performed by a RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). Several kits were then used for cDNA synthesis (Invitrogen) and subsequent purification, labelling and hybridization (Affymetrix). We used the Affymetrix "Human Genome U133 Plus 2.0 oligonucleotide GeneChips" platform.

Illumina Gene Chip-based gene expression

Short-term gene expression profiling was performed using Illumina HumanHT-12 v3 BeadChip (Illumina, Inc.) human whole-genome expression arrays. RNA integrity of isolated RNA was assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). The experiment is performed as described before [15]. For this experiment the following samples were used: RNA samples of three biological replicates for each condition, i.e. 2 hours or 24 hours treatments with vehicle, 10^{-8}M 1,25D3, 100 U/ml IFNβ, or 10^{-8}M 1,25D3 together with 100 U/ml IFNβ. The Illumina TotalPrep RNA Amplification Kit (Ambion) was used for RNA amplification of each sample according to manufacturer's instructions. In short, T7 oligo(dT) primer was used to generate single stranded cDNA followed by a second strand synthesis to generate double-stranded cDNA. In vitro transcription was done to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA was column purified and checked for quality by RNA 6000 Nano assay. A total of 750 ng of cRNA was hybridized for each array using standard Illumina protocol with streptavidin-Cy3 (GE healthcare) being used for detection. Slides were scanned on an iScan and analyzed using GenomeStudio v2010 (both from Illumina, Inc.).

Data analysis
Raw data was background subtracted using GenomeStudio v2010 (gene expression module 1.6), and processed using the Bioconductor R2.10.0 lumi-package [16]. Data was variance stabilization transformed and quantile normalized. Probes that were at least 5 times present in the experiments (detection p-value <0.01), were considered to be expressed and further analyzed. Differential expressed probes were identified using Bioconductor package 'limma', [17-18]. Selected Illumina IDs (genes correlated or anti-correlated with IFIT1 within a 5% interval) or Affymetrix IDs were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) and The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/).

DNA and mineralization assays
DNA and calcium measurements were performed as described previously [19]. Briefly, for DNA measurements cell lysates were incubated with heparin and Ribonuclease A for 30 minutes at 37°C. DNA was stained by adding ethidium bromide. For calcium measurements, cell lysates were incubated overnight with 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 in the cell lysates. DNA and calcium analyses were performed by using a Victor^2 plate reader (PerkinElmer Life and Analytical Science).

Quantification of mRNA expression
RNA isolation, cDNA synthesis and PCR reactions were performed as described previously [7, 20]. Oligonucleotide primer pairs, all being either on exon boundaries or spanning at least one intron, were purchased from Sigma-Aldrich. Gene expressions were corrected for GAPDH and normalized to 1. Primer sequences are listed in **Table 1**.

Gene nomenclature
Gene names and symbols were used as provided by the HUGO Gene Nomenclature Committee [21].

Statistics
The data provided are based on at least three independent experiments derived from at least 2 independent cultures. Values are means ± SEM. Significance was calculated using either the Student's t-test or for interactions the 2-way ANOVAs using GraphPad Prism 5.0. P-values < 0.05 were considered significant.

Table 1: Sequences of primer sets used for qPCR in this study. Most genes were detected using SYBR green; GAPDH, CYP24A1, CYP27B1, VDR and COL1A1 PCRs were performed with a specific probe (FAM-TAMRA).

Gene	Forward Primer	Reverse Primer	pmol/ reaction
GADPH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC	3.75
	Probe FAM-TAMRA: CGCCCAATACGACCAAATCCGTTGAC		3.75
IFIT1	TCCTTGGGTTCGTCTACAAATTG	TCAAAGTCAGCAGCCAGTCTCA	1.25
IFI44L	GGCAGAAGGAGCAGGACTGT	GGTTTACGGGAATTAAACTGATATCTGT	1.25
IFI6	GCTACCTGCTGCTCTTCACTTG	GCACTTTTTCTTACCTGCATCCTT	2.5
IFI27	CGGCCATTGCCAATGG	AGAGTCCAGTTGCTCCCAGTGA	2.5
OAS1	TGTGTGTCCAAGGTGGTAAAGG	CAACCAGGTCAGCGTCAGATC	5
OAS2	TCAGCGAGGCCAGTAATCTTG	TCAGCCATTGCCAGCATATTT	2.5
IFN	CTAGCACTGGCTGGAATGAGACTA	CCAGGACTGTCTTCAGATGGTTT	1.25
STAT1	GTCACCAAAAGAGGTCTCAA	AAACCTCGTCCACGGAATGA	5
CYP24A1	CAAACCGTGGAAGGCCTATC	AGTCTTCCCCTTCCAGGATCA	15
	Probe FAM-TAMRA: ACTACCGCAAAGAAGGCTACGGGCTG		7.5
CYP27B1	GGCAGAGTCTGAATTGCAAAT	CCGGGTCTTGGGTCTAACTG	10
	Probe FAM-TAMRA: CTTTGGCCCAGATCCTAACACATTTTGAGG		5
VDR	CCTCCAGTTCGTGTGAATGATG	TCATGTCTGAAGAGGTGATACAGTGAT	31.25
	Probe FAM-TAMRA: CCTTCCAGGCCCAACTCCAGACACA		5
COL1A1	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC	1.25
	Probe FAM-TAMRA: CCGGTGTGACTCGTGCAGCCATC		3.75
FBN2	AGCAGGCTCTGAAGGCGGGT	GTTGGGCCCTCGGAGCACG	2.5
LAMA1	GGACCCCAGTAACGCTGGGC	CAGGAAGTCGGGGGCCTGGT	5
PODN	CGCCTGACTTCCCGAGGGCT	GGAAGCGGGGTGCCAAGGTC	5

Results

We performed Affymetrix GeneChip analyses to identify genes and molecular networks in osteoblasts underlying the 1,25D3-enhanced mineralization. Previously we had shown that stimulation of mineralization by 1,25D3 is exerted via effects in the period prior to the actual mineralization. RNA was isolated at multiple time-points during human osteoblast (SV-HFO) differentiation prior to mineralization (days 3 and 7 of culture), at the onset of mineralization (day 12 of culture) and during full mineralization (day 19 of culture). Genes being at least 1.5 fold up- or down-regulated by 1,25D3 during the pre-mineralization period (days 3 and 7) were selected. 156 genes were selected of which 82 and 74 were up- and down-regulated, respectively. Bioinformatic analyses using DAVID of all regulated (both up- and down-regulated) genes revealed the GO-term 0009615 “response to virus” as the only biological process being significantly overrepresented with 9.8-fold enrichment (0.00077; Bonferroni multiple testing). When analyzing up- and down-regulated genes separately, no GO-term was significantly overrepresented in the list of up-regulated genes, while four GO-terms were significantly over-represented in the group with down-regulated genes. These

four GO-terms were “response to virus” (biological process), “centrosome” (cellular component), “microtubule organizing center” (cellular component) and “microtubule cytoskeleton” (cellular component). The last three GO-terms are based on the same 8 genes and therefore considered to represent the same cellular component. The GO-term 0009615 “response to virus” was 20.3-fold enriched (**Table 2**) and contained the following 9 interferon-related genes: DEAD Box polypeptide 58 (DDX58), myxovirus resistance 2 (MX2), radical S-adenosyl methionine domain containing 2 (RSAD2), interferon-induced protein 44 (IFI44), interferon induced with helicase C domain 1 (IFIH1), interferon, gamma-inducible protein 16 (IFI16), Signal Transducer and Activator of Transcription (STAT1), phospholipid scramblase 1 (PLSCR1), SAM domain and HD domain 1 (SAMHD1). Interestingly, besides the 9 genes annotated with “response to virus”, we identified at least 6 additional interferon-stimulated genes (ISG), that were down-regulated (**Table 3**): interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon-induced protein 44-like (IFI44L), 2',5'-oligoadenylate synthetase 1 and 2 (OAS1 and OAS2), interferon, alpha-inducible protein 27 (IFI27), and interferon alpha-inducible protein 6 (IFI6). This demonstrated that at least 20% of the down-regulated genes were related to IFNs.

Table 3: ISGs down-regulated by 1,25D3 at least 1.5 fold during the pre-mineralization period of osteoblasts, of which 9 genes are annotated with the GO-term “response to virus” (see **Table 2**).

Gene	regulation
DDX58	-1.90*
IFIT1	-1.86
IFI44L	-1.84
MX2	-1.80*
RSAD2	-1.78*
OAS1	-1.78
IFI44	-1.75*
OAS2	-1.67
IFIH1	-1.61*
IFI16	-1.61*
STAT1	-1.60*
IFI27	-1.58
PLSCR1	-1.55*
SAMHD1	-1.54*
IFI6	-1.51

* annotated to GO-term "response to virus"

Table 2: GO-term analyses of 74 genes being down-regulated by 1,25D3 during the pre-mineralization period of osteoblasts (days 3 and 7). Analyses were performed using DAVID 2008 (*david.abcc.ncifcrf.gov)*. Only GO-terms being significant ($p < 0.05$) after Bonferroni multiple testing are shown. The lower three GO-terms contain the same 8 genes.BP = Biological Process; CC = Cellular Component.

	GO-term	# of genes	fold enrichment	significance (Bonferroni)
BP	GO:0009615 - response to virus	9	20.3	< 0.0001
CC	GO:0005813 - centrosome	8	9.2	0.0021
CC	GO:0005815 - microtubule organizing center	8	8.2	0.0044
CC	GO:0015630 - microtubule cytoskeleton	10	4.8	0.017

Next we analyzed the expression of these 15 ISGs in more detail (**Figure 1**). All ISGs were consistently down-regulated during the pre-mineralization period (days 3 and 7, variation in change in expression of 17% and 22%, respectively). At day 12 of culture the down-regulation is less consistent across these 15 genes with a 42% variation in change of expression. At day 19 only a few genes were still down-regulated (**Figure 1**).

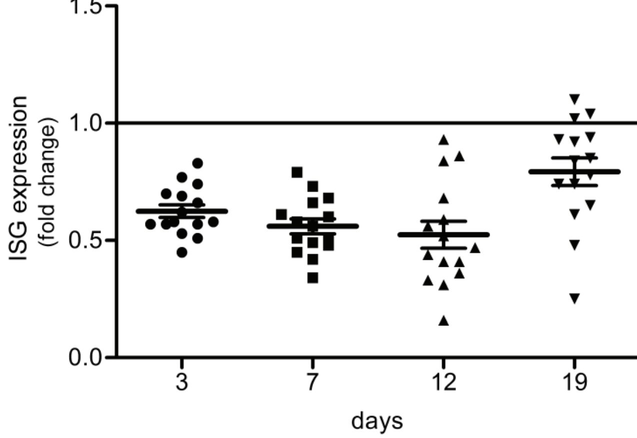


Figure 1. Expression profiles of ISGs in mineralizing human osteoblasts. In mineralizing osteoblasts, the regulation of ISGs by 1,25D3 was calculated as fold change compared to control at days 3, 7, 12 and 19, respectively. Black horizontal lines represent the average regulation of all ISGs included in that comparison.

We verified the expression of a set of ISGs (IFI27, IFI44L, IFI6, IFIT1, OAS1 and OAS2) by qPCR. All genes were down-regulated by 1,25D3 during the pre-mineralization stage (day 5) as well as during the onset of mineralization (day 12) confirming the GeneChip analyses, but were not regulated during mineralization (day 19) (**Figure 2**). qPCR analyses showed that 1,25D3 did not affect the expression of type-I (IFNα receptor 1 and 2) and type-II receptors (IFNγ receptor 1 and 2) as well as the genes coding for the various interferons, IFNA1, IFNB1 and IFNG. This also matched the Affymetrix GeneChip data (data not shown).

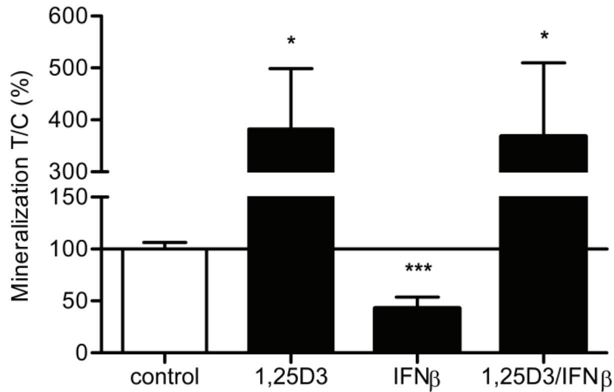


Figure 3. Mineralization of human osteoblasts upon co-treatment with IFNβ and 1,25D3. SV-HFO cells were treated continuously with or without 100 U/ml IFNβ, 10^{-8}M 1,25D3 or a combination of both. Mineralization was determined at day 14 of culture. Mineralization is depicted as percentage compared to control (set to 100%). * p-value < 0.05, *** p-value < 0.001.

Previously we have shown that 1,25D3 simulates [3, 7] while IFNβ inhibits mineralization [15]. Since 1,25D3 and IFNs have opposite effects on osteoblast mineralization and 1,25D3 down-regulates IFN target genes we further investigated a potential cross-talk. First, we tested the effect of the 1,25D3 and IFNβ combination on mineralization. As shown before, IFNβ inhibited mineralization while 1,25D3 stimulated mineralization (**Figure 3**). In the presence of 1,25D3 IFNβ was ineffective since mineralization was identical to that of 1,25D3 treatment alone. We recently published that 1,25D3 stimulated mineralization by increasing the amount of mature matrix vesicles during pre-mineralization phase [7]. IFNβ did not change basal as well as 1,25D3 stimulated matrix vesicle amount or maturation (data not shown). These data show that 1,25D3 is dominant over IFNβ with respect to osteoblast mineralization (**Figure 3**).

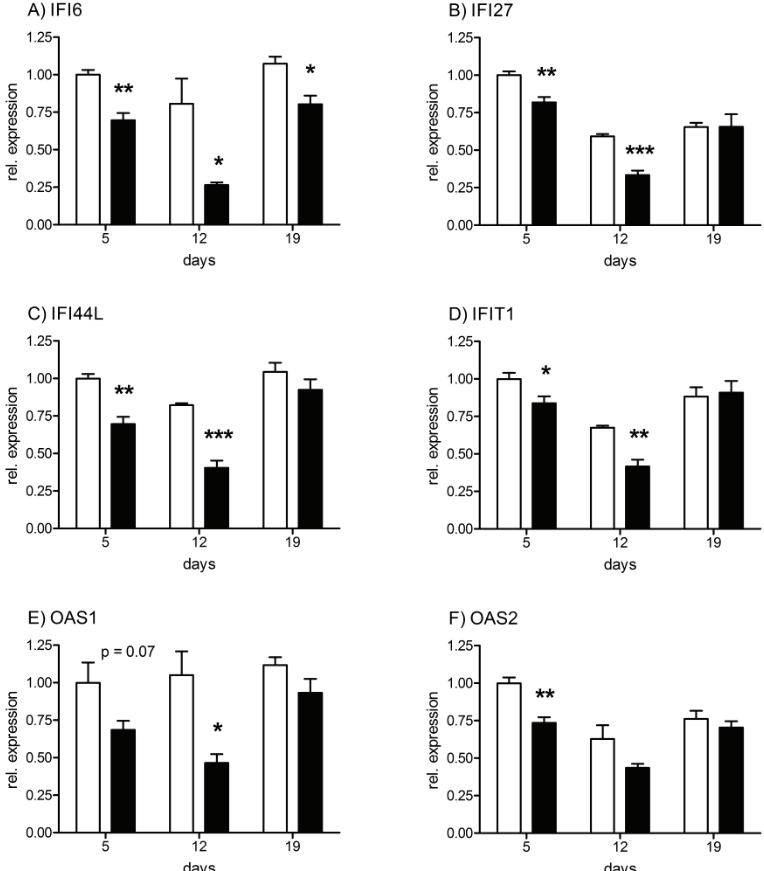


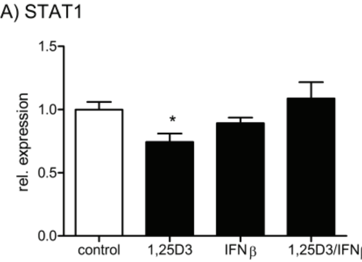
Figure 2. Expression analyses of ISGs in human osteoblasts treated with 1,25D3. SV-HFO cells were treated continuously with 10^{-8}M 1,25D3. Gene expression was determined at days 5, 12 and 19. Open bars represent control condition, black bars represent 1,25D3 condition. mRNA expression is corrected for GAPDH and normalized to control (set to 1). * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

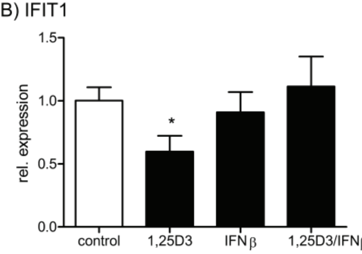
We demonstrated that IFNβ negatively affects mineralization within the first 72 hours of differentiation by inhibiting the expression of various extracellular matrix (ECM) genes [15]. Co-treatment analyses showed that 1,25D3 blocks the IFNβ inhibition of collagen, type I, alpha 1 (COL1A1), podocan (PODN), laminin alpha (LAMA1) and fibrillin 2 (FBN2) expression (**Table 4**).

Table 4: Regulation of the ECM-related genes COL1A1, FBN2, LAMA1 and PODN by 1,25D3, IFNβ and a combined treatment of both. SV-HFO were treated with 10nM 1,25D3 or 100 U/ml IFNβ or a combination of both for 72h. Osteoblasts were harvested at 72h, RNA isolated and RT-PCR performed. Values are GAPDH corrected, normalized to control = 1. * p-value < 0.05 (vs. control); ** p-value < 0.01 (vs. control) ; $^{\$}$ p-value < 0.05 (vs. IFNβ); $^{\$\$}$ p-value < 0.01 (vs. IFNβ).

	1,25D3	IFNβ	1,25D3 and IFNβ
COL1A1	1.54**	0.65 **	1.17 $^{\$\$}$
FBN2	1.15	0.72 *	1.19 $^{\$}$
LAMA1	1.23	0.79 $^{(0.05)}$	1.15 $^{\$}$
PODN	0.97	0.75 **	0.92

Since IFNβ inhibits mineralization via effects in the first 72 hours of osteoblast differentiation [15] we further analyzed the effects of 1,25D3 on ISGs in this period. At 24h 1,25D3 up-regulated 1,25D3 targets (BGLAP and CYP24A1) but did not influence ISGs (data not shown). STAT1, IFNβ and IFIT1 mRNA expression were significantly reduced by 1,25D3 at 72h. At this time point, the expression of these target genes was not changed anymore after treatment with IFNβ alone, but co-treatment prevented the 1,25D3 inhibition of STAT1 and IFIT1 expression but not IFNβ itself (**Figure 4A-C**). Focusing on 1,25D3-related genes, IFNβ alone but also in presence of 1,25D3 down-regulated CYP27B1 expression at 72 hours. At this time point IFNβ did not affect CYP24A1 and VDR expression (**Figure 5A-C**). From these data so far it is clear that 1,25D3 and IFNβ mutual affect each other's signaling cascades, and target gene expression.





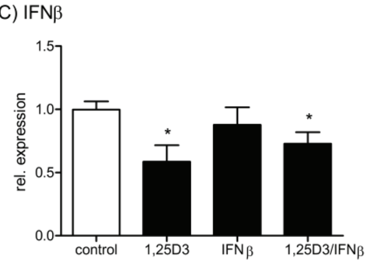
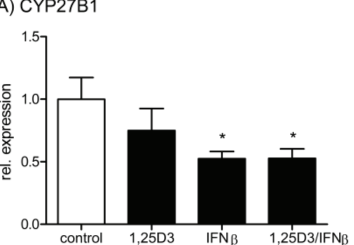
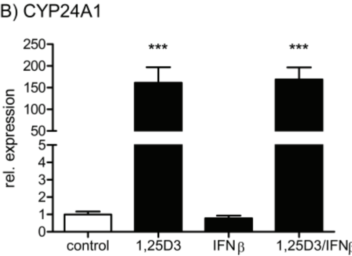


Figure 4. Expression analyses of ISGs in human osteoblasts. SV-HFO cells were treated continuously with or without 100 U/ml IFNβ, 10^{-8}M 1,25D3 or a combination of both. At day 12 of culture, RNA was isolated for expression analyses. mRNA expression is corrected for GAPDH and normalized to control (set to 1). * p-value < 0.05.





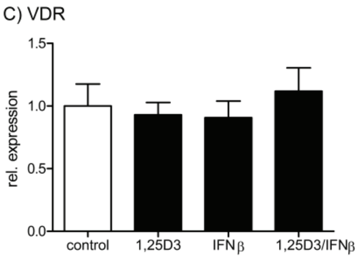
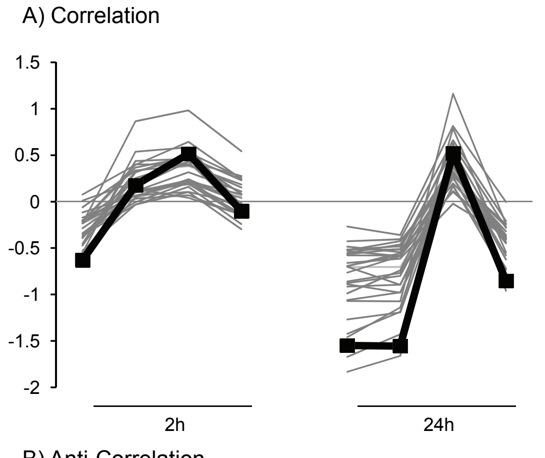


Figure 5. Expression analyses of 1,25D3 target genes in human osteoblasts. SV-HFO cells were treated continuously with or without 100 U/ml IFNβ, 10^{-8}M 1,25D3 or a combination of both. At day 12 of culture, RNA was isolated for expression analyses. mRNA expression is corrected for GAPDH and normalized to control (set to 1). * p-value < 0.05, *** p-value < 0.001.

To further analyze this cross-talk and find insights into the mechanism underlying these mutual effects of 1,25D3 and IFNβ we examined the effect of short-term (2 and 24 hours) vehicle, 1,25D3, IFNβ or 1,25D3 + IFNβ treatment on gene expression and in particular on these ISGs in early phase of osteoblast differentiation. IFIT1 is one of the strongest regulated genes by IFNβ and is widely used as IFNβ signaling read-out. We identified genes which are in a 5% interval (or 95%) correlated or anti-correlated with the expression profile of IFIT1 in all 8 conditions (i.e. the 4 treatments at two time points) (**Figure 6**). This identified 144 genes (117 correlated and 27

anti-correlated) (**Supplemental Table 1**). Repeating this correlation analysis with the IFNβ target genes IFI44L and DDX58 revealed similar genes being annotated in similar processes (data not shown). Analyses of those 144 genes with DAVID resulted in biological processes, which negatively regulate protein metabolic processes (GO-terms: GO:0032269, GO:0051248,). The average over-representation of those terms is 7.2 fold (<0.05, Bonferroni multiple testing). In the GO-term group of cellular component we found an overrepresentation of 2.2 fold with the GO-terms reflecting the organelle lumen (GO:0070013, GO:0043233, GO:0031974, GO:0031981).



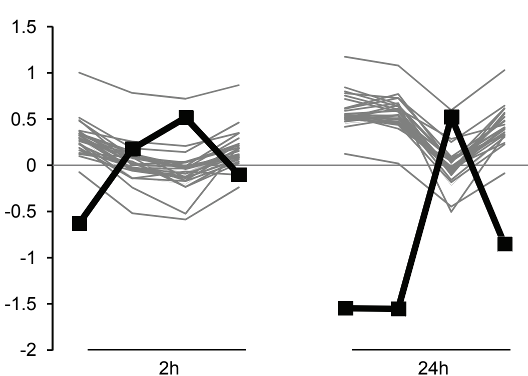


Figure 6. Pearson's correlation (A) and anti-correlation (B) of the expression profile of IFIT1. The black line shows the expression profile of IFIT1. The black markers represent from left to right the expression of IFIT1 in 1) control, 2) 1,25D3, 3) IFNβ, and 4) 1,25D3/IFNβ treatment condition at 2h and 24h. Grey lines represent in A) the top 30 genes correlated and in B) the 27 anti-correlating.

Analyses of the same set of genes within Ingenuity revealed that the top canonical pathways are "protein ubiquitination" and "activation of IRF and IFN signaling". Interestingly, the top physiological system development and function description was "Connective Tissue Development and Function" with various significant (p-value < 0.01) underlying processes such as "development of connective tissue cells", "osteoblast proliferation", "osteo-genesis of bone marrow stromal cells", "mineralization of bone marrow

stromal cells". The genes driving these processes are STAT1, fibroblast growth factor 2 (FGF2) and eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2).

Discussion

The current study provides evidence for interplay between 1,25D3 and interferon in regulation of human osteoblast activity and mineralization. IFNβ is already established as interaction partner of 1,25D3 in osteoclasts in which 1,25D3 induces IFNβ expression to control osteoclast differentiation and activity [22]. In osteoblasts, we clearly demonstrated that 1,25D3 prevents IFNβ-mediated inhibition of mineralization and expression of ECM genes. From previous studies we know that 1,25D3 stimulates mineralization via effects in the pre-mineralization period of osteoblast differentiation [7]. We identified the IFN system as potential interaction partner since during the pre-mineralization period 20% of all genes down-regulated by 1,25D3 were part of IFN signaling. IFNβ itself also acts in the pre-mineralization phase but in contrast to 1,25D3 it inhibits mineralization by down-regulating the gene expression of various ECM genes [15]. We tested whether IFNβ signaling could act as a control mechanism of 1,25D3-mediated stimulation of mineralization. Our data demonstrated that 1,25D3 signaling is dominant over IFNβ signaling in the control of mineralization and that the IFNβ inhibition of mineralization is completely overruled by 1,25D3. Additionally, we found that IFNβ decreased expression of ECM-related genes is completely prevented by 1,25D3.

From a mechanistic point of view it is of interest that ISGs are highly expressed at a basal level and that 1,25D3 was able to down-regulate their basal expression. This supports the idea of a basal IFNβ signaling being present in osteoblasts, which is supported by our observation of IFNβ expression by osteoblasts itself. It has been shown that basal IFNβ signaling maintains the expression of STAT1 [23]. Interestingly, STAT1-deficient mice show a strong increase in bone mass and it has been demonstrated that STAT1 is able to attenuate osteoblast differentiation via interactions with runt-related transcription factor 2 (RUNX2) [24-25]. 1,25D3 modifies STAT1 expression suggestive for a role of interaction between these transcription factors to explain the observed effects. In addition, it has been shown that vitamin-D-receptor (VDR) can interact with STAT1 resulting in modifications of transcriptional activities [26]. It is tempting to speculate about an interaction system between STAT1, VDR and RUNX2 in the 1,25D3 and IFNβ interplay controlling osteoblast differentiation and mineralization.

However, yet the precise mechanism that explains the combined effects on ECM protein gene expression and mineralization is unclear. On basis of our current observations it is unlikely that regulation of ISGs plays a prominent

role in the dominant 1,25D3 effect on mineralization. If ISGs would be the key-players of IFNβ-mediated inhibition of mineralization, down-regulation of these targets would lead to enhanced mineralization. This is indeed observed in the 1,25D3 alone condition when mineralization is paralleled by a decrease in ISG expression. However, in the co-treatment studies increased mineralization is paralleled by elevated expression of ISGs. Thus, albeit 1,25D3 and IFNβ affect the same processes and genes and mostly in an opposite manner, 1,25D3 is dominant in processes related to stimulation osteoblasts and bone formation and mineralization. Clues for processes underlying the IFNβ and 1,25D3 interplay in bone cell function and bone metabolism are derived from the gene expression analyses after combined treatment and the IFIT1 correlation analyses. Most interestingly, bioinformatic analyses annotated the selected genes to processes such as osteoblast proliferation and osteogenesis.

Like STAT1, EIF2AK2 was one of the genes correlated with IFIT1 expression and driving this association. EIF2AK2 is reported to interact with STAT1 and to increase STAT1 degradation [27-28]. Reduction of EIF2AK2 activity also reduced RUNX2 activity and murine osteoblast differentiation [29-30]. Considering this, it seems illogical that EIF2AK2 is up-regulated in human osteoblasts after IFNβ treatment which results in an inhibition of mineralization. Whether this is explained by species differences (i.e. murine versus human) is unclear but since EIF2AK2 is also involved in STAT1 degradation [30] and STAT1 is also up-regulated after IFNβ stimulation, one could speculate about a mechanism to control STAT1 effects on RUNX2-mediated transcription. In addition to STAT1 and EIF2AK2 a third gene was linked to osteoblast differentiation and correlated with IFIT1: FGF2. FGF2 disruption in mice leads to impaired bone formation [31], which is opposite to what is expected on basis of the IFNβ effect on osteoblast differentiation and mineralization. However, FGF2 inhibits mineralization by human dental pulp cells [32]and studies on human mesenchymal stem cells suggest that FGF2 may keep the cells in a more immature stage [33] that upon proper stimulation can be induced to differentiate [34]. Supporting for this is the role of FGF2 in maintaining stemness and the fact that miRNA profiling after induction of human osteoblast differentiation identified an increase in expression of miRNAs that putatively target FGF2 [35]. In addition, a link between FGF2 and RUNX2 activity or STAT1 levels cannot be excluded [31, 36]. Albeit not all interactions and mechanisms are yet explained, the current observations add to the complexity of networks regulating osteoblast differentiation and presence of feedback control systems.

Interestingly, IFNβ down-regulated CYP27B1, an enzyme which is necessary to synthesize 1,25D3, whereas the catabolic enzyme of 1,25D3, CYP24A1, is not regulated. The down-regulation of CYP27B1 is independent of the presence of 1,25D3 and one could speculate about the following local

mechanism in bone: IFNβ limits the concentrations of 1,25D3 in bone and therefore the extent of 1,25D3 mediated actions. This mechanism would also reduce 1,25D3-mediated actions on IFNβ signaling.

The interplay between IFNβ and 1,25D3 can be of significance in the broader context of bone metabolism. In addition, to our current observations in human osteoblasts there are data on IFNβ in the control of osteoclast formation a process also affected by 1,25D3 [37-39]. There are various studies suggesting interactions between osteoblasts and osteoclasts and the role of basal IFNβ therein [7, 22, 24, 40-43]. Data obtained in osteoclast studies show that IFNβ is playing a crucial role in osteoclast development. Receptor Activator of NF-κB Ligand (RANKL) stimulates IFNβ production in osteoclasts subsequently leading to an inhibition of osteoclastogenesis [43]. As we demonstrated that also osteoblasts express IFNβ and that IFN receptors are present, IFNβ can be part of the cross-talk between osteoclasts and osteoblasts with 1,25D3 controlling IFNβ action. Further studies are needed to precisely delineate this interplay between the seco-steroid hormone 1,25D3 and the immune modulator IFNβ in context of bone turnover. Important herein is also the relation between bone metabolism and the immune system and the impact of immune-related disorders on bone. Following this, combined therapy of IFNβ and Vitamin D is used to treat Multiple Sclerosis (MS) [44-46]. Vitamin D deficiency is a risk factor for MS [45, 47-48] and IFNβ has been shown to reduce various symptoms and disease activity [49]. MS is associated with low bone mass and a higher frequency of osteoporosis [50] and it is under debate whether IFNβ treatment is involved in those symptoms [51]. Focusing on this debate, we demonstrate that osteoblasts treated with IFNβ exhibit a decreased mineralization. Therefore, IFNβ could be involved in low bone mass or osteoporosis. But we also showed that osteoblasts simultaneously treated with 1,25D3 and IFNβ keep the stimulatory effect of 1,25D3 on mineralization. Therefore, one will appreciate that 1,25D3 prevents the deleterious effects of IFNβ on bone mineralization and a combinatory medication should protect MS patients from a debatable IFNβ-mediated low bone mass or osteoporosis. Alternatively, it warrants the significance of 1,25D3 sufficiency to maintain bone health in immune-related bone disorders.

Acknowledgements

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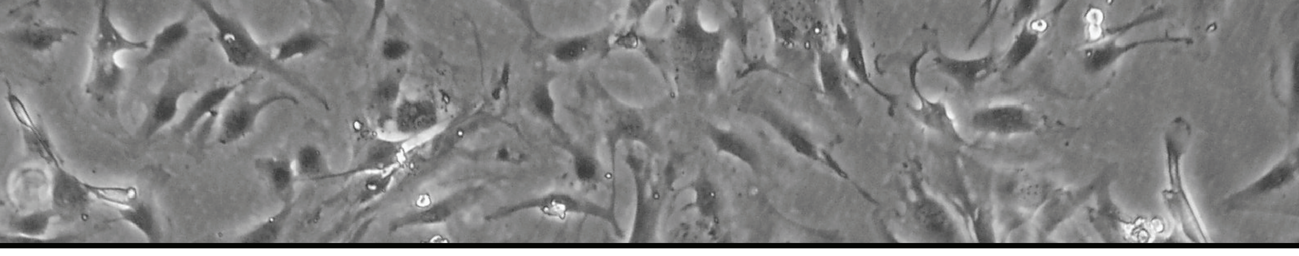

Chapter 4

1α,25-dihydroxyvitamin D_3 stimulates activin A production to fine-tune osteoblast-induced mineralization

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Submitted

Abstract

In healthy bones mineralization has to be tightly controlled to avoid pathological phenotypes. In this study we investigated interactions between 1α,25(OH)$_2$D$_3$ (1,25D3) and activin A in the regulation of osteoblast induced mineralization. In human osteoblast cultures we demonstrated that besides stimulation of mineralization, 1,25D3 also induced activin A, a strong inhibitor of mineralization. Simultaneously, follistatin (FST), the natural antagonist of activin A, was down-regulated by1,25D3. This resulted in an increase in activin activity during 1,25D3 treatment. We also showed that in 1,25D3-treated osteoblasts, mineralization can be further increased when activin A activity was abrogated by adding exogenous FST. This observation implies that, besides stimulation of mineralization, 1,25D3 also controls activin A-mediated inhibition of mineralization. Besides activin A, 1,25D3 also induces osteocalcin (BGLAP), another inhibitor of mineralization. Inactivating osteocalcin by blocking its carboxylation also increased 1,25D3-induced mineralization. Interaction between these two systems became evident from the synergistic increase in BGLAP expression upon blocking activin activity in 1,25D3-treated cultures. In conclusion, we demonstrate that 1,25D3 stimulation of mineralization by human osteoblasts is suppressed by concomitant induction of inhibitors of mineralization. Mineralization induction by 1,25D3 is tightly controlled via interplay with activin A and osteocalcin. Finally, this complex regulation of mineralization substantiates the significance of tight control of mineralization to prevent excessive mineralization and consequently reduction in bone quality and strength.

Introduction

1α, 25-dihydroxyvitamin D$_3$ (1,25D3) plays an important role in calcium homeostasis in humans. Defects in 1,25D3 synthesis or signaling lead to rickets in children or osteomalacia in adults, and are both characterized by, among other phenotypes, impaired bone mineralization [1]. 1,25D3 enhances mineralization of the extracellular matrix (ECM) of bone tissue that is produced by human osteoblasts [2]. Controlled bone mineralization is crucial to avoid the development of pathological over-mineralization such as in marble bone disease [3]. 1,25D3 is a strong stimulator of bone formation but various studies have shown that 1,25D3 induces inhibitory mechanisms to avoid excessive mineralization, e.g. the induction of osteopontin [4-7]. Another established mechanism that avoids excessive mineralization is the induction of osteocalcin (BGLAP), which is a known inhibitor of mineralization [8-13]. We recently published that 1,25D3 also interferes with the IFNβ system, which is a powerful inhibitor of mineralization as well [14].

Several members of the transforming growth factor β (TGFβ) superfamily, including TGFβ, modulate osteoblast differentiation and mineralization.

Depending on the stage of osteoblast differentiation, TGFβ either promotes or inhibits osteoblast development and mineralization [15-18]. TGFβ has been shown to interact with 1,25D3 to stimulate differentiation in osteoblasts and bone marrow stromal cells [19-21]. Another member of the TGFβ superfamily, Activin A, inhibits osteoblast-induced mineralization by modifying processes including ECM composition and maturation [22-23]. Although TGFβ and activin A bind to different cell surface receptors, they both activate SMAD2/3 signaling [24]. The effects of activin A are counteracted by follistatin (FST). FST binds activin A extracellularly and thereby prevents interaction of activin A with its receptors [25]. Both activin A and follistatin are produced by osteoblasts [23].

Although 1,25D3 and activin A have opposite effects on mineralization, they both exert their effects during the phase of ECM production and maturation (pre-mineralization period) in human osteoblasts. Additionally, Vitamin D Response Elements (VDRE) were identified within the promoter of genes encoding human activin A and the activin A receptor type 1B (ACVR1B) [26]. These findings prompted us to scrutinize a potential interplay between 1,25D3 and activin A to control osteoblast mineralization.

Material and Methods

Cell culture

The human pre-osteoblast cell line SV-HFO [27] was cultured as described previously [23]. To induce osteoblast differentiation leading to production of a mineralized extracellular matrix cells were cultured in αMEM (Gibco BRL, Life Technologies) supplemented with freshly added 10 mM β-glycerophosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich), which was refreshed every 2 or 3 days. Other used additives were 10^{-8}M $1\alpha,25(OH)_2D_3$ (LeoPharma), 25ng/ml Activin A (R&D Systems), 500ng/ml FST (PeproTech, Rocky Hill, NJ, USA) and 25 μM warfarin (Sigma Aldrich). Bone marrow-derived human mesenchymal stem cells (MSCs; Lonza) were cultured as described previously [28]. Osteogenic differentiation of the MSCs and treatment with 1,25D3 was identical to that of SV-HFO cell cultures (see above) but medium was replaced every 3 or 4 days. Osteoblast differentiation was divided in 3 functionally distinct periods: week 1, pre-mineralization (days 0-7); week 2, onset of mineralization (days 7-14) and week 3, full mineralization (day 14-21).

DNA, protein and mineralization assays

DNA, protein and calcium measurements were performed as described previously [29-30].

Quantification of mRNA expression

RNA isolation, cDNA synthesis and PCR reactions were performed as described previously [29, 31]. Oligonucleotide primer pairs, all being either on exon boundaries or spanning at least one intron, were purchased from Sigma-Aldrich (**Table 1**). Gene expressions were corrected for GAPDH and normalized to 1. Gene names and gene symbols were used as provided by the HUGO Gene Nomenclature Committee [32].

Table 1: Primer and probe sequences used for qPCR. CLEC3B and SMAD7 were detected using SYBR green; GAPDH, INHBA and BGLAP PCRs were performed with a specific FAM-TAMRA labeled probe.

Gene	Forward Primer	Reverse Primer	pmol/ reaction
GADPH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC	3.75
	Probe FAM-TAMRA: CGCCCAATACGACCAAATCCGTTGAC		3.75
INHBA	CCTCGGAGATCATCACGTTTG	GGCGGATGGTGACTTTGGT	3.0
	Probe FAM-TAMRA: CTGACAGGTCACTGCCTTCCTTGGAAATCT		2.0
CLEC3B	CACCACCGAGCCACCAA	GGTGTCCAGACGGCTCTTGA	2.5
SMAD7	TGTGAATCTTACGGGAAGATCAAC	ACTCTAGTTCGCAGAGTCGGCTAA	2.5
BGLAP	CAGGAGGGCAGCGAGGTA	TGGGGCTCCCAGCCA	25.0
	Probe FAM-TAMRA: TGATACAGGTAGCGCCTG		5.0

Quantification of activin A and follistatin

Quantification of human activin A and FST were performed as described previously [23]. Briefly, 48 hours after replacement, conditioned medium was collected at days 5, 12 and 19 of culture for activin A and FST measurements. Activin A was measured, using the activin A DuoSet ELISA kit (R&D Systems) and FST was determined using the FST quantikine ELISA kit (R&D Systems).

SMAD3 luciferase signaling (CAGA box reporter assay)

Activin signaling was measured using a HEK293F luciferase reporter cell line under control of a Smad3 sensitive $(CAGA)_{12}$ promoter element [33]. The $(CAGA)_{12}$MLP promoter was amplified out of the pGL3$(CAGA)_{12}$-lux vector using the following two primers: forward 5'-acgggaggtacttggagcggccgc-3' and reverse 5'-ggtggctttaccaacagtaccgg-3'. The PCR product was cloned into a lentiviral pLenti6 vector (Invitrogen) that contained a firefly luciferase reporter. Lentivirus production was performed as described earlier [34]. HEK293F (Invitrogen) cells were transduced and after transduction, cells were expanded for three passages under selection of 8 mg/mL blasticidin (Sigma Aldrich) resulting in a stable HEK293F-$(CAGA)_{12}$-luc cell line. For the luciferase reporter assay 22.000 HEK293F-$(CAGA)_{12}$-luc cells were seeded in a white semi-transparent 96-well plate (µClear white wells flat bottom, Greiner Bio-One) in 100 ml DMEM including 10% HI-FCS. The next

day, cells were stimulated with 100 ml conditioned medium derived from SV-HFO or MSC cultures. Conditioned medium (control or 10^{-8}M 1,25D3) was collected on day 5 of SV-HFO or MSC cultures. As positive control, 2ng/ml activin A (R&D Systems) and as negative control, medium from wells without cells was used. After 24 hours medium was removed and 25 µl/well lysis buffer was added for 20 minutes (Promega). Following addition of 25 µl/well Steady-Glo (Invitrogen) for 5 minutes, luminescence was measured for 6 seconds on the multichannel plate reader (Wallac Victor^2 1420 multilabel counter, PerkinElmer Life and Analytical Science).

Statistics

Data were presented only if multiple independent experiments showed similar results and each experiment consisted of at least 3 cultures. Values are the means ± SEM. For statistical analyses we used GraphPad Prism 5. Significance was calculated using the Student's *t* test and interaction was tested by 2-way-ANOVA, if applicable.

Results

In order to study the modulation of the activin A/FST system by 1,25D3 in relation to human osteoblast mineralization and differentiation, we used a human pre-osteoblasts cell line (SV-HFO) and human MSCs. In the SV-HFO model we measured Activin A and FST levels during the three phases of osteoblast differentiation (pre-mineralization, onset of mineralization and full mineralization) in the presence of 1,25D3. 1,25D3 significantly increased activin A production during pre-mineralization and onset of mineralization (**Figure 1A**).

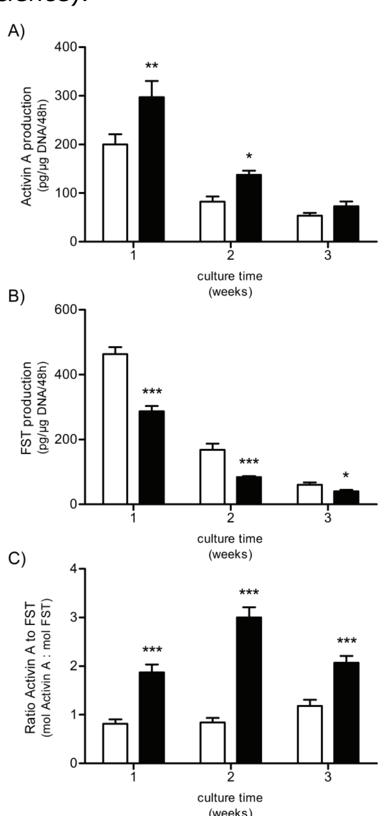


Figure 1. 1,25D3 induces activin A and reduces FST production in human pre-osteoblasts. SV-HFO cells were treated continuously either with vehicle (control; open bars) or 10-8 M 1,25D3 (black bars). In the 1st, 2nd and 3rd week medium was collected to determine secreted (A) activin A and (B) FST by ELISA. Production was corrected for DNA levels of the respective cell culture. C) Molar ratio between activin A and FST of control and 1,25D3 cultures in the 1st, 2nd and 3rd week . * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Activin A transcript (Inhibin beta A (INHBA)) levels corresponded to the activin A protein levels (data not shown). Additionally, 1,25D3 significantly decreased the production of the activin A antagonist FST at all time points during osteoblast differentiation (**Figure 1B**). Therefore, in 1,25D3 treated osteoblasts the ratio of activin A/FST shifted towards activin A (**Figure 1C**) suggesting 1,25D3-mediated stimulation of activin A signaling. We observed similar effects in osteogenic human MSCs, in which 1,25D3 treatment induced activin A production (**Figure 2A**) and inhibited FST production (**Figure 2B**) resulting in a shift towards increased activin A signaling (**Figure 2C**) during pre-mineralization.

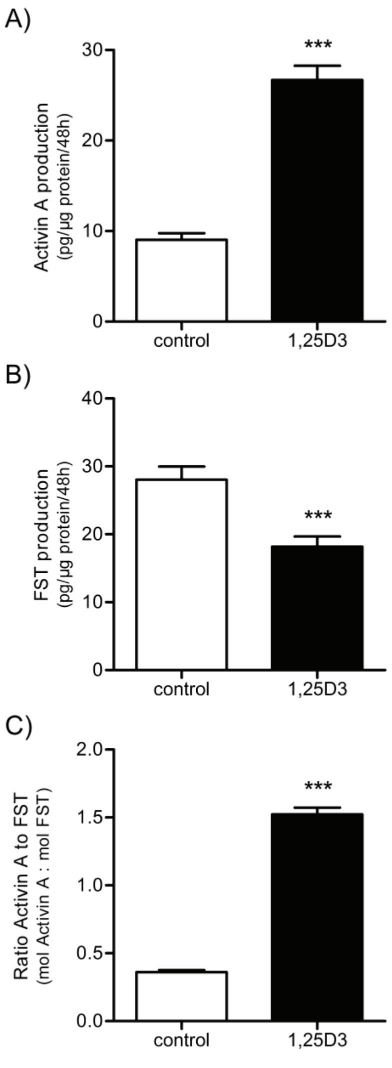


Figure 2. 1,25D3 induces activin A and reduces FST production in human MSCs. MSCs were treated continuously with vehicle (control; open bars) or 10^{-8} M 1,25D3 (black bars). In the 1st week of culture, medium was collected to determine secreted (A) activin A and (B) FST by ELISA. Production was corrected for protein levels of the respective cell culture. C) Molar ratio between activin A and FST of control and 1,25D3 cultures within the 1st week (day 5). *** p-value < 0.001.

Based on the 1,25D3-increased activin A/FST ratio, we expected elevated activin A signaling in 1,25D3-treated osteoblasts. We used two methods to investigate potential 1,25D3 changes of activin A signaling in osteoblasts. First, CAGA box reporter cells (indicative of SMAD3 signaling) were treated with conditioned medium of control and 1,25D3-treated osteoblasts. Conditioned medium of 1,25D3-treated osteoblasts (SV-HFO and osteogenic MSC) significantly enhanced SMAD3 signaling compared to controls (**Figures 3A and B**).

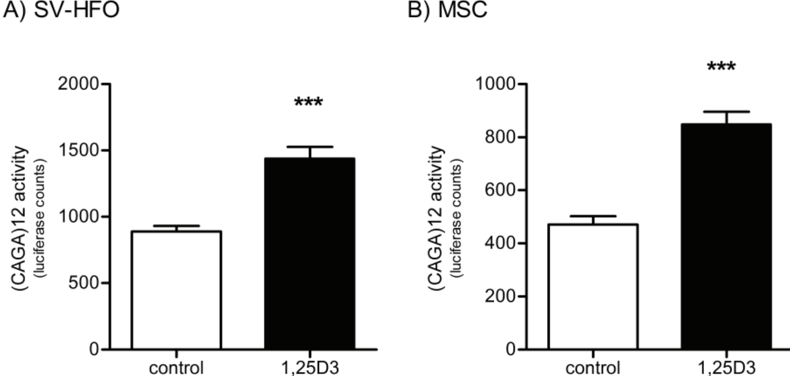


Figure 3. 1,25D3 induces activin A signaling in SV-HFO and MSC. We used a CAGA box luciferase reporter assay to determine the bioactivity of activin A. HEK293F CAGAluc cells were supplemented with conditioned medium from SV-HFO cells (A) or MSC cultures (B), which was collected on day 5 of control (white bars) and 10^{-8} M 1,25D3-treated (black bars) cultures. The bioactivity of endogenous activin A levels was determined 24h later by luciferase activity. *** p-value < 0.001. CPS = counts per second.

Secondly, we analyzed the expression of tetranectin (CLEC3B) and SMAD7, two genes that previously were identified as activin A targets being down- and up-regulated, respectively [23-24, 35]. 1,25D3 treatment of osteoblast resulted in down-regulation of CLEC3B and up-regulation of SMAD7 (**Figures 4A and B**).

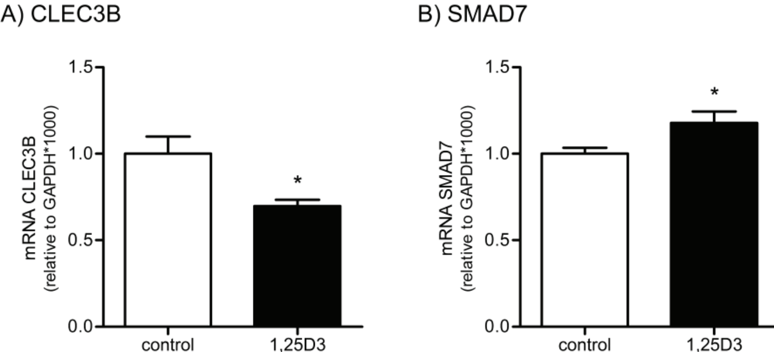


Figure 4. 1,25D3 regulates the expression of activin A target genes CLEC3B and SMAD7. At day 5 of control (open bars) and 10^{-8} M 1,25D3-treated (black bars) cultures, RNA was harvested and the gene expression of CLEC3B (A) and SMAD7 (B) was determined by qPCR (relative to GAPDH mRNA). * p-value < 0.05.

The data so far appear counterintuitive in that the stimulator of mineralization, 1,25D3, stimulates the production and signaling activity of an inhibitor of mineralization, activin A. This led us to hypothesize that activin A limits the stimulatory effect of 1,25D3 on osteoblast mineralization. To test

this we examined the impact of neutralizing activin A signaling by FST during 1,25D3-stimulated mineralization. The presence of FST significantly augmented 1,25D3-stimulated mineralization (**Figure 5A**). This indicates indeed that activin A limits 1,25D3-induced mineralization.

To take the concept further that 1,25D3 concomitantly stimulates mineralization and the synthesis of mineralization inhibitors, we examined BGLAP mRNA expression. BGLAP is strongly induced by 1,25D3 and can be γ-carboxylated involving the co-enzyme vitamin K. Moreover, it has been reported to inhibit *de novo* mineralization by delaying nucleation of hydroxyapatite [8, 10] and BGLAP deficient mice have a mineral maturation defect [11]. The negatively charged γ-carboxy-residues are important for the binding of calcium [36]. We examined the effect of the vitamin K inhibitor warfarin, which prevents γ-carboxylation [37] of BGLAP, on 1,25D3-stimulated mineralization. As shown in **Figure 5B**, warfarin treatment significantly increased 1,25D3-induced mineralization supporting the concept that 1,25D3 simultaneously stimulates mineralization and expression of a mineralization inhibitor.

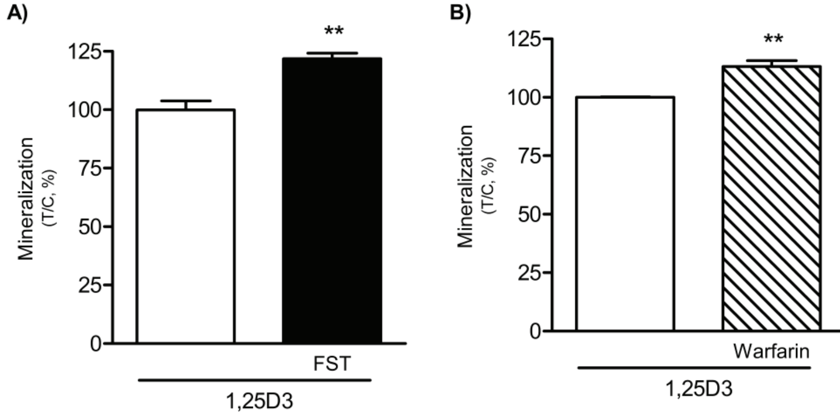
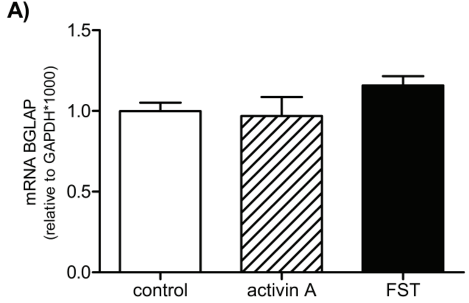


Figure 5. Co-treatment of 1,25D3 with FST or warfarin enhanced osteoblast mineralization. SV-HFO cells were either treated with 10^{-8} M 1,25D3 alone (white bars) and in combination with 500ng/ml FST (A; black bars) or in combination with 2.5^{-7} M warfarin (B; black bars). Mineralization was assessed during the 3rd week of differentiation. Values are corrected for DNA content and depicted in percentage treatment over control (T/C). ** p-value < 0.01.

Next, to assess the presence of a fine-tuning mechanism in the expression of these inhibitors of mineralization, we examined the effect of activin A on BGLAP expression. Neither activin A treatment of osteoblasts nor the inhibition of endogenous activins (i.e. activin A produced by osteoblasts (**Figure 1**)) by FST altered BGLAP expression (**Figure 6A**). However, 1,25D3-stimulated BGLAP expression is significantly inhibited by activin A and inhibition of endogenous activin A by FST further increased 1,25D3 stimulation of BGLAP expression (**Figure 6B**).



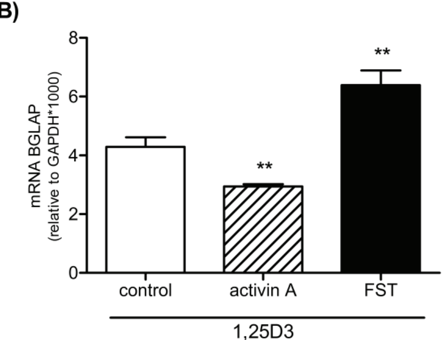


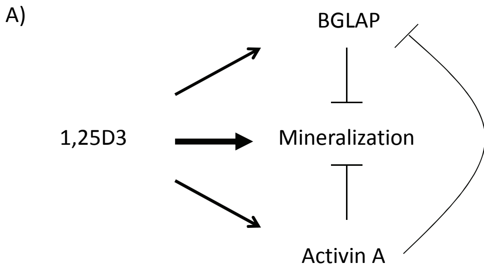
Figure 6. Activin A and FST affect BGLAP mRNA expression only in 1,25D3-treated cultures. SV-HFO cells were either treated with 25ng/ml activin A or 500 ng/ml FST (A) or the same treatments in the presence of 10^{-8} M 1,25D3 (B). At day 5 of culture, total RNA was isolated and BGLAP mRNA expression was determined (relative to GAPDH mRNA expression). Values are expressed as fold change versus control. ** p-value < 0.01.

Discussion

The current study extends our knowledge on the role of 1,25D3 in osteoblast function and bone metabolism and its interaction with locally produced factors such as activin A. This study demonstrates a tightly controlled regulation of 1,25D3-induced mineralization by co-induction of inhibitors of mineralization. Controlled mineral deposition into the ECM is crucial for optimal bone quality and strength. The importance of physiological 1,25D3 levels on bone is exemplified by mutations in the CYP27B1 gene. CYP27B1 catalyzes the hydroxylation of 25(OH)D3 to 1,25D3 and individuals with a mutation in CYP27B1 develop vitamin-D-dependent rickets [38]. It has been reported that in a mouse model for rickets a reduced extent of mineral deposition led to a greater extensibility and lower stiffness of fibrils [39], highlighting the importance of an optimal degree of mineralization for healthy bones. Indeed, besides bone-weakening pathologies due to limited mineralization, bones can also be over-mineralized. One example is marble bone disease, which is characterized by an increased fracture risk due to uncontrolled mineralization ultimately leading to decreased flexibility of bones [3].

We demonstrate that 1,25D3-induced stimulation of mineralization is accompanied by the parallel induction of activin A expression, a strong inhibitor of mineralization [23]. Furthermore, the inhibition of activin A by

FST led to a significantly stronger 1,25D3 stimulation of mineralization. These observations led to the model that activin A acts as control mechanism to prevent pathological over-mineralization by 1,25D3. We extended this by analyzing the BGLAP gene. Blocking γ-carboxylation of osteocalcin by warfarin led to a stronger 1,25D3-stimulated mineralization. These data demonstrate that 1,25D3 induces two inhibitors simultaneously with the stimulation of mineralization. Based on our findings, we came up with a model to explain the role of 1,25D3, activin A and BGLAP in the prevention of excessive osteoblast mineralization (**Figure 7**). We were able to show that in the presence of 1,25D3, activin A significantly inhibited BGLAP expression in osteoblasts (**Figure 7A**). In line with this, inhibition of activin A signaling by FST synergistically enhanced 1,25D3-induced BGLAP gene expression in osteoblasts (**Figure 7B**). This shows interaction between activin A and BGLAP to control 1,25D3-enhanced mineralization. We postulate that this interaction reflects an additional mechanism to safeguard the control of mineralization and this observation substantiates the significance of controlled mineralization.



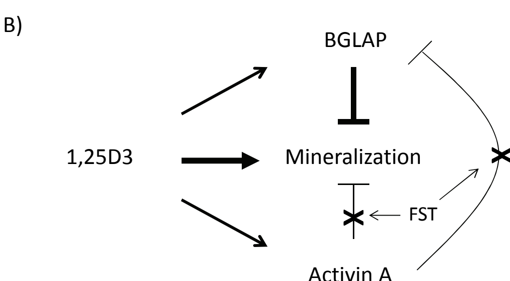


Figure 7. Model for 1,25D3 actions on inhibitors of mineralization during the pre-mineralization period. A) Besides stimulating mineralization, 1,25D3 induces BGLAP and activin A expression, both inhibitors of mineralization. Only in 1,25D3-treated cells activin A also inhibits BGLAP gene expression. B) Inhibition of endogenous activin A by FST in 1,25D3-treated osteoblasts diminishes activin A-mediated inhibition of BGLAP, leading to further up-regulation of BGLAP transcripts.

Several studies report on the interaction between 1,25D3 and the activin A/FST system [40-41]. In human and murine leukemic cells, 1,25D3 inhibits activin-A-induced erythroid differentiation [42-43], but it remains unclear whether these effects are directly through regulation of activin A signaling. A possible direct interaction between 1,25D3 and activin A is implicated by *in silico* analyses showing potential VDREs in the promoter of the human genes encoding activin A (INHBA) and the activin A receptor (ACVR1B) [26]. Albeit

that the functionality of those VDREs was not validated, the observation of 1,25D3-induced activin A production in human alveolar macrophages [44] and in human osteoblasts (this study) supports the presence of functional VDREs. Furthermore, we show that the shift in the activin A/FST ratio by 1,25D3 in human osteoblasts resulted in elevated activin signaling. This indicates a direct interplay between 1,25D3 and activin A during osteoblast differentiation. Activin A signals via SMAD2/3 phosphorylation which can be inhibited by the activin A target gene SMAD7 [45]. It has been shown that upon 1,25D3 stimulation, SMAD3 is able to interact with VDR leading to an enhanced transactivation of the BGLAP promoter [46-47]. Interestingly, co-expression with SMAD7 inhibits the SMAD3/VDR interaction leading to reduced BGLAP transactivation [48]. These findings suggest a close relationship between SMAD2/3 signaling and BGLAP transactivation, which could be supported by our observation that activin A signaling inhibits BGLAP gene expression. Whether these SMADs play a role within this inhibitory interaction needs to be elucidated in future studies.

Our data provide evidence for tightly-controlled 1,25D3 effects to avoid pathological over-mineralization. In a broader perspective this is not only of significance for bone but also other tissues like the vascular system that can calcify under certain triggers. Since in these tissues calcification leads to pathologies, various mechanisms are in place to avoid the initiation of this process. Interestingly, 1,25D3 is linked to vascular calcifications and activin A has been shown to inhibit vascular smooth muscle cell calcification [23, 49]. In addition, matrix gla protein, a γ-carboxylated protein just like BGLAP, has been reported to inhibit vascular calcification [50-51].

In conclusion, our data demonstrate that 1,25D3 simultaneously with stimulating mineralization induces the expression of the inhibitor of mineralization activin A. Additionally activin A attenuates 1,25D3-induced BGLAP expression. This implies that in case of reduced activin A levels, which are not sufficient to limit 1,25D3-stimulated mineralization, the expression of another inhibitor, BGLAP, increases. This would shift the 1,25D3-induced inhibition of mineralization from activin A towards osteocalcin. The advantage of this seemingly redundant process is that multiple interactive mechanisms allow for fine-tuning of osteoblast-induced mineralization and that in case of deficiency, compensation by other factors is possible and excessive mineralization is prevented.

Acknowledgements

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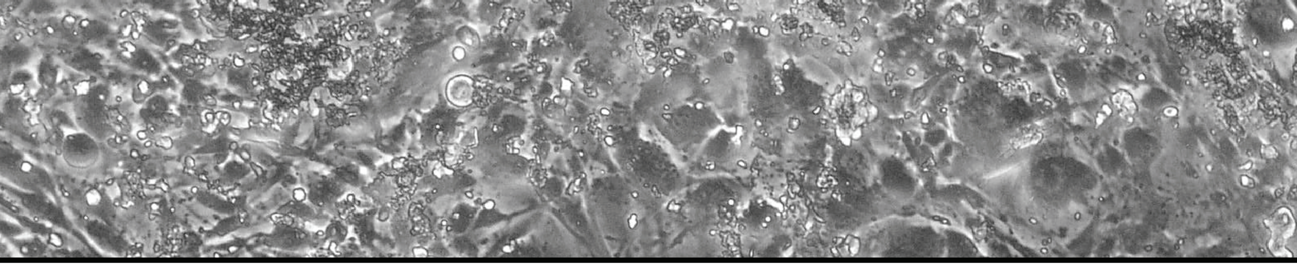

Chapter 5

1α,25-dihydroxyvitamin D_3 and rosiglitazone synergistically enhance osteoblast-mediated mineralization

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Submitted

Abstract

Both vitamin D receptor (VDR) and peroxisome proliferator-activated receptor γ (PPARγ) are ligand-activated nuclear transcription factors that are instrumental for bone health. While 1α,25-dihydroxyvitamin D3 (1,25D3), the ligand for VDR, is essential for the development and maintenance of healthy bone, PPARγ agonists cause detrimental skeletal effects. Recent studies have revealed evidence for a cross-talk between 1,25D3- and PPAR-α /-δ ligand-mediated signaling but there is a current lack of knowledge regarding cross-talk between signaling of 1,25D3 and the PPARγ ligand mediated signaling. In this study we investigated the cross-talk between 1,25D3- and PPARγ agonist rosiglitazone-mediated signaling in human osteoblasts. 1,25D3 slightly but significantly induced expression of the primary PPARγ target gene ANGPTL4 but did not influence FABP4. 1,25D3 did not change rosiglitazone regulation of ANGPTL4 and FABP4. The other way around, rosiglitazone reduced CYP24A1 gene expression but this didn't change CYP24A1 induction by 1,25D3.The findings regarding CYP24A1 gene expression are in line with the observation that 1,25D3 levels in medium were not affected by rosiglitazone. Furthermore, rosiglitazone significantly inhibited 1,25D3-induction of BGLAP while rosiglitazone alone did not change BGLAP. Additionally, 1,25D3 and rosiglitazone increase osteoblast alkaline phosphatase activity and synergistically stimulated extracellular matrix mineralization. In conclusion, these data provide evidence for a cross-talk between rosiglitazone- and 1,25D3-mediated signaling leading to an acceleration of extracellular matrix mineralization. The data suggest that the reduction of the mineralization inhibitor BGLAP and the increased differentiation status underlie the increased mineralization.

Introduction

Being one of the major factors in calcium homeostasis, vitamin D is essential for the development and maintenance of healthy bones. 1α,25-dihydroxyvitamin D3 (1,25D3), the biologically most active vitamin D receptor (VDR) agonist, is synthesized by subsequent vitamin D 25-hydroxylase (CYP2R1) and 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) activity in the liver and kidney, respectively [1]. Bone formation is indirectly influenced by 1,25D3 since it promotes calcium uptake in the intestine and promotes calcium reabsorption in the kidneys. Also direct effects of 1,25D3 on osteoblasts have been established. 1,25D3 stimulates mineralization of human osteoblast cultures [2-3] and modulates gene expression of various osteoblast differentiation and mineralization-related genes such as alkaline phosphatase (ALPL), osteocalcin (BGLAP) and osteopontin (SPP1) [2, 4-5]. 1,25D3 also enhances osteoblast-produced matrix vesicle maturation [6]. To prevent uncontrolled 1,25D3 action and potentially hypercalcemia, 1,25D3

stimulates its own degradation by up-regulating the expression of 24,25D3-hydroxylase (CYP24A1) [7-8].

The synthetic peroxisome proliferator-activated receptor γ (PPARγ) agonist rosiglitazone belongs to the thiazolidinediones (TZD) and is an insulin sensitizer widely prescribed to type 2 diabetic patients. Recent clinical studies have revealed detrimental effects of rosiglitazone on the aging skeleton [9]. Underlying mechanisms include rosiglitazone-mediated suppression of bone formation and stimulation of bone resorption caused by a) stimulation of osteoclast differentiation from hematopoietic precursor cells [10], b) increased osteoblast and osteocyte apoptosis [11-15], c) preferential differentiation of mesenchymal stem cells into adipocytes at the expense of osteoblasts in the bone marrow [16-19], or d) modulation of hormones important for bone metabolism, e.g. leptin [20], insulin [21-22], insulin-like growth factor I [23], or estrogen [24]. Recently, we published a pro-apoptotic effect of rosiglitazone causing a premature onset of mineralization in human osteoblasts and vascular smooth muscle cells [25].

Both VDR and PPARγ are nuclear receptors that form heterodimers with the retinoid X receptor (RXR). Moreover, recent studies have revealed evidence for a cross-talk between 1,25D3- and PPAR-α/-δ-ligand-mediated signaling involving a stimulatory effect of 1,25D3 on PPAR expression that is dependent on VDR [26-27]. However, there is a current lack of knowledge regarding cross-talk between 1,25D3- and PPARγ-ligand-mediated signaling. In this study we therefore investigated the interaction between 1,25D3- and PPARγ-ligand-mediated signaling in the context of osteoblast differentiation and mineralization.

Material and Methods

Cell culture

The human pre-osteoblast cell line SV-HFO [28] was cultured as described previously [29]. To induce osteoblast differentiation, medium (αMEM; Gibco BRL, Life Technologies) was supplemented with freshly added 10 mM β-glycerophosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich) and replaced every 2 or 3 days. As supplements 10 nM 1,25D3 (Leo Pharmaceuticals), 100 nM rosiglitazone (Cayman Chemicals) or a combination of both were used. Cells were harvested at different time points during culture.

DNA, ALP and mineralization assays

SV-HFO were harvested at the onset of mineralization (respective of culture, day 9 or 12) and DNA, ALP and calcium measurements were performed as described previously [30]. All measurements were performed using a Victor^2 plate reader (PerkinElmer Life and Analytical Science).

Quantification of mRNA expression

Cultures continuously treated with control, 10nM 1,25D3, 100 nM rosiglitazone or a combination were harvested during the pre-mineralization period (day 5 or 7) and at the onset of mineralization (day 9 or 12) (depending on the experiment). RNA isolation, cDNA synthesis and PCR reactions were performed as described previously [6, 31]. Oligonucleotide primer pairs, all being either on exon boundaries or spanning at least one intron, were purchased from Sigma-Aldrich (listed in **Table 1**). Gene names and symbols were used as provided by the HUGO Gene Nomenclature Committee [32].

Table 1: Sequences of primer sets used for qPCR in this study. PPARγ, ANGPTL4 and FABP4 were detected using SYBR green; GAPDH, CYP24A1 and BGLAP PCRs were performed with an additional FAM-TAMRA-labeled probe.

Gene	Forward Primer	Reverse Primer	pmol/ reaction
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC	3.75
	Probe: CGCCCAATACGACCAAATCCGTTGAC		3.75
CYP24A1	CAAACCGTGGAAGGCCTATC	AGTCTTCCCCTTCCAGGATCA	15
	Probe: ACTACCGCAAAGAAGGCTACGGGCTG		7.5
BGLAP	CAGGAGGGCAGCGAGGTA	TGGGGCTCCCAGCCA	25
	Probe: TGATACAGGTAGCGCCTG		5
ANGPTL4	GACAAGAACTGCGCCAAGAG	AGTACTGGCCGTTGAGGTTG	1.25
FABP4	TACTGGGCCAGGAATTTGAC	GGACACCCCCATCTAAGGTT	1.25

Quantification of 1,25D3

SV-HFO conditioned medium samples from the cultures at the onset of mineralization (day 9 or 12, respectively of experiment) were analyzed for 1,25D3 concentration by using 1,25-dihydroxyvitamin D RIA (IDS; immunodiagnostic systems) according to manufacturer's instructions.

Statistics

The data provided are based on multiple independent experiments derived from four independent cultures. Values are means ± SEM. Significance was calculated using the Student's t-test and 2-way ANOVA. P-values lower than 0.05 were considered as statistically significant.

Results

We investigated the effects of 1,25D3 on endogenous osteoblastic PPARγ signaling by measuring transcript levels of established primary PPARγ target genes in rosiglitazone, 1,25D3 and 1,25D3/rosiglitazone co-treated human osteoblasts during the pre-mineralization period and at the onset of mineralization. At both time points rosiglitazone increased expression levels of the primary PPARγ target genes angiopoietin-4 (ANGPTL4**; Figure 1A and C**) and fatty acid binding protein 4 (FABP4; **Figures 1B and D**). 1,25D3

induced ANGPTL4 expression during the pre-mineralization period and during the onset of mineralization (**Figure 1A and C**). Treatment with 1,25D3 did not change expression levels of FABP4 (**Figure 1B and D**) and co-treatment with rosiglitazone did not reveal any significant interactions on both PPARγ targets (**Figures 1A-D**).

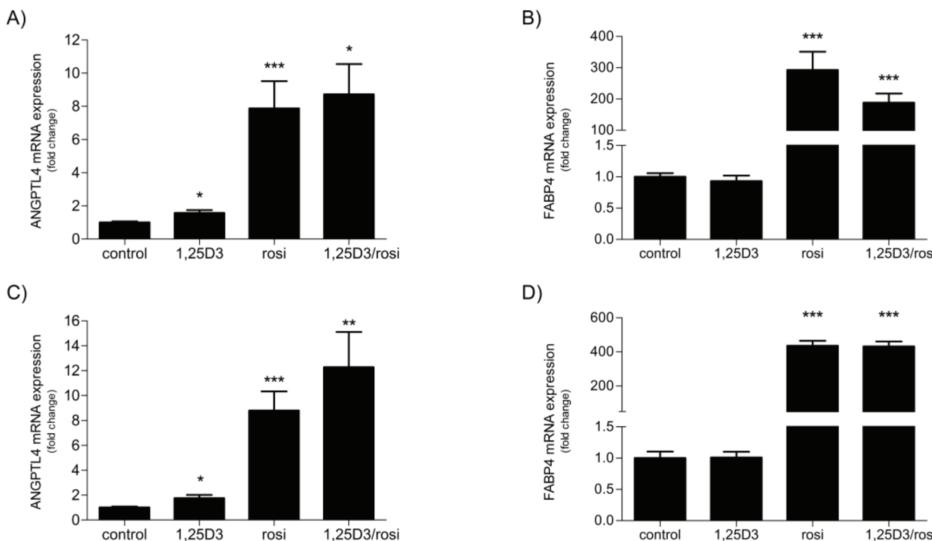


Figure 1. 1,25D3 on primary PPARγ target genes in human osteoblasts. SV-HFO were cultured with control, 1,25D3, rosiglitazone (rosi) and a co-treatment of 1,25D3/rosi. Cultures were harvested in the 1st week of differentiation (A and B) and at the onset of mineralization (C and D) to isolate RNA. mRNA expression of ANGPTL4 (A and C) and FABP4 (B and D) were determined by qPCR. Expression is relative to GAPDH and depicted in fold change to control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significantly different from control cultures.

To investigate the effects of rosiglitazone on endogenous osteoblastic 1,25D3 signaling, we measured the transcript levels of the well-established primary 1,25D3 target gene CYP24A1 in SV-HFO cultures treated with rosiglitazone, 1,25D3 or a combination of rosiglitazone/1,25D3 during the pre-mineralization period and at the onset of mineralization. CYP24A1 expression was significantly increased by 1,25D3 at both time points (**Figure 2A and B**). Interestingly, rosiglitazone reduced basal but not 1,25D3-induced CYP24A1 expression during pre-mineralization (**Figure 2A**) but not at the onset of mineralization (**Figure 2B**). To determine whether rosiglitazone affects 1,25D3 levels as has been reported before *in vitro* and *in vivo* [27, 33], we analyzed conditioned medium collected at the onset of mineralization from cultures treated with rosiglitazone, 1,25D3 and the combination thereof. We found the expected increase in 1,25D3 levels in cultures treated with 1,25D3 but the presence of rosiglitazone didn’t affect these levels (**Figure 2C**).

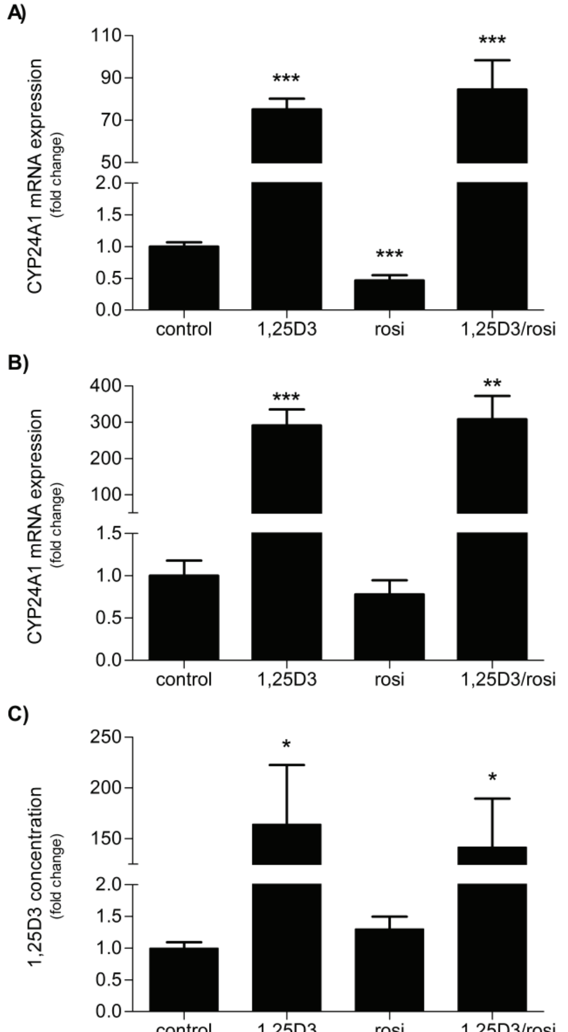


Figure 2. Rosiglitazone effects on CYP24A1 expression and activity. SV-HFO were cultured with control, 1,25D3, rosiglitazone (rosi) and a co-treatment of 1,25D3/rosi. Cultures were harvested in the 1st week of differentiation (A) and at the onset of mineralization (B) and RNA was isolated. mRNA expression of CYP24A1 was determined by qPCR. Expression is relative to GAPDH and depicted in fold change to control. C) SV-HFO were cultured until the onset of mineralization with control, 1,25D3, rosiglitazone (rosi) or a co-treatment of 1,25D3/rosi. At this time point medium was collected and 1,25D3 levels were quantified by 1,25-dihydroxyvitamin D3 RIA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ significantly different from control cultures.

Another well-established 1,25D3 target gene and is reported to inhibit mineralization is BGLAP. We investigated the rosiglitazone and 1,25D3 effects on BGLAP. BGLAP expression was increased by 1,25D3 during pre-mineralization and mineralization (**Figure 3A and B**). Rosiglitazone did not affect BGLAP expression during the pre-mineralization period (**Figure 3A**) but significantly reduced the 1,25D3-induced BGLAP expression at the onset of mineralization (**Figure 3B**).

Finally, we investigated the effects of rosiglitazone and 1,25D3 and their combination on the phenotype of human pre-osteoblasts. DNA content was similar between 1,25D3-treated cultures and controls and was significantly

reduced in all cultures treated with rosiglitazone (**Figure 4A**). Next, 1,25D3 and rosiglitazone both increased ALP activity and their combination showed an additive effect for ALP activity, reflecting enhanced differentiation compared to cultures with single treatments (**Figure 4B**). Finally, 1,25D3 as well as rosiglitazone increased mineralization in SV-HFO cultures (**Figure 4C**). Furthermore, the combination of both compounds showed a synergistic stimulation of mineralization (**Figure 4C**).

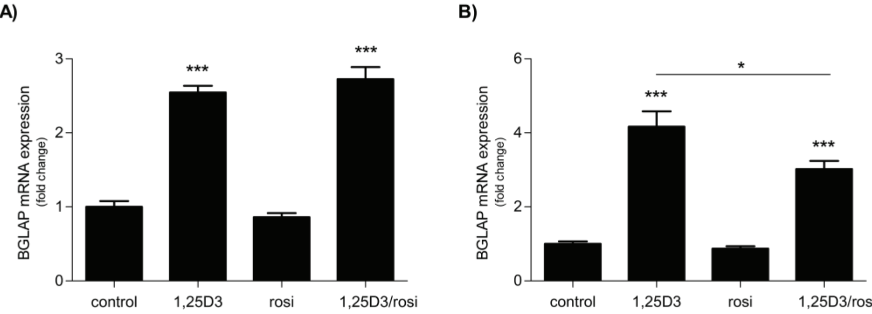
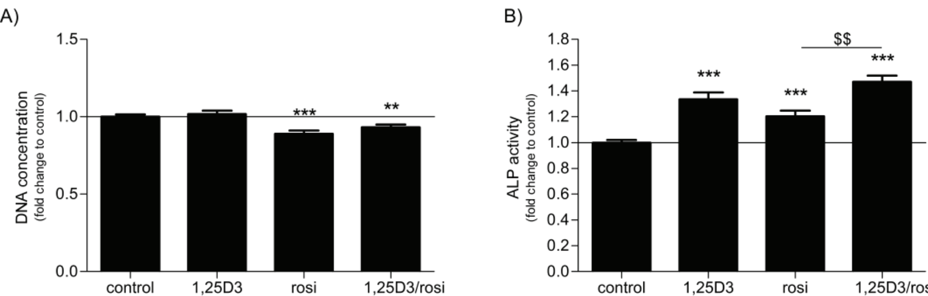


Figure 3. Rosiglitazone effects on BGLAP expression. SV-HFO were cultured with control, 1,25D3, rosiglitazone (rosi) and a co-treatment of 1,25D3/rosi. Cultures were harvested in the 1st week of differentiation (A) and at the onset of mineralization (B) and RNA was isolated. mRNA expression of BGLAP was determined by qPCR. Expression is relative to GAPDH and depicted in fold change to control. * $p < 0.05$ and *** $p < 0.001$ significantly different from control cultures. Interaction effects determined by 2-way ANOVA resulted in a p-value of 0.058.



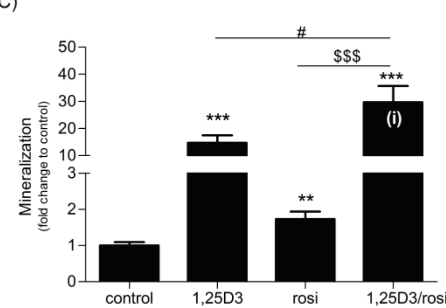


Figure 4. Rosiglitazone and 1,25D3 increase osteoblast differentiation and synergistically enhance mineralization. SV-HFO osteoblasts were cultured with control, 1,25D3, rosiglitazone (rosi) and a co-treatment of 1,25D3/rosi until the onset of mineralization followed by analyses of A) ALP activity as read-out for differentiation, B) DNA content as a measure of cell growth and C) calcium measurement reflecting mineralization. Values represent fold-changes to respective controls from two independent experiments. ** $p < 0.01$ and *** $p < 0.001$ significantly different from control cultures. # $p < 0.05$ significantly different from 1,25D3 cultures. $$ $p < 0.01$ and $$$ $p < 0.001$ significantly different from rosiglitazone cultures. (I) $p < 0.05$ for 2- Anova significance of interaction.

Discussion

The current study demonstrates for the first time that the PPARγ agonist rosiglitazone and the VDR ligand 1,25D3 synergistically stimulate extracellular matrix mineralization by human osteoblasts. The current observations demonstrating synergism suggest that VDR and PPARγ mediated stimulation of mineralization share common mechanisms. It is likely that these compounds target common transcriptional networks underlying these physiological processes. VDR and PPARγ share the retinoid X receptor (RXR) as a common heterodimeric partner. RXR could have been a limiting factor in case of concomitant activation of VDR and PPARγ but the observed synergism suggest that RXR is not a limiting factor.

The precise mechanisms shared by VDR and PPARγ to stimulate mineralization remain yet to be delineated. However, an interesting gene and potential mechanism for regulation of mineralization by 1,25D3 and rosiglitazone is BGLAP. BGLAP is an inhibitor of mineralization and is induced by 1,25D3 [34] and reducing BGLAP activity increases 1,25D3 potency to stimulate mineralization (Woeckel et al, submitted, Chapter 4). We show that rosiglitazone reduces 1,25D3-induced BGLAP expression, which is in line with studies reporting inhibitory effects of PPAR signaling on BGLAP expression. For example, TZD treatment of bone marrow stromal cells which stably express PPARγ showed suppression of BGLAP expression [18]. Additionally, murine bone marrow cells from PPARγ-deficient heterozygous mice exhibited increased BGLAP expression and a reintroduction of PPAR-γ suppressed BGLAP expression [35]. In humans, 1,25D3 strongly induces BGLAP expression, which inhibits *de novo* mineralization by delaying nucleation of hydroxyapatite [36-37]. In addition, BGLAP deficient mice have a mineral maturation defect [38]. Based on these studies and our osteoblast mineralization studies, it is tempting to speculate that the synergistic stimulation of 1,25D3-induced mineralization by rosiglitazone is partly based on the reduction of 1,25D3-induced BGLAP expression. Additionally, the increased differentiation status of co-treated osteoblasts, as assessed by alkaline phosphatase activity, could play a role in the observed increased mineralization. Furthermore, rosiglitazone and 1,25D3 induce apoptosis and can have anti-proliferative effects [11, 39-40], which can be another shared mechanism. The more so, we have recently shown a role for apoptosis in mineralization [41-42]. Unraveling potential interactions at the level of apoptosis are subject to further studies.

We also studied regulation of CYP24A1 expression as a potential level of interaction. CYP24A1 encodes for the enzyme 24-hydroxylase, the rate limiting step in 1,25D3 degradation and thereby limiting the 1,25D3 activity [43]. 1,25D3 is the most potent inducer of CYP24A1 but we found that in absence of 1,25D3 rosiglitazone is also able to reduce CYP24A1 expression in human osteoblasts. Observations regarding CYP24A1 expression have been

reported in melanoma cells, in which the non-specific PPAR ligands bezafibrate and alpha-linolenic acid reduced the 1,25D3-mediated elevation of CYP24A1 [27]. We noted that the inhibitory effect of rosiglitazone was minute compared to the strong induction by 1,25D3, which was confirmed by unaltered 1,25D3 levels. Nevertheless, these data suggests that rosiglitazone is able to modify 1,25D3 metabolism. This was demonstrated by Chakreeyarat *et al.* who recently reported increased circulating levels of 25-hydroxyvitamin D_3 in postmenopausal women treated with TZDs [33]. Whether the observed elevated 25-hydroxyvitamin D3 also led to increased levels of 1,25D3 was not investigated. Taken together, further studies have to be completed to identify mechanisms of rosiglitazone-mediated modification of 1,25D3 metabolism in osteoblasts. However, although rosiglitazone can affect CYP24A1 mRNA expression the current data exclude that reduced 1,25D3 metabolism explains the increased mineralization.

Finally, we demonstrate that 1,25D3 can regulate primary PPARγ target gene ANGPTL4 suggesting that PPARγ signaling is targeted by 1,25D3. This small effect of 1,25D3 on PPARγ signaling is in line with recent studies that showed no or minor effects of 1,25D3 treatment on PPARγ expression and signaling in various melanoma cell lines and other cell lines not derived from the skin [27, 44]. Whether the regulation of ANGPTL4 plays a role in the synergstic-enhanced mineralization is unlikely as in combination treatment the effect of PPARγ activation by rosiglitazone is dominant. Nevertheless, the 1,25D3 effect on ANGPTL4 together with the rosiglitazone effect on CYP24A2 expression further points to cross-regulation of PPARγ and VDR transcriptional cascades.

In summary, we show that rosiglitazone and 1,25D3 synergistically increase mineralization by human osteoblasts. In these osteoblasts we observed an advanced differentiation status and reduced BGLAP expression. These data demonstrate interplay between two nuclear hormone receptor signaling cascades that share at least dimerization with RXR in the control of osteoblast activity.

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General Discussion

The significance of $1\alpha,25(OH)_2D_3$ (1,25D3) for bone are known since the 1920s, but nearly 100 years later the mechanisms of 1,25D3 on bone formation and mineralization are still poorly understood. For decades it has been believed that the effects of 1,25D3 on bone are only indirect via alterations in calcium homeostasis. The cloning of the vitamin D receptor (VDR), the subsequent localization of this receptor in bone cells, and first observations regarding 1,25D3 effects on cultured bone cells positioned bone as a direct target for vitamin D. In addition, data by our laboratory demonstrated that bone cells are able to synthesize 1,25D3 enabling it to act as a factor in an auto- or paracrine manner [1]. Overall, these studies indicate direct involvement of 1,25D3 on bone formation.

This thesis was focused on direct effects of 1,25D3 on osteoblast differentiation and mineralization. Firstly, we wanted to scrutinize the 1,25D3-mediated process of enhanced mineralization. And secondly, factors never act independently but are part of a regulatory network and act in interaction with other factors. With the long term aim to describe the effect of 1,25D3 on bone cells in a systems biological manner we first focused on the interaction of 1,25D3 with other factors in the regulation of osteoblast differentiation and mineralization. Within this chapter the main findings are summarized and discussed in a broader perspective.

Effects on mineralization are osteoblast differentiation phase-dependent

During osteoblast differentiation various functional phases can be identified including proliferation, ECM production and maturation and mineralization. In the human osteoblast model that we used, the proliferation and ECM formation phases are identified as the pre-mineralization phase, which is supported by gene profiling data of human osteoblasts showing an increase in expression of a broad range of ECM proteins (Chapter 1).

We were able to identify the pre-mineralization phase to be crucial for 1,25D3 effects on mineralization. This is in line with previous data in rat osteoblasts showing that the effect of 1,25D3 depends on timing and phase of osteoblast differentiation [2]. The importance of the pre-mineralization period for the regulation of mineralization is not unique for 1,25D3. Similar time dependent effects on mineralization have been reported with FGF8 [3]. Furthermore, we were able to show that both IFNβ and activin A modulate ECM formation during the pre-mineralization period and therefore negatively influence mineralization (Chapter 2, [4-5]). Interestingly, we identified a complex IFNβ response pattern during osteoblast differentiation (Chapter 2).

MSCs with an osteogenic trigger did not respond to IFNβ but when these cells first were induced to differentiate into the osteogenic lineage they responded to IFNβ by inhibiting mineralization. Next, we identified that pre-osteoblasts are sensitive to IFNβ but that this is lost upon further differentiation towards osteoblasts (Chapter 2). These observations revealed a restricted time window of IFNβ sensitivity at the early stage of osteoblast differentiation. In a broader perspective, these findings related to 1,25D3 and IFNβ together with our previous data on activin A [5] and glucocorticoid [6] as well as observations in literature [2] stress the significance of timing of treatment for the eventual effect on osteoblast differentiation and mineralization.

The role of matrix vesicles in osteoblast mineralization

The onset of mineralization is determined by various factors such as ECM composition, production of MVs, enzymes and concentration of calcium and phosphate ions. The main mechanism of initiating mineralization is the formation of hydroxyapatite arising from crystallization of calcium ions and inorganic phosphate (Pi) groups [7]. Pi groups emerge as breakdown products of pyrophosphates and this conversion is driven by ALPL [8-11]. It is thought that the hydroxyapatite deposition is driven by MVs whose membranes are abundant with calcium- and phosphate transporters aiding in the achievement of high intra-vesicular concentrations of calcium and phosphate ions [12-13]. This is supported by the fact that in the membrane of MVs collagen-binding proteins (i.e. annexins) are located. These proteins stimulate calcium-ion uptake into MVs upon binding to collagen [14-15]. Together with high local ALPL concentrations all pre-requisites are present to initiate hydroxyapatite formation [16-18]. Indeed, electron microscopic images revealed that a) MVs are embedded in a collagenous network [19] and b) with MV maturation intravesicular hydroxyapatite is exposed to the extravesicular environment [16].

In line with other studies on MVs derived from cartilage, 1,25D3 increased the amount of ALPL positive MVs produced by osteoblasts (Chapter 1, [20-25]). This finding is supported by previous data obtained in our laboratory that in the pre-mineralization period 1,25D3 enhances the expression of ALPL in osteoblast cultures [26]. We postulate that for mineralization initiation a certain threshold amount of ALPL-positive MVs needs to be produced and incorporated in the ECM. Under the influence of 1,25D3 a higher quantity of ALPL positive MVs is produced and the mineralization threshold is reached earlier, reflected in our model systems by an earlier onset of mineralization (Chapter 1). Interestingly, IFNβ as inhibitor of mineralization did not modify MV production or maturation and did not affect the 1,25D3-stimulated MV production (Chapter 2). In view of the observation that IFNβ exerts inhibitory effects through reduced expression of various ECM genes, one could

speculate that due to an altered ECM composition MVs cannot bind properly to the ECM. Alternatively, since IFNβ reduced COL1A1 production (Chapter 2), the induction of calcium uptake upon binding to collagen might be disrupted.

Inhibitors of osteoblast mineralization in a 1,25D3 environment

Various bone pathologies characterized by under- or over-mineralization highlight the importance of optimal mineralization to gain healthy bones [27-29]. A good example is marble bone disease in which excessive mineralization leads to brittle bones [30]. To prevent pathologies based on mineralization abnormalities several control mechanism are established. First, mineralization can be stimulated by controlling ALPL, which is necessary for the breakdown of the mineralization inhibiting factor pyrophosphate [8-10] and by this process also provides Pi, which is needed for crystal formation [11]. 1,25D3 stimulates the expression of ALPL and the amount of ALPL positive MVs (Chapter 1). Additionally, 1,25D3 increases the expression of COL1A1, the most abundant collagen in the ECM (Chapter 3, [31]). At the same time, 1,25D3 prevents excessive mineralization by several mechanisms. For example, 1,25D3 inhibits the formation of hydroxyapatite by reducing the gene expression of ISBP [32-34]. 1,25D3 also induces the non-collagenous ECM proteins BGLAP and SPP1 which both inhibit mineralization [35-43]. On top of that and independent of bone, 1,25D3 strongly induces its own degradation by stimulating CYP24A1 encoding the enzyme 24-hydroxylase [1, 44-45].

In Chapter 2 we identified a new inhibitor of mineralization: IFNβ. We found that IFNβ exerts its inhibitory effects on mineralization via down-regulation of COL1A1 and various other matrix genes. In mineralizing osteoblasts, ISGs are highly expressed leading to the notion that basal IFNβ signaling is present in osteoblasts. It has been demonstrated before that basal IFNβ signaling exists in fibroblasts [46]. Since 1,25D3 down-regulated IFNβ signaling in mineralizing osteoblasts, we first assumed IFNβ as a fine-tuning mechanism of mineralization. We were not able to hold on to this assumption since activation of IFNβ signaling in a 1,25D3 environment did not modify the extent of mineralization. In addition, we found that 1,25D3 prevented the IFNβ-mediated inhibitory effects on ECM genes (Chapter 3). Rather, based on the findings in Chapter 2 we hypothesize that IFNβ-mediated actions target the HSC niche and that the effects of mineralization are indirect.

In Chapter 4 we found that 1,25D3 induces the production of activin A leading to enhanced activin A signaling in mineralizing osteoblasts. Activin A is a strong inhibitor of mineralization [5] and therefore the idea of activin A as another 1,25D3-induced inhibitor of mineralization arose. Interestingly, inhibition of endogenous activin A production in 1,25D3-treated osteoblasts

led to a synergistic increase in BGLAP expression. This interaction between activin A levels and BGLAP expressions may elude towards a back-up mechanism in controlling osteoblast mineralization: in mineralizing osteoblasts 1,25D3 induces both BGLAP and activin A production to avoid over-mineralization. When activin A signaling lacks, BGLAP expression is further enhanced to antagonize the potentially excessive mineralization.

In **Figure 1** an overview of the 1,25D3 actions leading to increased mineralization is summarized. We were able to characterize activin A as a novel player in 1,25D3-enhanced mineralization. The effects of IFNβ and rosiglitazone on mineralization in a 1,25D3 environment are intriguing but the mechanisms behind their involvement require further scrutiny. However, IFNβ is under debate as risk factor for osteoporosis [47]. Treatment with IFNβ and occurrence of secondary osteoporosis in multiple sclerosis and clinical trials revealed that an additional treatment with 1,25D3 is beneficial for patients [48-50]. Therefore, in case of diseases where co-treatment with IFNβ and 1,25D3 is performed, the 1,25D3-mediated prevention of the deleterious effects on mineralization by IFNβ requires further scrutiny.

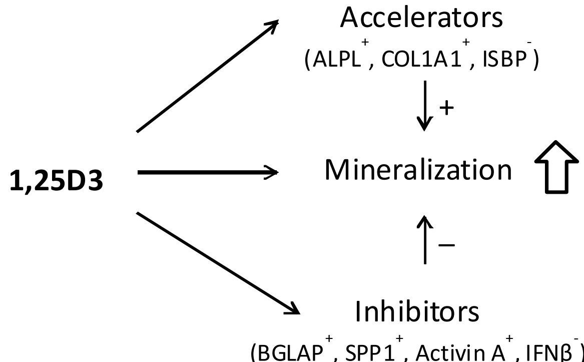

Figure 1. Summary of 1,25D3-mediated actions on various accelerators and inhibitors leading to enhanced osteoblast mineralization. A "+" symbol behind gene name indicates stimulation by 1,25D3 and genes with a "-" symbol are inhibited by 1,25D3.

Interaction of 1,25D3-mediated signaling with transcription factors in osteoblasts

In chapter 3 we demonstrated that 1,25D3 down-regulated STAT1, a major transcription factor of IFNβ signaling, at both mRNA and protein level in human osteoblasts. Additionally, interferon target genes whose transcription are mediated via STAT1 were found to be down-regulated by 1,25D3 suggesting reduced STAT1 activity. 1,25D3-mediated signaling is accomplished by binding to its receptor VDR, which then translocalizes into the nucleus to heterodimerize with RXR ultimately leading to transcription initiation of target genes. Interestingly from a regulatory feedback

perspective, STAT1 has been reported to antagonize 1,25D3 transcriptional activity by binding to VDR in monocytes-macrophages [51]. In mice, STAT1 also targets RUNX2, a key-transcription factor of osteoblast differentiation. Upon interaction, STAT1 attenuates the translocation of RUNX2 into the nucleus and thereby inhibiting its activities [52]. Among these, it may stimulate RUNX2-mediated stabilization of the interaction of VDR on the VDRE of the BGLAP promoter [53]. Since STAT1 is able to modulate RUNX2 and 1,25D3 signaling, STAT1 may be an interesting factor during osteoblast differentiation. On basis of these data it is tempting to postulate that 1,25D3-mediated down-regulation of STAT1 is to prevent IFNβ antagonizing 1,25D3 action as well as RUNX2-mediated transcription. A slight shift in concentration/activation of those transcription factors may have an impact on the extent of mineralization. This exemplifies the complexity of interactions between RUNX2, VDR and STAT1 including regulatory loops during osteoblast differentiation and eventually to fine-tune mineralization at the protein level. Further experiments have to elucidate the complex interaction between these transcription factors.

In chapter 4 we describe that activin A signaling is present in 1,25D3-treated osteoblasts. Activin A and TGFβ signal via activation of SMAD3 and subsequent heterodimerization with SMAD2 [54]. In the presence of 1,25D3, SMAD3 is able to bind to VDR resulting in stabilization of the transcriptional complex on the BGLAP promoter and thus fortifying BGLAP transcription [55-56]. This interaction of SMAD3/VDR can be interrupted by SMAD7 [57]. In chapter 4 we have shown that in 1,25D3-treated human osteoblasts, increased activin A signaling was present and SMAD7 was up-regulated. In addition, BGLAP expression was inhibited by endogenous activin A signaling. Based on our observations and the described effects of SMAD7 on the VDR/SMAD3-mediated promoter activity of BGLAP [57], we speculate that a balance of SMAD3/VDR complexes and SMAD7 could be involved in 1,25D3-mediated control of mineralization. However, it should be emphasized that this is only based on gene expression data and whether this holds at the protein level and functional interactions between these transcription factors exists, has to be further elucidated. However, our group has previously demonstrated functional interaction between TGFβ signaling and 1,25D3 and VDR in osteoblasts [58].

In chapter 5 we studied the effects of rosiglitazone on 1,25D3-enhanced mineralization. Rosiglitazone signals via PPARγ, but cross-activations of PPARα and PPARδ are possible. The described effects of rosiglitazone on bone are contradictory. The described effects on osteoblasts range from inhibition of differentiation [59-61], promotion of apoptosis [62-63] to stimulation of differentiation [64]. In our study, rosiglitazone did not affect mineralization but in cultures co-treated with rosiglitazone and 1,25D3 we observed a synergistic increase in mineralization, most likely explained by a

rosiglitazone-mediated down-regulation of 1,25D3 induced BGLAP expression and advanced differentiation status. The role of PPARγ needs to be further elucidated.

By focusing only on the described transcription factors (VDR, RUNX2, SMAD3 and STAT1) it is already obvious how complex the process of 1,25D3-mediated transcription is and how many factors are involved. RUNX2 and SMAD3 are both able to stabilize the VDR on the VDRE in the BGLAP promoter whereas STAT1 and SMAD7 are counteracting 1,25D3-mediated transcriptions. This complex process of mediating transcription activities complicates the analyses of studies using different stimuli (IFNβ, activin A, rosiglitazone) in a 1,25D3 environment. Additionally, one should realize that obtained results during osteoblast differentiation heavily depend on the timing and duration of treatment regimens.

The interaction of VDR with the mentioned transcription factors should be focus of future studies. It is a big challenge to describe these interactions in a quantitative systems biological manner. Thereby, the focus should be the identification of dominant pathways, crucial regulatory steps and neat balances in osteoblast differentiation and bone formation.

Modification of 1,25D3 metabolism in osteoblasts

In bone, the expression of the genes encoding for enzymes of the 1,25D3 metabolism, CYP27B1 and CYP24A1, is correlated [65]. 1α-hydroxylase (encoded by CYP27B1) catalyses the synthesis of 1,25D3. Since 1,25D3 induces the CYP24A1 genexpression, encoding for the 24-hydroxylase, the degradation of 1,25D3 is stimulated. The importance of physiological 1,25D3 levels on bone is demonstrated by the mutation of the CYP27B1 gene. Subjects with a mutation of that gene develop vitamin-D-dependent rickets [27]. It has been reported that in a mouse model for rickets decreased mineral deposition led to a greater extensibility and lower stiffness of fibrils [29]. The direct involvement of 1,25D3 in bone formation has been demonstrated by the fact that 1,25D3 can be produced by osteoblasts [1, 66] and that various isoforms of 1,25D3 are able to stimulate mineralization as well [67]. During our studies looking at inhibitors of osteoblast mineralization, we postulate that IFNβ could modulate 1,25D3 levels by down-regulating CYP27B1 during the pre-mineralization period and by up-regulating CYP24A1 during the onset of mineralization (Chapter 3). Modifications of 1,25D3-induced CYP24A1 expression have also been described upon PTH or EGF treatment EGF [68-69]. In our experiments we used 1,25D3 to treat osteoblasts, therefore, the significance of CYP27B1 inhibition by IFNβ could not be determined. IFNβ and 1,25D3 have opposing effects on mineralization and it is possible that IFNβ could minimize 1,25D3-mediated effects by either reducing 1,25D3 production or faster degradation

to reinforce its own effects. Unfortunately, we were not able to identify these underlying processes and thus the significance of potential modification of 1,25D3 metabolism remains unresolved. Nevertheless, it would be interesting to repeat this set of experiments using 25(OH)D3 instead to elucidate the significance of the IFNβ-mediated CYP27B1 down-regulation.

Final Remarks & Conclusion

The discussion points raised in the general discussion of this thesis highlight the complexity of osteoblast differentiation and mineralization. The observed effects of 1,25D3, IFNβ and activin A demonstrated that stimulating or inhibiting effects on mineralization are dependent on specific time windows within the pre-mineralization period during osteoblast differentiation (Chapter 1, 2, [2, 5]). From a molecular point of view, in human osteoblasts 1,25D3-mediated signaling interferes with several other signaling pathways (i.e. IFNβ-, activin A- and rosiglitazone-mediated signaling) (Chapter 3, 4 and 5). We postulate that 1,25D3 interferes at least with activin A-mediated signaling to optimally control mineralization. Besides activin A, BGLAP is another inhibitor of mineralization which is strongly induced by 1,25D3. Interestingly, BGLAP expression is not only modified by activin A but also by rosiglitazone in 1,25D3-treated osteoblast (Chapter 4 and 5). Whether these modifications lead to alterations of mineralization needs to be further scrutinized. Possible modifications of other known inhibitors of mineralization (e.g. osteopontin) should be considered to be included in those studies. With regards to IFNβ, we found indications that it may play an important role in adhesion of HSC to its niche (Chapter 2). Further studies to prove this hypothesis and the role of 1,25D3 therein need to be conducted. Our studies were mainly based on assessing gene transcription level and phenotypical outcome, it would be of great interest to verify the obtained results at the protein level.

In this thesis, we tested only two stimuli at the same time *in vitro*, but one should realize that *in vivo* a variety of triggers exist next to each other. One can imagine that a complex interplay between those transcription factors exists and that interfering with this complex interplay alters mineralization. Quantitative protein or transcript measurements of involved transcription factors would benefit from system biological approaches to investigate these interactions.

In conclusion, the findings in this thesis demonstrated direct effects of 1,25D3 on osteoblast-mediated mineralization but also indirectly through regulation of inhibitors of the same process. The observations shed light on the complex interactions of stimulators and inhibitors of mineralization and further stresses the intricate nature of osteoblast differentiation and mineralization.

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Summary

Vitamin D is a hormone, which controls calcium homeostasis in the body and is essential for maintenance of healthy bones. Based on its beneficial effects on bone, vitamin D is widely used as food supplement to strengthen bone and prevent osteoporosis in elderly. Osteoporosis is a disease of bone characterized by low bone mineral density, subsequently resulting in an increased risk of fractures. Additionally, taking into account that the world's population becomes older, the prevention and treatment of osteoporosis is of great importance.

Vitamin D's effect on bone is based on several processes. Vitamin D indirectly regulates bone formation and mineralization via the control of serum calcium levels. But it also became evident that vitamin D can directly stimulates osteoblast-mediated extracellular matrix (ECM) production and mineralization. The mechanism by which vitamin D regulates mineralization is not yet revealed, despite the fact that vitamin D effects on human health are very well studied.

We aimed to gain *novel insights into vitamin D effects on osteoblasts and mineralization.*

In **chapter 1** we demonstrated that vitamin D directly affects mineralization by an accelerated production of matrix vesicles (MV). These vesicles are believed to initiate mineralization and we proposed a concept that 1α,25$(OH)_2D_3$ (1,25D3) accelerates the deposition of mature MVs, which subsequently leads to an earlier onset of mineralization. We further demonstrated that the basis of 1,25D3-enhanced mineralization is established before mineralization is initiated. 1,25D3 only enhanced mineralization when administered prior to the onset of mineralization highlighting that underlying mechanisms are targeted during the pre-mineralization period. In addition, the duration of treatment turned out to be more important than a specific time window in reaching enhanced mineralization. These data suggest that during early osteoblast differentiation long-lasting processes were targeted. We tested for 1,25D3-mediated effects on ECM production and found that besides type I Collagen (COL1A1), the majority of genes annotated to the GO-term "extracellular matrix" were not regulated by 1,25D3. Although 1,25D3 did not regulate most of the ECM genes, we report in **chapter 3** that 1,25D3 prevents down-regulation of the same ECM genes by interferon (IFN) β. This suggests that although 1,25D3 did not regulate ECM genes, it may protect them from being down-regulated.

We further focused on the mechanisms behind 1,25D3-enhanced mineralization, using gene chip profiling. During the analyses we found many IFN target genes being down-regulated by 1,25D3 during the pre-

mineralization period. Following these observations, in **chapter 2** we studied the effects of IFNβ on human osteoblast differentiation and mineralization in detail. Human osteoblasts responded to IFNβ by up-regulating known IFN target genes (ISG) such as STAT1, IFIT1, IFI44L but also by stimulating its own expression. We were the first to show that IFNβ treatment of osteoblasts led to inhibition of mineralization. Like stimulation of mineralization by 1,25D3, inhibition by IFNβ was dependent on the time window during osteoblast differentiation. A one week IFNβ treatment from day 0 onwards led to a 40-60% decrease of mineralization whereas a one week treatment from day 7 onwards only led to a 20-30% reduction. IFNβ treatment of human mesenchymal stem cells (MSC) triggered towards osteoblast differentiation did not result in an inhibition of mineralization. When these MSC were first induced to become osteoblasts (osteogenic commitment), an inhibition of mineralization was observed upon IFNβ treatment. These studies highlighted that during early differentiation, osteoblasts are particularly sensitive to IFNβ, leading to inhibition of mineralization. Interestingly, a single IFNβ treatment in the early stage of osteoblast differentiation was as effective in reducing mineralization as was continuous treatment. This is likely explained by osteoblast desensitization upon repeated treatments with IFNβ. Next, we focused on the mechanism underlying IFNβ inhibition of mineralization. We demonstrated that IFNβ has severe inhibitory effects on mineralization by modifying the ECM produced by osteoblasts. We showed that IFNβ decreased gene expression of ECM-related genes, such as COL1A1, fibronectin (FN1), fibulin 1 (FBLN1), fibrillin 2 (FBN2) and laminin 1 (LAMA1) in the early stage of osteoblast differentiation. Additionally, ECM produced by IFNβ-treated osteoblasts contained less collagen protein. Besides effects on ECM genes we also found that IFNβ stimulated gene expression of osteopontin (SPP1), annexin2 (ANXA2), and hyaluronan synthase 1 (HAS1), which are important factors in the adhesion of hematopoietic stem cells (HSC) in the HSC niche. This puts the effects of IFNβ on early osteoblasts in a different perspective suggesting a HSC supportive phenotype. Further studies are needed to understand the role of IFNβ on early osteoblasts and within the HSC niche.

After investigating the effects of IFNβ on osteoblasts and mineralization, we focused on the possible interaction between 1,25D3 and IFNβ with respect to mineralization. In **chapter 3** we showed that 20% of the genes being down-regulated by 1,25D3 were ISGs. 1,25D3 down-regulated ISGs predominantly during the pre-mineralization period suggesting possible interactions of 1,25D3 and IFNβ with regard to mineralization. Although IFNβ did not affect 1,25D3-stimulated mineralization, 1,25D3 in turn prevented down-regulation of ECM genes mediated by IFNβ. Therefore, 1,25D3 demonstrated dominance over IFNβ with regard to bone formation and mineralization. Next, we performed gene expression profiling during early osteoblast differentiation.

We focused on genes, which correlated or anti-correlated with interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) after 2 and 24 hours in control, 1,25D3, IFNβ treated and 1,25D3 / IFNβ co-treated osteoblasts. This approach allowed us to specifically identify processes driven by IFNβ and 1,25D3 in osteoblast differentiation. We identified 144 genes of which 117 correlated and 27 anti-correlated with IFIT1. Bioinformatic analyses showed that these genes were associated with negative regulation of protein metabolic processes and with the cellular component "organelle lumen". Analyses using IPA (Ingenuity Systems, www.ingenuity.com) revealed "protein ubiquitination" as process being associated to those genes. Interestingly, these systems pathway analyses associated the majority of the 144 genes to "Connective Tissue Development" with various underlying processes including "osteoblast differentiation". Although we clearly demonstrated a 1,25D3-IFNβ cross-talk with respect to osteoblast mineralization and we identified genes being part of this cross-talk, further scrutiny is needed to fully elucidate the mechanisms behind it.

While analyzing the 1,25D3 gene-chip profiling of osteoblast differentiation, we detected that transcripts of activin A (INHBA) were induced by 1,25D3 during early osteoblast differentiation. Activin A is a strong inhibitor of mineralization and it was shown previously that 1,25D3, although stimulating mineralization itself, it also induces inhibitors of mineralization (such as BGLAP). We explored this relationship with activin A further in **chapter 4**. We demonstrated that during early osteoblast differentiation, 1,25D3 induced activin A protein and reduced follistatin production, which is the endogenous activin A inhibitor. These modifications allowed for increased activin A signaling demonstrated by a luciferase-reporter construct and respective regulations of the activin A target genes SMAD7 and CLEC3B. Since activin A is an inhibitor of mineralization we hypothesized that 1,25D3 stimulated mineralization is increased by blocking endogenous activin A signaling. Indeed co-treatment of follistatin with 1,25D3 led to a stronger increase of mineralization compared to 1,25D3 alone, implying that besides stimulation of mineralization, 1,25D3 also controls mineralization by induction of activin A-mediated inhibition of mineralization. We achieved similar effects on mineralization by treating osteoblasts with warfarin, which prevents carboxylation of the established inhibitor of mineralization osteocalcin (BGLAP), rendering it less active. Interestingly, blocking activin A signaling in 1,25D3-treated cultures led to a synergistic increase in BGLAP, suggesting modulatory actions of activin A on BGLAP in a 1,25D3-stimulated environment. These results support a control mechanism involving interplay of activin A and osteocalcin by which 1,25D3 prevents excessive osteoblast-mediated mineralization.

This PhD project was part of the NucSys training network, which focused on nuclear receptors. **Chapter 5** resulted from cooperation within this network.

Vitamin D receptor (VDR) and peroxisome proliferator-activated receptor γ (PPARγ) are ligand-activated nuclear transcription factors that both form heterodimers with the retinoid X receptor (RXR). PPARγ's ligand rosiglitazone was already used as a diabetes-related drug and was reported to increase fractures and negatively affect bone mass as potential side-effects. *In vitro*, rosiglitazone accelerated osteoblast differentiation and enhanced mineralization, however, at the expense of mitochondrial dysfunction and early cell death. We tested the effects of rosiglitazone on 1,25D3-treated osteoblasts. At the onset of mineralization, we observed increased ALPL activity in co-treated cultures compared to control cultures. This suggests an advanced differentiation status of co-treated osteoblasts. Additionally, we found that stimulation of osteoblasts with 1,25D3 and rosiglitazone synergistically enhanced mineralization. 1,25D3 induced the expression of BGLAP in osteoblasts. Interestingly, at the onset of mineralization co-treatment with rosiglitazone led to a significant decrease in BGLAP expression. In **chapter 4** we observed increased mineralization by inhibiting BGLAP. Consequently, we hypothesize that the increased mineralization in 1,25D3/rosiglitazone-treated cultures is based on reduced expression of BGLAP.

In **chapter 6** the most important findings of this thesis were put in context with current knowledge. To conclude, this thesis brought novel insights into 1,25D3-control of osteoblast differentiation and function and the eventual mineralization of the ECM produced. We provide evidence that timing of the 1,25D3 and IFNβ treatment is crucial for the effect on mineralization and we have provided working models for mechanisms and processes behind 1,25D3-enhanced mineralization. The production of more mature MVs highlighted the importance of these subcellular structures and may stimulate research with respect to the initiation process of mineralization. We identified IFNβ as a novel inhibitor of mineralization and suggested a potential role of IFNβ on early osteoblasts leading to a phenotype suggestive for support of HSC. The interaction of 1,25D3 with mineralization inhibitors such as BGLAP, activin A and IFNβ as well as with rosiglitazone shed new light on the complex process of mineralization and possible interplays of VDR with other transcription factors such as RUNX2, SMAD3, STAT1 or even PPARγ. This thesis provides important new insights into the action of 1,25D3 in human osteoblasts and mineralization and interaction with several counter-regulatory factors. However, future research is needed to further unravel these intricate processes. In particular, the future holds a big challenge to describe these interactions in a quantitative systems biological manner and thereby to identify dominant and crucial regulatory steps in osteoblast differentiation and bone formation as potential targets for therapy in skeletal diseases.

Samenvatting

Het hormoon vitamine D regelt de calciumhomeostase van het lichaam en is essentieel voor de instandhouding van gezonde botten. Op basis van hun positieve effecten op het skelet worden vitamine D-supplementen aangeraden om botten te versterken en osteoporose te voorkomen. Osteoporose is een botziekte die wordt gekenmerkt door lage botmassa en verhoogd risico op fracturen wat voornamelijk voorkomt bij oudere mensen. Omdat de wereldbevolking steeds ouder wordt heeft het voorkomen en behandelen van osteoporose een groeiende belangstelling.

De effecten van vitamine D op bot worden op verschillende manieren bewerkstelligd. Vitamine D reguleert de botvorming op een indirecte manier, namelijk via de controle van calcium in de circulatie, in samenwerking met het parathyroid hormoon. Maar er zijn ook indicaties dat vitamine D direct de productie van osteoblast-gemedieerde extracellulaire matrix (ECM) en mineralisatie stimuleert. Hoewel de effecten van vitamine D op de calcium huishouding en het skelet uitgebreid zijn bestudeerd, is het mechanisme achter vitamine D-gestimuleerde mineralisatie nauwelijks bekend.

Het algemene doel in dit proefschrift was het verkrijgen van *nieuwe inzichten in de vitamine D-gestimuleerde osteoblast mineralisatie*.

In **hoofdstuk 1** hebben wij laten zien dat vitamine D door een verhoogde productie van matrix vesicles (MV) een directe invloed heeft op de mineralisatie. Een theorie is dat de rijping van MVs de mineralisatie initieert. Op basis van de gevonden data, concluderen wij dat $1\alpha,25(OH)_2D_3$ (1,25D3) de incorporatie van rijpe MVs versnelt, wat vervolgens tot een vervroegde mineralisatie leidt. Verder hebben wij aangetoond dat de oorzaak voor de 1,25D3-gestimuleerde mineralisatie in de fase voor de mineralisatie plaatsvindt. 1,25D3 stimuleert de mineralisatie alleen wanneer het voor het begin van de mineralisatie werd toegevoegd. Deze observatie verduidelijkt dat onderliggende mechanismes voor 1,25D3-gestimuleerde mineralisatie zich in de pre-mineralisatiefase bevinden. In lijn met deze bevinding concludeerden wij dat voor de stimulatie van de mineralisatie de duur van 1,25D3 behandeling belangrijker is dan een bepaald behandelingstijdstip. Deze resultaten bevestigen dat langdurige processen tijdens de osteoblastdifferentatie het aangrijpingspunt zijn voor 1,25D3. Een van deze processen zou de productie of maturatie van de ECM kunnen zijn. Wij hebben de 1,25D3 effecten op ECM productie getest en hebben aangetoond dat de meeste genen die met de GO-term “extracellular matrix” geannoteerd zijn niet door 1,25D3 werden beïnvloed. Een uitzondering hierop vormt type I collageen (COL1A1). Hoewel het merendeel van ECM genen niet door 1,25D3 gereguleerd is, hebben wij in **hoofdstuk 3** beschreven, dat 1,25D3 een verlaagde expressie van ECM genen door interferon (IFN) β voorkomt. Dit

suggereert dat hoewel 1,25D3 de ECM genen niet reguleert, deze door 1,25D3 wel kunnen worden beschermd.

Om ons doel de mechanismen achter de 1,25D3 gestimuleerde mineralisatie te onderzoeken, hebben we o.a. ‘gene chip profiling’ toegepast. Tijdens de analyses hiervan vonden wij in de pre-mineralisatiefase opmerkelijk veel IFN-gerelateerde genen die door 1,25D3 verlaagd tot expressie werden gebracht. Op basis van deze observatie hebben wij in **hoofdstuk 2** de effecten van IFNβ op humane osteoblastdifferentiatie en mineralisatie bestudeerd. Humane osteoblasten reageerden op IFNβ door de genexpressies van bekende IFNβ gestimuleerde genen (ISG), zoals STAT1, IFIT1, IFI44L en IFNβ zelf, te stimuleren. Als eerste hebben wij aangetoond dat een IFNβ behandeling van osteoblasten tot een remming van mineralisatie leidt. Evenals de stimulatie van mineralisatie door 1,25D3, was de remming door IFNβ tijdsafhanklijk. Een zevendaagse behandeling, beginnend vanaf dag 0, leidde tot een 40-60% reductie van de mineralisatie. Dezelfde behandelingsduur, beginnend vanaf dag 7, leidde tot een reductie van slechts 20-30%. IFNβ behandeling van humane mesenchymale stamcellen (MSCs), die osteoblastdifferentiatie ondergaan, veroorzaakte geen reductie in mineralisatie. Een remming van de mineralisatie werd pas duidelijk nadat MSCs 48 uur na de osteoblastinductie met IFNβ werden behandeld. Deze resultaten verduidelijken dat de vroege fase van osteoblastdifferentiatie bijzonder gevoelig is voor IFNβ behandeling en dus ook voor uiteindelijke remming van mineralisatie. Opmerkelijk is dat een enkele IFNβ behandeling aan het begin van de osteoblast differentiatie voldoende is om de maximale remming van mineralisatie te bereiken. Een verklaring zou kunnen zijn dat voortdurend met IFNβ behandelde osteoblasten minder gevoelig voor de stimulus worden. Verder bekeken wij de mogelijke mechanismen die de IFNβ remming zouden kunnen veroorzaken. We hebben aangetoond dat IFNβ de door osteoblasten geproduceerde ECM aantast. Dit gebeurt tijdens de vroege fase van osteoblastdifferentiatie door een verlaagde genexpressie van meerdere ECM-gerelateerde genen zoals COL1A1, fibronectin (FN1), fibulin 1 (FBLN1), fibrillin 2 (FBN2) en laminin 1 (LAMA1). Bovendien bestaat de door IFNβ-beïnvloede en de door osteoblast-geprocudeerde ECM uit minder collageeneiwit. Belangrijk om te noemen is dat IFNβ de genexpressie van osteopontin (SPP1), annexin 2 (ANXA2) en hyaluron synthase 1 (HAS1) verhoogt. Deze genen zijn belangrijke factoren voor de aantrekking van hematopoietische stamcellen (HSC) naar hun niche. Deze observatie zet de bevindingen van IFNβ in vroege osteoblasten in een ander perspectief en suggereert een HSC niche-ondersteunend fenotype. Verdere studies zijn noodzakelijk om de taken van IFNβ in vroege osteoblasten met betrekking tot de HSC niche beter te begrijpen.

In **hoofdstuk 3** hebben wij laten zien dat maar liefst 20% van alle genen, die door 1,25D3 een verlaagde expressie laten zien, gerelateerd zijn aan

IFNβ. Aangezien 1,25D3 en IFNβ een tegenovergesteld effect hebben op osteoblast mineralisatie, is gekeken naar de interactie tussen 1,25D3 en IFNβ ten opzichte van mineralisatie. IFNβ had geen invloed op 1,25D3 gestimuleerde mineralisatie, maar 1,25D3 beschermde meerdere ECM genen tegen IFNβ-gemedieerde genexpressie verlaging. Daaruit concludeerden wij, dat ten aanzien van regulatie van botvorming en mineralisatie 1,25D3 een overheersende rol speelt ten opzichte van IFNβ . Om verder inzicht in de mineralisatie te verkrijgen hebben wij een genexpressieprofiel gemaakt van osteoblasten in de pre-mineralisatiefase na behandeling met 1,25D3, IFNβ of een combinatie van beide. Voor de analyses hiervan hebben wij voornamelijk genen bestudeerd die of sterk gecorreleerd of anti-gecorreleerd waren met interferon-induced protein with tetratricopeptide repeats 1 (IFIT1). Omdat we voor deze benadering kozen, konden wij specifieke processen bestuderen, die door IFNβ en 1,25D3 in osteoblasten gestuurd worden. We hebben 144 genen geïdentificeerd, waarvan 117 correleerden en 27 anti-correleerden met IFIT1. Bioinformatische analyses associeerden deze genen met de negatieve regulatie van eiwit metabolische processen en met het cellulaire onderdeel „organelle lumen‟. Analyses die wij met Ingenuity Pathway Analysis (IPA) uitvoerden, associeerden dezelfde genenset met „protein ubiquitination‟. Opmerkelijk is dat IPA het merendeel van de 144 genen met „Connective Tissue Development" associeerde en onderliggend daaraan een scala aan processen waaronder ook osteoblast differentiatie. Hoewel we duidelijk een samenspel tussen 1,25D3 en IFNβ ten aanzien van mineralisatie hebben laten zien en een aantal onderliggende genen konden identificeren, is verdere diepgang noodzakelijk om de precieze mechanismen te ontrafelen.

Tijdens de analyse van het 1,25D3 gene-chip profiel tijdens osteoblastdifferentiatie vonden wij een door 1,25D3 verhoogde expressie van het activine A transcript (INHBA). Activine A is een bekende, sterke remmer van mineralisatie. 1,25D3 stimuleert mineralisatie, maar er zijn ook rapporten, die aantonen dat 1,25D3 remmers van de mineralisatie induceert (waaronder osteocalcine (BGLAP)). Wij bestudeerden de relatie tussen 1,25D3 en activine A in **hoofdstuk 4**. 1,25D3 verhoogde de productie van activine A en verminderde de productie van follistatine, een endogene remmer van activine A, tijdens de pre-mineralisatiefase. Deze verschuivingen in de activine A en follistatine ratio resulteerde in een verhoogde activine A signalering in de cel, die we met behulp van een luciferase-reporter construct hebben aangetoond. Ook werden de verwachte veranderingen in de genexpressies van de activine A-gereguleerde genen SMAD7 en CLEC3B gevonden. Omdat activine A een remmer van mineralisatie is, hadden wij de hypothese opgesteld dat de 1,25D3-gestimuleerde mineralisatie tevens door stimulatie van activine A geremd wordt. Een behandeling met 1,25D3 en follistatine samen resulteerde in een verdere verhoging van de 1,25D3-gestimuleerde mineralisatie, wat onze hypothese bevestigde. We

vermoedden dat 1,25D3 door de inductie van activine A de mate van mineralisatie controleert. Vergelijkbare resultaten hebben wij behaald in andere proeven met een andere remmer van de mineralisatie, BGLAP. Warfarine verminderde in de aanwezigheid van 1,25D3 de activiteit van BGLAP door het blokkeren van de γ-carboxylering, wat een verhoging van de mineralisatie tot gevolg had. Opmerkelijk was dat het blokkeren van activine A signalering in 1,25D3-behandelde kweken tot een synergistische verhoging van BGLAP expressie leidde. Activine A remt de BGLAP expressie in 1,25D3 behandelde osteoblasten. Deze bevindingen onderbouwen de aanwezigheid van een controle mechanisme met betrekking tot activine A en BGLAP in 1,25D3-behandelde osteoblasten. Op deze manier zou een ongecontroleerde excessieve osteoblast mineralisatie door 1,25D3 voorkomen kunnen worden.

Dit proefschrift maakte deel uit van het 'NucSys training network', waarbinnen de focus op nucleaire receptoren lag. **Hoofdstuk 5** is gebaseerd op een samenwerkingsproject binnen dit netwerk. De vitamine D receptor (VDR) en de peroxisome proliferator-activated receptor γ (PPARγ) zijn ligand-geactiveerde nucleaire transcriptiefactoren, die allebei met de retinoid X receptor (RXR) heterodimeriseren. Een ligand van PPARγ, rosiglitazone, was al in gebruik als geneesmiddel voor diabetes. In klinische studies zijn echter een verhoogd risico voor fracturen en een negatief effect op de botmassa als mogelijke bijwerkingen van rosiglitazone beschreven. *In vitro* studies hebben aangetoond dat rosiglitazone de osteoblast differentiatie versnelt en de mineralisatie verhoogt ten koste van mitochondriale afwijkingen en een vroege celdood. We waren geïnteresseerd in de effecten van rosiglitazone op 1,25D3-behandelde osteoblasten. Aan het begin van de mineralisatie konden wij in 1,25D3 en rosiglitazone- behandelde osteoblasten, vergeleken met onbehandelde cellen, een verhoogde ALPL activiteit meten, wat duidt op een versnelde differentiatie. Verder resulteerde de combinatiebehandeling van 1,25D3 en rosiglitazone in een synergistische verhoging van mineralisatie ten opzichte van 1,25D3 alleen. Aan het begin van de mineralisatie vonden wij dat de genexpressie van BGLAP, een bekende 1,25D3 target, in osteoblasten die met rosiglitazone en 1,25D3 werden behandeld, significant lager was dan in 1,25D3-behandelde osteoblasten. In **hoofdstuk 4** lieten we zien dat een remming van BGLAP tot een verdere verhoging van 1,25D3-gemedieerde mineralisatie leidde. Op basis van deze gegevens werd een concept opgesteld, waarin de verhoging van mineralisatie na 1,25D3/rosiglitazone behandeling op een verlaagde BGLAP genexpressie of functie gebaseerd kan zijn. Verder onderzoek is nodig om dit concept te onderbouwen.

In **hoofdstuk 6** zijn de belangrijkste bevindingen uit dit proefschrift afgezet tegen de huidige aanwezige kennis. Samenvattend heeft dit proefschrift nieuwe inzichten verschaft in de 1,25D3-gemedieerde controle van osteoblastdifferentiatie, -functie en uiteindelijk mineralisatie van de ECM. Wij hebben aanwijzingen dat de timing van 1,25D3 en IFNβ behandeling van

cruciaal belang zijn voor de effecten op osteoblast mineralisatie. Verder introduceerden wij werkmodellen voor achterliggende mechanismen en processen van 1,25D3-gestimuleerde mineralisatie. De productie van rijpe MVs ondersteunt het belang van deze subcellulaire structuren en zal verder onderzoek ten aanzien van de initiatie van mineralisatie stimuleren. We hebben IFNβ als nieuwe remmer van de mineralisatie geïdentificeerd en suggereren bovendien een mogelijke taak van IFNβ bij de interactie van vroege osteoblasten met HSCs. De ware complexiteit van de processen die aan 1,25D3-gestimuleerde mineralisatie ten grondslag liggen, wordt pas duidelijk, als de interactie van 1,25D3 met mineralisatieremmers (bijv. BGLAP, activine A en IFNβ, maar ook rosiglitazone) en mogelijke interacties van VDR met andere transcriptiefactoren (bijv. RUNX2, SMAD3, STAT1 of zelfs PPARγ) in beschouwing wordt genomen.

Samenvattend verschaft dit proefschrift nieuwe inzichten in de effecten van 1,25D3 op humane osteoblastdifferentiatie, mineralisatie evenals de interactie met een aantal tegengesteld werkende regulatieve factoren. Verder onderzoek zou meer inzicht in deze ingewikkelde processen moeten brengen. Het is een grote uitdaging voor toekomstig onderzoek om deze interacties op een kwantitatieve en meer systeembiologische manier te bestuderen. Hiermee zouden overheersende en cruciale regulatieve mechanismen tijdens de osteoblastdifferentiatie en botvorming geïdentificeerd kunnen worden. Uiteindelijk zouden deze nieuwe inzichten aangrijpingspunten kunnen vormen voor toekomstige therapieën bij diverse botziekten waaronder osteoporose.

Zusammenfassung

Das Hormon Vitamin D reguliert die Kalzium Homeostase des Körpers und ist essentiell für die Instandhaltung von gesunde Knochen. Aufgrund seiner positiven Effekte auf den Knochen wird Vitamin D als Ergänzungsmittel empfohlen, um die Knochen zu stärken und Osteoporose vorzubeugen. Osteoporose ist eine Knochenkrankheit, die durch eine geringe Knochenmasse und ein erhöhtes Risiko von Knochenbrüchen charakterisiert ist. Hauptsächlich sind ältere Menschen von Osteoporose betroffen. Auch aufgrund der stets älter werdenden Weltbevölkerung ist eine Vorbeugung und Behandlung von Osteoporose von großer Wichtigkeit.

Die Effekte von Vitamin D auf den Knochen basieren auf verschiedenen Prozessen. Vitamin D reguliert die Knochenbildung auf eine indirekte Art und Weise: über die Kontrolle von Kalzium im Serum. Aber es gibt auch Hinweise, dass Vitamin D die Produktion von Osteoblast-vermittelter Extracellular Matrix (ECM) und die Mineralisierung direkt stimuliert. Obwohl die Effekte von Vitamin D auf die menschliche Gesundheit gut untersucht sind, sind die Mechanismen hinter der Vitamin D stimulierten Mineralisierung kaum bekannt.

Unser Ziel war es *neue Erkenntnisse in die Vitamin D verstärkte Mineralisierung* zu bekommen.

In **Kapitel 1** haben wir zunächst gezeigt, dass Vitamin D durch eine erhöhte Produktion von Matrix Vesikeln (MV) einen direkten Einfluss auf die Mineralisierung nimmt. Eine Theorie besagt, dass reife MVs die Mineralisierung initiieren. Wir stellten ein Konzept auf, dass $1,25(OH)_2D_3$ (1,25D3) das Ablagern von reifen MVs beschleunigt, welches dadurch zu einer verfrühten Mineralisierung führt. Des Weiteren haben wir gezeigt, dass die Ursache von der 1,25D3 stimulierten und verstärkten Mineralisierung in der Phase vor der Mineralisierung zu suchen ist. 1,25D3 hat die Mineralisierung nur dann verstärkt, wenn es vor der Mineralisierung zu den Zellen gegeben wurde. Diese Beobachtung verdeutlicht, dass sich unterliegende Mechanismen in der Prä-Mineralisierungsphase befinden. Im Einklang mit den Ergebnissen folgern wir, dass für die Verstärkung der Mineralisierung die Dauer der 1,25D3 Behandlung wichtiger ist als ein bestimmtes Zeitfenster. Diese Ergebnisse bestätigen, dass langfristige Prozesse während der Osteoblastdifferenzierung die Ziele sind. So ein Prozess könnte die Produktion oder die Reifung von der ECM sein. Wir haben die 1,25D3 Effekte auf die ECM Produktion getestet und haben herausgefunden, dass die meisten Gene, die mit dem GO-Begriff „Extracellular Matrix" verbunden sind, nicht durch 1,25D3 beeinflusst werden. Eine Ausnahme ist Typ I Kollagen (COL1A1). Obwohl die Mehrheit von ECM Genen nicht durch 1,25D3 reguliert wird, haben wir in **Kapitel 3** beschrieben, dass 1,25D3 eine

Herunterregulierung der Genexpression von ECM Genen durch Interferon (IFN) β verhindert. Dies deutet darauf, dass, obwohl 1,25D3 die ECM Gene nicht selbst reguliert, diese jedoch vor einer alternativen Regulierung beschützen kann.

Unser Ziel waren die Mechanismen hinter der 1,25D3 verstärkten Mineralisierung, die wir durch Gene Chip Profiling aufspüren wollten. Während der Analysen haben wir in der Prä-Mineralisierungsphase auffällig viel IFN-verbundende Gene, die durch 1,25D3 eine niedrigere Genexpression aufzeigten, gefunden. Aufgrund dieser Beobachtung haben wir in **Kapitel 2** die Effekte von IFNβ auf humane Osteoblastdifferenzierung und Mineralisierung untersucht. Humane Osteoblasten reagierten auf IFNβ mit einer Erhöhung der Genexpression von bekannten IFNβ verbundenen Genen (ISG), zum Beispiel STAT1, IFIT1, IFI44L aber auch IFNβ selbst. Zuerst haben wir gezeigt, dass eine IFNβ Behandlung von Osteoblasten zu einer Hemmung der Mineralisierung führt. Genauso wie die Verstärkung der Mineralisierung durch 1,25D3 war die Hemmung durch IFNβ zeitabhängig. Eine einwöchige Behandlung, von Tag 0 an beginnend, führte zu einer 40-60%igen Reduzierung der Mineralisierung. Eine einwöchige Behandlung, die am 7. Tag begann, führte jedoch nur zu einer Reduzierung von 20-30%. Eine IFNβ Behandlung von Mesenchymalen Stammzellen (MSC), die in Osteoblasten differenziert wurden, hatte keine Auswirkung auf die Mineralisierung.

Wenn die MSCs jedoch zuerst den Stimulus zur Osteoblastdifferenzierung bekommen haben und danach 48 Stunden später mit IFNβ behandelt wurden, wurde die Mineralisierung gehemmt.

Diese Ergebnisse verdeutlichen, dass die frühe Phase von Osteoblastdifferenzierung besonders sensibel für eine IFNβ Behandlung und damit auch die Mineralisierungshemmung ist.

Auffallend ist, dass eine einzelne IFNβ Behandlung dieselbe Hemmung erzielt wie eine mehrmalige IFNβ Behandlung. Ein Grund dafür könnte eine Desensibilisierung der Osteoblasten sein. Des Weiteren haben wir nach den möglichen Mechanismen der Hemmung gesucht. Wir konnten zeigen, dass IFNβ die durch Osteoblasten produzierte ECM angreift. Dies geschieht während der frühen Phase der Osteoblastdifferenzierung durch eine verminderte Genexpression von mehreren ECM verbundenen Genen (zum Beispiel: Fibronectin (FN1), Fibulin 1 (FBLN1), Firbrillin 2 (FBN2) and Laminin 1 (LAMA1)).

Darüber hinaus beinhaltet die durch IFNβ beeinflusste und durch Osteoblasten produzierte ECM weniger Kollagen-Protein. Interessanterweise konnten wir auch aufzeigen, dass die IFNβ Behandlung auch in eine Erhöhung der Genexpression von Osteopontin (SPP1), Annexin 2 (ANXA2) und Hyaluron Synthase 1 (HAS1) führte. Diese Gene sind wichtige Faktoren für die Haftung

von hämatopoietischen Stammzellen (HSC) an ihrer Nische. Diese Beobachtung bringt die erzielten Resultate der IFNβ Behandlung von frühen Osteoblasten in eine andere Perspektive und weist auf einen HSC-unterstützenden Phänotypen hin. Weitere Studien müssen folgen, um die Rolle von IFNβ in frühen Osteoblasten mit Bezug auf die HSC Nische zu verstehen.

Nachdem wir die Effekte von IFNβ auf Osteoblasten und Mineralisierung besser verstanden haben, haben wir begonnen das Zusammenspiel zwischen 1,25D3 und IFNβ zu untersuchen. In **Kapitel 3** haben wir gezeigt, dass 20% aller durch 1,25D3 herunterregulierten Gene mit dem IFNβ Signalweg verbunden sind. 1,25D3 verminderte hauptsächlich in der Prä-Mineralisierungsphase die Genexpression von ISGs. Wir vermuteten ein Zusammenspiel zwischen 1,25D3 und IFNβ mit Beziehung zur Mineralisierung.

IFNβ hatte keinen Einfluss auf die durch 1,25D3 verstärkte Mineralisierung aber 1,25D3 beschützte mehrere ECM verbundene Gene vor einer durch IFNβ vermittelten Herunterregulierung der Genexpression. Daraus folgerten wir, dass in Beziehung zur Knochenbildung 1,25D3 dominant über IFNβ ist. Um tiefergehende Einsichten zu erlangen, haben wir anschließend ein Genexpressionsprofil der Prä-Mineralisierungsphase erstellt.

Während der Analyse haben wir unser Augenmerk auf Gene gerichtet die entweder mit dem Expressionsprofil von interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) korrelieren oder anti-korrelieren, gerichtet.

An zwei Zeitpunkten, 2 und 24 Stunden, sammelten wir Osteoblastkulturen, die entweder mit Kontrolle, 1,25D3, IFNβ oder einer Kombination aus 1,25D3 und IFNβ behandelt waren, ein.

Diese Annäherung erlaubte uns die Identifizierung von spezifischen Prozessen, die durch IFNβ und 1,25D3 in Osteoblasten beeinflusst werden. Wir haben 144 Gene identifiziert, von denen 117 korrelierten und 27 anti-korrelierten mit IFIT1.

Bioinformatische Analysen assoziierten diese Gene mit der negativen Regulierung von proteinmetabolische Prozessen und den zellulären Komponenten „organelle lumen". Weitere Analysen mit IPA (Ingenuity Systems, www.ingenuity.com) assoziierten dieselben Gene mit „Protein Ubiquination". Interessanterweise hat IPA die Mehrheit der 144 Gene mit „Connective Tissue Development" verbinden können. Darunter fallen eine Vielzahl von Prozessen worunter auch Osteoblastdifferenzierung ist. Obwohl wir das Zusammenspiel zwischen 1,25D3 und IFNβ mit Beziehung zur Mineralisierung verdeutlich haben und auch eine Anzahl unterliegende Gene identifiziert haben, müssen noch weitere Studien erfolgen, um den genauen Mechanismus zu identifizieren.

Während der Analysen des 1,25D3 Gene Chip Profils der Osteoblastdifferenzierung haben wir eine durch 1,25D3 verursachte Erhöhung des Activin A Transkripts (INHBA) beobachtet. Activin A ist ein bekannter starker Hemmer der Mineralisierung. 1,25D3 verstärkt die Mineralisierung, aber es gibt auch Literatur die zeigt, dass 1,25D3 Mineralisierungshemmer induziert (zum Beispiel BGLAP). Wir haben das Zusammenspiel zwischen 1,25D3 und Activin A im **Kapitel 4** untersucht.

In der Prä-Mineralisierungsphase erhöht 1,25D3 die Produktion von Activin A und vermindert die Produktion von Follistatin, ein endogener Hemmer von Activin A. Diese Verschiebungen im Activin A/Follistatin Verhältnis führte zu einer erhöhten Activin A Signalweiterleitung, was wir anhand eines Luziferasekonstruktes bewiesen haben. Des Weiteren wurden die erwarteten Veränderungen in der Genexpression von den durch Activin A regulierten Genen SMAD7 und CLEC3B beobachtet. Weil Activin A ein Mineralisierungshemmer ist, haben wir die Vermutung aufgestellt, dass die 1,25D3 verstärkte Mineralisierung zugleich durch Activin A gehemmt wird.

Eine gleichzeitige Behandlung von Osteoblasten mit 1,25D3 und Follistatin resultierte in eine noch stärkere Mineralisierung als durch 1,25D3 allein, was unsere Hypothese bestätigte. Wir spekulierten, dass 1,25D3 durch die Induktion von Activin A den Grad der Mineralisierung kontrolliert. Vergleichbare Ergebnisse haben wir in anderen Experimenten mit einen anderen Mineralisierungshemmer, Osteocalcin (BGLAP), erzielt. Durch das Verhindern der γ –Carboxylierung von Osteocalcin hemmt Warfarin dessen Aktivität und eine Behandlung von Osteoblasten mit Warfarin führte folgerichtig auch zu einer Mineralisierungsverstärkung.

Interessanterweise hat das Blockieren der Activin A Signalweiterleitung in 1,25D3 behandelte Osteoblasten zu einer synergistischen Erhöhung der BGLAP Genexpression geführt. Daraus folgt, dass Activin A die BGLAP Genexpression in 1,25D3 behandelte Osteoblasten hemmt. Diese Ergebnisse unterstreichen die Anwesenheit eines Kontrollmechanismuses, mit Activin A und BGLAP als Teilnehmer, in 1,25D3 behandelte Osteoblasten. Dieser Kontrollmechanismus könnte eine unkontrollierte, exzessive Mineralisierung durch 1,25D3 verhindern.

Diese Doktorarbeit war Unterteil des NucSys Training Netzwerks, welches auf nukleare Rezeptoren fokussiert ist.

Kapitel 5 basiert auf einer Zusammenarbeit innerhalb dieses Netzwerkes. Der Vitamin D Rezeptor (VDR) und der Peroxisome Proliferator-activated Rezeptor γ (PPARγ) sind durch Liganden aktivierte nukleare Transkriptionsfaktoren, die beide mit dem Retinoid X Rezeptor (RXR) heterodimerisieren. Ein Ligand von PPARγ ist Rosiglitazone, welcher bereits als Arzneimittel für Diabetes verschrieben wurde. In klinischen Studien sind ein erhöhtes Risiko für Knochenbrüche und ein negativer Effekt auf die

Knochenmasse als mögliche Nebenwirkungen des Arzneimittels beschrieben. *In vitro* hat Rosiglitazone die Osteoblastdifferenzierung beschleunigt und die Mineralisierung verstärkt, jedoch wurden auch mitochondriale Störungen und ein verfrühter Zelltod festgestellt. Unser Ziel war die Untersuchung von Effekten von Rosiglitazone auf 1,25D3 behandelte Osteoblasten.

Am Beginn der Mineralisierung haben wir, im Vergleich zur Kontrolle, eine erhöhte ALP Aktivität festgestellt. Dies weist auf eine bereits weiter fortgeschrittene Differenzierung von Osteoblasten, die mit 1,25D3 und Rosiglitazone behandelt sind, hin. Weiter resultierte dieselbe Doppelbehandlung in eine synergistische Verstärkung der Mineralisierung im Vergleich zur 1,25D3 verstärkten Mineralisierung. 1,25D3 erhöht die Genexpression von BGLAP und am Beginn der Mineralisierung zeigten wir, dass die Genexpression von BGLAP in Osteoblasten, die mit 1,25D3 und Rosiglitazone behandelt waren, signifikant niedriger war als in 1,25D3 behandelte Osteoblasten. In **Kapitel 4** hat die Hemmung von BGLAP zu einer weiteren Verstärkung der 1,25D3 stimulierten Mineralisierung geführt. Dadurch stellten wir die folgende Hypothese auf: Die Verstärkung der Mineralisierung in Rosiglitazone/1,25D3 Osteoblastkulturen könnte auf eine verminderte BGLAP Genexpression basiert zurück zu führen sein. Weitere Studien müssten durchgeführt werden, um diese Ergebnisse zu bestätigen.

Im **Kapitel 6** sind die wichtigsten Ergebnisse aus dieser Doktorarbeit mit Hinsicht auf den wissenschaftlichen Forschungsstand diskutiert. Zusammenfassend hat diese Doktorarbeit neue Erkenntnisse in der die durch 1,25D3 vermittelte Kontrolle von Osteoblastdifferenzierung, Funktion und schlussendlich auch Mineralisierung der ECM gebracht. Wir haben gezeigt, dass das Timing einer 1,25D3 und IFNβ Behandlung von großer Wichtigkeit für die schlussendlichen Effekte auf die Mineralisierung ist. Des Weiteren haben wir Arbeitsmodelle für unterliegende Mechanismen und Prozesse der 1,25D3 verstärkten Mineralisierung aufgestellt. Die verstärkte Produktion von reifen MVs durch 1,25D3 unterstützt die Wichtigkeit dieser subzellulären Strukturen und könnte ein Ausgangspunkt zu neuen Studien mit Bezug zur Initiierung der Mineralisierung sein. Auch haben wir IFNβ als neuen Hemmer der Mineralisierung identifiziert. Außerdem haben wir in frühen Osteoblasten Anzeichen gefunden, die auf eine Rolle von IFNβ bei der Anhaftung von HSCs in ihrer Nische hinweisen. Die Komplexität der Prozesse, die der 1,25D3 verstärkten Mineralisierung unterliegen, wird deutlich wenn man das Zusammenspiel von 1,25D3 mit Mineralisierungshemmern (z. B. BGLAP, Activin A, IFNβ oder auch Rosiglitazone) oder das mögliche Zusammenspiel von VDR mit anderen Transskriptionsfaktoren (z. B. RUNX2, SMAD3, STAT1 oder PPARγ) betrachtet.

Diese Doktorarbeit hat neue Erkenntnisse in die Handlungen von 1,25D3 auf humane Osteoblasten, der Mineralisierung und dem Zusammenspiel mit einer Anzahl von entgegenwirkenden regulatorischen Faktoren gebracht. Weitere

Studien müssen tiefere Einblicke in die komplizierten Prozesse bringen. Es ist eine große Herausforderung für zukünftige Studien, das Zusammenspiel mit systembiologischen Techniken quantitativ zu erforschen. Hierdurch könnten bedeutende und kritische Mechanismen für die Osteoblastdifferenzierung und Knochenbildungen identifiziert werden. Diese Mechanismen könnten mögliche Angriffspunkte für zukünftige Therapien von Knochenkrankheiten sein.

Abbreviations

1,25D3	1α,25-dihydroxyvitamin D3
$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D3
$25(OH)D_3$	25-hydroxyvitamin D3
ACVR1B	activin A receptor type 1B
ALPL	alkaline phosphatase
ANGPTL4	angiopoietin-like 4
ANXA2	annexin 2
BGLAP	osteocalcin
BMP	bone morphogenic proteins
BMU	basic multicellular unit
$(CAGA)_{12}$	12 tandem copies of the CAGA Smad binding element
CD45	cluster of differentiation 45
c-Fms	colony stimulating factor 1 receptor
CLEC3B	tetranectin
COL1A1	collagen type 1 alpha 1
CPS	counts per second
CYP24A1	24-hydroxylase
CYP27B1	1α-hydroxylase
CYP2R1	25-hydroxylase
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBP	vitamin D binding protein
DCN	Decorin
DDX52	DEAD Box polypeptide 58
dex	dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
ECM	extracellular matrix
EGF	Epidermal growth factor
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
ELF-97	phosphatase substrate
ELISA	enzyme-linked immunosorbent assay
FABP4	fatty acid binding protein 4
FACS	fluorescence activated cell sorting
FBLN1	fibulin 1
FBN2	fibrillin 2
FDA	Food and Drug Administration
FGF2	fibroblast growth factor 2
FGF8	fibroblast growth factor 8
FN1	fibronectin
FST	follistatin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GO	gene ontology
HA	hyoluronan
HAS1	hyaluronan synthase 1
HAS3	hyaluronan synthase 3

HEK293F	sub-type of human embryonic kidney 293 cells
HI-FCS	Heat Inactivated Fetal Calf Serum
HSC	hematopoietic stem cell
HUGO	Human Genome Organisation
IBSP	bone sialoprotein
ICAM1	inter-cellular-adhesion molecule 1
IFI	interferon-induced proteins
IFI27	interferon, alpha-inducible protein 27
IFI44	interferon-induced protein 44
IFI44L	interferon-induced protein 44-like
IFI6	interferon alpha-inducible protein 6
IFIH1	interferon induced with helicase C domain 1
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
IFN	Interferon
IFNA1	interferon alpha 1
IFNAR1	IFN α/β receptor 1
IFNAR2	IFN α/β receptor 2
IFNB1	interferon beta 1
IFNG	interferon gamma
IFNGR	IFN γ receptor
IFNα	interferon alpha
IFNβ	interferon beta
IFNγ	interferon gamma
IL	interleukin
INHBA	Inhibin, beta A
IPA	Ingenuity Systems Pathway Analysis
IRF	interferon regulatory factor
ISG	interferon target genes
ISGF3	interferon-stimulated gene factor 3
LAMA1	laminin alpha 1
MC3T3	nontransformed murine preosteoblastic cell line
M-CSF	macrophage colony stimulating factor
MG63	human osteosarcoma cell line
MS	multiple sclerosis
MSC	mesenchymal stem cells
MV	matrix vesicle
MX2	myxovirus resistance 2
Nhost	normal human osteoblast cell line
OAS1	2',5'-oligoadenylate synthetase 1
OAS2	2',5'-oligoadenylate synthetase 2
OPG	osteoprotegerin
OPN	osteopontin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pi	inorganic phosphate
PIAS1	protein inhibitor of activated STAT 1
PLSCR1	phospholipid scramblase 1
PNP	para-nitrophenol

PNPP	*para*-Nitrophenylphosphate
PODN	Podocan
PPARA	peroxisome proliferator-activated receptor α
PPARγ	peroxisome proliferator-activated receptor γ
PPARD	peroxisome proliferator-activated receptor δ
PPi	pyrophosphates
PTH	parathyroid hormone
PTPN11	Tyrosine-protein phosphatase non-receptor type 11
PTPN2	Tyrosine-protein phosphatase non-receptor type 2
QPCR	quantitative real time polymerase chain reaction
RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
rosi	rosiglitazone
RSAD2	radical S-adenosyl methionine domain containing 2
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
RXR	retinoid X receptor
SAMDH1	SAM domain and HD domain 1
SDF1	stromal-cell-derived factor 1
SMAD2	mothers against decapentaplegic homolog 2
SMAD3	mothers against decapentaplegic homolog 3
SMAD6	mothers against decapentaplegic homolog 6
SMAD7	mothers against decapentaplegic homolog 7
SOCS1	suppressor of cytokine signaling 1
SOCS3	suppressor of cytokine signaling 3
SP7	osterix
SPP1	osteopontin
STAT1	signal transducers and activators of transcription 1
STAT2	signal transducers and activators of transcription 2
SV-HFO	simian virus 40 immortalized - human fetal osteoblast cell line
T/C	treatment over control
TEM	transmission electron microscopy
TGF	transforming growth factor
TLR	toll-like receptor
TZD	thiazolidinediones
VCAM1	vacular-cellular-adhesion molecule 1
VDR	vitamin D receptor
VDRE	vitamin D response elements
αMEM	Minimum Essential Medium *Alpha*

Supplementary Data

Chapter 1

Table S1: 470 genes being regulated by 1,25D3 during the mineralization period (days 12 and 19).

Gene Symbol	Gene Title	fold change	Probe Set ID
ZNF462	zinc finger protein 462	2.23	226575_at
SRRM2	serine/arginine repetitive matrix 2	2.22	207435_s_at
ASH1L	ash1 (absent, small, or homeotic)-like (Drosophila)	2.21	222667_s_at
FLJ13725	hypothetical protein FLJ13725	2.15	45749_at
EP300	E1A binding protein p300	2.05	202221_s_at
NFIC	nuclear factor I/C (CCAAT-binding transcription factor)	2.05	226377_at, 226895_at
NCOR2	nuclear receptor co-repressor 2	1.96	207760_s_at
DNCLI2	dynein, cytoplasmic, light intermediate polypeptide 2	1.96	203590_at
SRRM1	serine/arginine repetitive matrix 1	1.94	201224_s_at
CASP2	caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2)	1.91	209811_at
SESN2	sestrin 2	1.90	223195_s_at
GNAS	GNAS complex locus	1.90	217673_x_at
SPTAN1	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	1.90	215235_at
TEB4	similar to S. cerevisiae SSM4	1.88	201737_s_at
BGN	biglycan	1.86	213905_x_at
LARP	likely ortholog of mouse la related protein	1.84	212193_s_at, 210966_x_at
CIC	capicua homolog (Drosophila)	1.84	212784_at
FBXO13	F-box only protein 13	1.84	227203_at
TPR	translocated promoter region (to activated MET oncogene)	1.82	201730_s_at
GOSR1	golgi SNAP receptor complex member 1	1.80	204630_s_at
PURB	purine-rich element binding protein B	1.80	226762_at
XTP2	HBxAg transactivated protein 2	1.79	211948_x_at
RRBP1	ribosome binding protein 1 homolog 180kDa (dog)	1.79	201204_s_at
SHARP	SMART/HDAC1 assciated repressor protein	1.78	1556059_s_at
BNC2	basonuclin 2	1.77	230722_at
CBLL1	Cas-Br-M (murine) ecotropic retroviral transforming sequence-like 1	1.77	220018_at
ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	1.77	218501_at
CDC42BPB	CDC42 binding protein kinase beta (DMPK-like)	1.77	217849_s_at
KIAA1190	hypothetical protein KIAA1190	1.76	226484_at
EIF2C2	eukaryotic translation initiation factor 2C, 2	1.76	225827_at
SMARCC2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	1.76	201321_s_at
LOC340318	hypothetical protein LOC340318	1.75	213360_s_at
DKFZp762 N1910	hypothetical protein DKFZp762N1910	1.75	222264_at
MAP1B	microtubule-associated protein 1B	1.75	226084_at
KIAA0460	KIAA0460 protein	1.74	212553_at
IKBKAP	inhibitor of kappa light polypeptide gene enhancer in B-cells,	1.74	202491_s_at

	kinase complex-associated protein		
GAL3ST4	galactose-3-O-sulfotransferase 4	1.73	219815_at
DDX6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	1.72	1562836_at
SLC6A8	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	1.72	202219_at
BRD2	bromodomain containing 2	1.72	208685_x_at
BCL9L	B-cell CLL/lymphoma 9-like	1.71	228065_at
GK001	GK001 protein	1.70	217814_at
KIAA1005	KIAA1005 protein	1.70	213959_s_at
PRKCSH	protein kinase C substrate 80K-H	1.69	200707_at, 214080_x_at
PML	promyelocytic leukemia	1.69	211013_x_at, 209640_at
TTYH3	tweety homolog 3 (Drosophila)	1.69	224674_at
NLGN2	neuroligin 2	1.69	226288_s_at
SKI	v-ski sarcoma viral oncogene homolog (avian)	1.69	229265_at
EHD2	EH-domain containing 2	1.69	45297_at, 221870_at
ZNF574	zinc finger protein 574	1.68	218762_at
UBE1	ubiquitin-activating enzyme E1 (A1S9T and BN75 temperature sensitivity complementing)	1.68	200964_at
ACTN1	actinin, alpha 1	1.68	211160_x_at
LOC201194	hypothetical protein LOC201194	1.68	227377_at
CHERP	calcium homeostasis endoplasmic reticulum protein	1.68	202230_s_at
NCOA5	nuclear receptor coactivator 5	1.66	225145_at
PELP1	proline-, glutamic acid-, leucine-rich protein 1	1.66	215354_s_at
LOC284058	hypothetical protein LOC284058	1.66	225117_at
ASXL2	additional sex combs like 2 (Drosophila)	1.66	218659_at
SF3A1	splicing factor 3a, subunit 1, 120kDa	1.66	201356_at
ANTXR1	anthrax toxin receptor 1	1.65	224694_at
LOC339047	hypothetical protein LOC339047	1.65	214870_x_at
CSK	c-src tyrosine kinase	1.64	202329_at
BCOR	BCL6 co-repressor	1.64	219433_at
SET	SET translocation (myeloid leukemia-associated)	1.64	200630_x_at
UBE2R2	ubiquitin-conjugating enzyme E2R 2	1.64	223014_at
EMILIN1	elastin microfibril interfacer 1	1.64	204163_at
PDE4A	phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunce homolog, Drosophila)	1.64	204735_at
BAG3	BCL2-associated athanogene 3	1.63	217911_s_at
ATBF1	AT-binding transcription factor 1	1.63	235785_at
HIP1	huntingtin interacting protein 1	1.63	226364_at
LPIN3	lipin 3	1.63	225031_at
DKFZp761I2123	hypothetical protein DKFZp761I2123	1.62	54970_at
MARK4	MAP/microtubule affinity-regulating kinase 4	1.62	55065_at
TIP120A	TBP-interacting protein	1.61	243910_x_at
PHACTR2	phosphatase and actin regulator 2	1.61	204048_s_at
EIF4G3	eukaryotic translation initiation factor 4 gamma, 3	1.61	201936_s_at
KIAA0179	KIAA0179	1.61	212846_at
CAD	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	1.61	202715_at
MRC2	mannose receptor, C type 2	1.60	37408_at
CREB3L1	cAMP responsive element binding protein 3-like 1	1.60	213059_at

FLNA	filamin A, alpha (actin binding protein 280)	1.60	200859_x_at, 213746_s_at
C20orf67	chromosome 20 open reading frame 67	1.60	221762_s_at
RAI	RelA-associated inhibitor	1.60	218849_s_at
MGC39830	hypothetical protein MGC39830	1.60	228248_at
SON	SON DNA binding protein	1.59	226465_s_at
TNRC11	trinucleotide repeat containing 11 (THR-associated protein, 230kDa subunit)	1.59	216071_x_at
PIP5K1C	phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	1.59	212518_at
PHF15	PHD finger protein 15	1.59	212660_at
GTF2I	general transcription factor II, i	1.59	210891_s_at
KIAA1450	KIAA1450 protein	1.59	225924_at
BAT2	HLA-B associated transcript 2	1.58	208132_x_at
FLJ35036	hypothetical protein FLJ35036	1.58	225512_at
PRKAG1	protein kinase, AMP-activated, gamma 1 non-catalytic subunit	1.58	227527_at
GNA11	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	1.58	213766_x_at
TBC1D4	TBC1 domain family, member 4	1.58	203386_at
ZNF281	zinc finger protein 281	1.58	222619_at
STAG1	stromal antigen 1	1.58	202294_at
EHMT1	euchromatic histone methyltransferase 1	1.58	225461_at
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	1.57	208794_s_at, 213720_s_at
LAMC1	laminin, gamma 1 (formerly LAMB2)	1.57	200771_at
C6orf63	chromosome 6 open reading frame 63	1.57	226490_at
D2S448	Melanoma associated gene	1.57	212013_at
HCFC1	host cell factor C1 (VP16-accessory protein)	1.57	202474_s_at
KIAA0999	KIAA0999 protein	1.57	204155_s_at
FLJ10315	hypothetical protein FLJ10315	1.57	218770_s_at
RIPX	rap2 interacting protein x	1.56	213939_s_at
CCNK	cyclin K	1.56	225824_at
FLJ38725	hypothetical protein FLJ38725	1.56	228937_at
TPX2	TPX2, microtubule-associated protein homolog (Xenopus laevis)	1.56	210052_s_at
FBXO9	F-box only protein 9	1.56	212218_s_at
FBN1	fibrillin 1 (Marfan syndrome)	1.56	202766_s_at
C6.1A	c6.1A	1.56	221196_x_at
SLC38A1	solute carrier family 38, member 1	1.56	218237_s_at
PURA	purine-rich element binding protein A	1.55	204021_s_at
USP31	ubiquitin specific protease 31	1.55	226035_at
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.55	201983_s_at, 201984_s_at
MYH10	myosin, heavy polypeptide 10, non-muscle	1.55	212372_at
RNF153	ring finger protein 153	1.55	226394_at
KIAA1724	selenoprotein I, 1	1.55	224888_at
COL6A2	collagen, type VI, alpha 2	1.54	209156_s_at
BIRC4	baculoviral IAP repeat-containing 4	1.54	225859_at, 225858_s_at
BRD3	bromodomain containing 3	1.54	203825_at
ELOVL4	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	1.54	219532_at
ZNF325	zinc finger protein 325	1.54	219571_s_at
EIF4G1	eukaryotic translation initiation factor 4 gamma, 1	1.54	208625_s_at

PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	1.53	228006_at
LOC132671	LOC132671	1.53	229331_at
UREB1	upstream regulatory element binding protein 1	1.53	208598_s_at
KIAA0194	KIAA0194 protein	1.53	34221_at
BAT3	HLA-B associated transcript 3	1.53	213318_s_at
SALL2	sal-like 2 (Drosophila)	1.53	213283_s_at
KLC2	likely ortholog of kinesin light chain 2	1.53	218906_x_at
CLSTN1	calsyntenin 1	1.53	201561_s_at
LOC146174	hypothetical protein LOC146174	1.53	230296_at
ODAG	ocular development-associated gene	1.53	214718_at
USP7	ubiquitin specific protease 7 (herpes virus-associated)	1.52	201499_s_at
UBE2L3	ubiquitin-conjugating enzyme E2L 3	1.52	200676_s_at
ETV6	ets variant gene 6 (TEL oncogene)	1.52	225764_at
GSPT1	G1 to S phase transition 1	1.51	225276_at
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	1.51	203332_s_at
HSPA4	heat shock 70kDa protein 4	1.51	208814_at
BMP1	bone morphogenetic protein 1	1.51	202701_at
EP400	E1A binding protein p400	1.51	212376_s_at
KIAA0310	KIAA0310	1.51	215696_s_at
COL5A1	collagen, type V, alpha 1	1.51	203325_s_at
DNM2	dynamin 2	1.50	202253_s_at
KIAA0143	KIAA0143 protein	1.50	212150_at
RBAF600	retinoblastoma-associated factor 600	1.50	211950_at
SCA2	spinocerebellar ataxia 2 (olivopontocerebellar ataxia 2, autosomal dominant, ataxin 2)	1.50	202622_s_at
RBM10	RNA binding motif protein 10	1.50	215089_s_at
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	1.50	203505_at
NCOR1	nuclear receptor co-repressor 1	1.50	200854_at
TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)	1.50	203085_s_at
CTSD	cathepsin D (lysosomal aspartyl protease)	1.50	200766_at
FLJ10647	hypothetical protein FLJ10647	-1.52	221710_x_at
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	-1.52	201227_s_at
C15orf12	chromosome 15 open reading frame 12	-1.52	221688_s_at
CSRP2	cysteine and glycine-rich protein 2	-1.52	207030_s_at
NEK2	NIMA (never in mitosis gene a)-related kinase 2	-1.52	211080_s_at
FLJ32421	hypothetical protein FLJ32421	-1.52	241908_at
HLA-G	HLA-G histocompatibility antigen, class I, G	-1.52	211530_x_at
LANCL1	LanC lantibiotic synthetase component C-like 1 (bacterial)	-1.52	202019_s_at
NCB5OR	NADPH cytochrome B5 oxidoreductase	-1.52	219079_at
ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	-1.52	205176_s_at
C20orf43	chromosome 20 open reading frame 43	-1.52	234926_s_at
HSPC133	HSPC133 protein	-1.52	221570_s_at
EIF2B3	eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	-1.52	218488_at
ULBP2	UL16 binding protein 2	-1.52	221291_at
GAS5	growth arrest-specific 5	-1.52	224741_x_at
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	-1.52	210453_x_at
C21orf33	chromosome 21 open reading frame 33	-1.52	210667_s_at

PRIM1	primase, polypeptide 1, 49kDa	-1.52	205053_at
LYPLA2	lysophospholipase II	-1.52	202292_x_at
C6orf162	chromosome 6 open reading frame 162	-1.52	213312_at
MRPL51	mitochondrial ribosomal protein L51	-1.53	223086_x_at
FLJ14346	hypothetical protein FLJ14346	-1.53	212995_x_at
JAK1	Janus kinase 1 (a protein tyrosine kinase)	-1.53	1552611_a_at
BID	BH3 interacting domain death agonist	-1.53	227143_s_at
KIAA1648	KIAA1648 protein	-1.53	244189_at
CDA11	CDA11 protein	-1.53	223231_at
SAE1	SUMO-1 activating enzyme subunit 1	-1.53	1555618_s_at
PSMC2	proteasome (prosome, macropain) 26S subunit, ATPase, 2	-1.53	201068_s_at
HSPCA	heat shock 90kDa protein 1, alpha	-1.53	211968_s_at
JWA	cytoskeleton related vitamin A responsive protein	-1.53	200760_s_at
MRPS18B	mitochondrial ribosomal protein S18B	-1.53	208907_s_at
SS18L2	synovial sarcoma translocation gene on chromosome 18-like 2	-1.53	218283_at
SARA2	SAR1a gene homolog 2 (S. cerevisiae)	-1.53	1554482_a_at
MRPL1	mitochondrial ribosomal protein L1	-1.53	223154_at
SKP2	S-phase kinase-associated protein 2 (p45)	-1.53	203626_s_at, 210567_s_at
NEDD5	neural precursor cell expressed, developmentally down-regulated 5	-1.54	1554747_a_at
KCTD5	potassium channel tetramerisation domain containing 5	-1.54	222645_s_at
MUTED	muted homolog (mouse)	-1.54	226543_at
BAG1	BCL2-associated athanogene	-1.54	211475_s_at
PSME4	proteasome (prosome, macropain) activator subunit 4	-1.54	212220_at
FLJ11753	glycosyltransferase-like 1	-1.54	238585_at
Ufm1	ubiquitin-fold modifier 1	-1.54	222502_s_at
ARL4A	ADP-ribosylation factor-like 4A	-1.54	205020_s_at
MRS2L	MRS2-like, magnesium homeostasis factor (S. cerevisiae)	-1.54	228542_at
MAT2A	methionine adenosyltransferase II, alpha	-1.54	200769_s_at
PPP1CB	protein phosphatase 1, catalytic subunit, beta isoform	-1.54	228222_at
FLJ22688	hypothetical protein FLJ22688	-1.54	221187_s_at
KIAA0141	KIAA0141 gene product	-1.54	227056_at
ATPIF1	ATPase inhibitory factor 1	-1.54	218671_s_at
MRPL48	mitochondrial ribosomal protein L48	-1.54	218281_at
CLIC4	chloride intracellular channel 4	-1.54	201559_s_at
PSMA2	proteasome (prosome, macropain) subunit, alpha type, 2	-1.54	201317_s_at
AK2	adenylate kinase 2	-1.54	212173_at
SGTA	small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha	-1.54	201396_s_at
HSPC152	hypothetical protein HSPC152	-1.55	217774_s_at
SLC25A14	solute carrier family 25 (mitochondrial carrier, brain), member 14	-1.55	211855_s_at
CETN3	centrin, EF-hand protein, 3 (CDC31 homolog, yeast)	-1.55	209662_at
MGC45386	Similar to RIKEN cDNA 1110033O09 gene	-1.55	227008_at
KIAA1143	KIAA1143 protein	-1.55	227493_s_at
MCM4	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	-1.55	212142_at
DKFZP547E1010	DKFZP547E1010 protein	-1.55	209927_s_at
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	-1.55	202647_s_at
C10orf86	chromosome 10 open reading frame 86	-1.55	211376_s_at
PRO1853	hypothetical protein PRO1853	-1.55	223560_s_at

ATF7	activating transcription factor 7	-1.55	228829_at
FLJ10036	hypothetical protein FLJ10036	-1.55	218349_s_at
USP1	ubiquitin specific protease 1	-1.55	202412_s_at
SYNGR1	synaptogyrin 1	-1.55	210613_s_at
RARSL	arginyl-tRNA synthetase-like	-1.55	232902_s_at
HMGB1	high-mobility group box 1	-1.55	216508_x_at
DRB1	developmentally regulated RNA-binding protein 1	-1.55	228578_at
DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)	-1.55	203258_at
PEX19	peroxisomal biogenesis factor 19	-1.55	201706_s_at
STK17B	serine/threonine kinase 17b (apoptosis-inducing)	-1.56	205214_at
APOC1	apolipoprotein C-I	-1.56	213553_x_at
SNX4	sorting nexin 4	-1.56	205329_s_at
C9orf156	chromosome 9 open reading frame 156	-1.56	222195_s_at
RPL31	ribosomal protein L31	-1.56	200963_x_at
C13orf12	chromosome 13 open reading frame 12	-1.56	217769_s_at
CCNE2	cyclin E2	-1.56	211814_s_at
EIF4E	eukaryotic translation initiation factor 4E	-1.56	201435_s_at
HNRPC	heterogeneous nuclear ribonucleoprotein C (C1/C2)	-1.56	200751_s_at
MGST3	microsomal glutathione S-transferase 3	-1.56	201403_s_at
HIG1	likely ortholog of mouse hypoxia induced gene 1	-1.56	221896_s_at
RPS7	ribosomal protein S7	-1.56	200082_s_at
ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C, isoform 1	-1.56	202874_s_at
ENTPD8	ectonucleoside triphosphate diphosphohydrolase 8	-1.56	1554327_a_at
MGC9084	hypothetical protein MGC9084	-1.56	213528_at
GAJ	GAJ protein	-1.56	223700_at
SNRPG	small nuclear ribonucleoprotein polypeptide G	-1.57	205644_s_at
MGC40397	hypothetical protein MGC40397	-1.57	226349_at
SLC25A26	solute carrier family 25, member 26	-1.57	225862_at
NICE-3	NICE-3 protein	-1.57	1555226_s_at
NQO1	NAD(P)H dehydrogenase, quinone 1	-1.57	201467_s_at, 210519_s_at
FLJ11305	hypothetical protein FLJ11305	-1.57	225149_at
M17S2	membrane component, chromosome 17, surface marker 2 (ovarian carcinoma antigen CA125)	-1.57	201383_s_at
SNRPN	small nuclear ribonucleoprotein polypeptide N	-1.57	206042_x_at
TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	-1.57	1558136_s_at
VPS24	vacuolar protein sorting 24 (yeast)	-1.57	222436_s_at
GSR	glutathione reductase	-1.58	205770_at
ATP8B2	ATPase, Class I, type 8B, member 2	-1.58	216873_s_at
PSMB4	proteasome (prosome, macropain) subunit, beta type, 4	-1.58	202243_s_at
PSMA4	proteasome (prosome, macropain) subunit, alpha type, 4	-1.58	203396_at
GNG12	guanine nucleotide binding protein (G protein), gamma 12	-1.58	1555240_s_at
ADORA2B	adenosine A2b receptor	-1.58	205891_at
HIST1H3H	histone 1, H3h	-1.58	206110_at
BAG4	BCL2-associated athanogene 4	-1.58	219624_at
KIAA0117	KIAA0117 protein	-1.58	212591_at
TCFL4	transcription factor-like 4	-1.58	217910_x_at
HSPC150	HSPC150 protein similar to ubiquitin-conjugating enzyme	-1.58	223229_at
RPS9	ribosomal protein S9	-1.58	214317_x_at
SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	-1.58	202690_s_at

HBLD1	HESB like domain containing 1	-1.58	226007_at
CRIP2	cysteine-rich protein 2	-1.58	208978_at
PRG1	proteoglycan 1, secretory granule	-1.59	201858_s_at
HSPC129	hypothetical protein HSPC129	-1.59	1555106_a_at
C11orf15	chromosome 11 open reading frame 15	-1.59	222507_s_at
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	-1.59	214995_s_at
HSPC121	butyrate-induced transcript 1	-1.59	217777_s_at
ZDHHC4	zinc finger, DHHC domain containing 4	-1.59	220261_s_at
NEUGRIN	mesenchymal stem cell protein DSC92	-1.59	224281_s_at
PIR	Pirin	-1.59	207469_s_at
DKFZp434 N2030	hypothetical protein DKFZp434N2030	-1.59	228378_at
HADHA	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, alpha subunit	-1.59	208629_s_at
	enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit		
PLEKHJ1	pleckstrin homology domain containing, family J member 1	-1.59	218290_at
GTF2F2	general transcription factor IIF, polypeptide 2, 30kDa	-1.59	209595_at
CHCHD5	coiled-coil-helix-coiled-coil-helix domain containing 5	-1.59	223479_s_at
EXOSC3	exosome component 3	-1.59	223490_s_at
GRINL1A	glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	-1.60	212241_at
FLJ22609	hypothetical protein FLJ22609	-1.60	219458_s_at
NUP43	nucleoporin 43kDa	-1.60	238474_at
MRPS22	mitochondrial ribosomal protein S22	-1.60	223448_x_at
HIATL2	hippocampus abundant gene transcript-like 2	-1.60	224078_at
C9orf150	chromosome 9 open reading frame 150	-1.60	227443_at
LEREPO4	likely ortholog of mouse immediate early response, erythropoietin 4	-1.60	201593_s_at
HINT1	histidine triad nucleotide binding protein 1	-1.60	207721_x_at, 200093_s_at, 208826_x_at
NFYB	nuclear transcription factor Y, beta	-1.60	218129_s_at
C9orf10	chromosome 9 open reading frame 10	-1.60	200767_s_at
cig5	viperin	-1.60	213797_at
FLJ20604	hypothetical protein FLJ20604	-1.60	219133_at
RRAS2	related RAS viral (r-ras) oncogene homolog 2	-1.61	208456_s_at
LOC147727	hypothetical protein LOC147727	-1.61	235014_at
COPS8	COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis)	-1.61	202143_s_at
SEPT8	septin 8	-1.61	209000_s_at
MRPL39	mitochondrial ribosomal protein L39	-1.61	218558_s_at
HAX1	HS1 binding protein	-1.61	201145_at
BBS4	Bardet-Biedl syndrome 4	-1.61	212745_s_at
SARA1	SAR1a gene homolog 1 (S. cerevisiae)	-1.61	210790_s_at
C3orf4	chromosome 3 open reading frame 4	-1.61	208925_at
TALDO1	transaldolase 1	-1.61	226227_x_at, 226835_s_at, 224915_x_at
RPS27L	ribosomal protein S27-like	-1.61	218007_s_at
C10orf84	chromosome 10 open reading frame 84	-1.61	222617_s_at
CUGBP1	CUG triplet repeat, RNA binding protein 1	-1.61	1555467_a_at
NIPSNAP3A	nipsnap homolog 3A (C. elegans)	-1.62	224436_s_at

NARG2	NMDA receptor-regulated gene 2	-1.62	235189_at
NDUFV3	NADH dehydrogenase (ubiquinone) flavoprotein 3, 10kDa	-1.62	226209_at
ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	-1.62	1555419_a_at
DEFA1	defensin, alpha 1, myeloid-related sequence	-1.62	205033_s_at
NPHP1	nephronophthisis 1 (juvenile)	-1.62	238844_s_at
F-LANa	carcinoma related gene	-1.62	218333_at
MRPS10	mitochondrial ribosomal protein S10	-1.62	222522_x_at, 224247_s_at
FLJ22353	hypothetical protein FLJ22353	-1.62	1554077_a_at
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	-1.62	200641_s_at
M6PR	mannose-6-phosphate receptor (cation dependent)	-1.62	200900_s_at
COX6C	cytochrome c oxidase subunit Vic	-1.62	201754_at
MGC874	hypothetical protein MGC874	-1.63	224177_s_at
POP5	processing of precursor 5, ribonuclease P/MRP subunit (S. cerevisiae)	-1.63	204839_at
C15orf15	chromosome 15 open reading frame 15	-1.63	217915_s_at, 222465_at
WDR1	WD repeat domain 1	-1.63	210935_s_at
GTSE1	G-2 and S-phase expressed 1	-1.63	204315_s_at
MGC14141	hypothetical protein MGC14141	-1.64	225568_at
BIC	BIC transcript	-1.64	229437_at
VMP1	likely ortholog of rat vacuole membrane protein 1	-1.64	224917_at
LOC147965	hypothetical protein LOC147965	-1.64	227951_s_at
FLJ22729	hypothetical protein FLJ22729	-1.64	219146_at
SLC39A6	solute carrier family 39 (zinc transporter), member 6	-1.64	1555460_a_at
SRA1	steroid receptor RNA activator 1	-1.64	224130_s_at
DYRK4	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4	-1.64	212954_at
HT007	uncharacterized hypothalamus protein HT007	-1.64	221622_s_at
GFER	growth factor, augmenter of liver regeneration (ERV1 homolog, S. cerevisiae)	-1.64	204659_s_at
PAIP2	poly(A) binding protein interacting protein 2	-1.65	222983_s_at
CIP29	cytokine induced protein 29 kDa	-1.65	224914_s_at
SHFM1	split hand/foot malformation (ectrodactyly) type 1	-1.65	202276_at
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	-1.65	1554768_a_at
C6orf175	chromosome 6 open reading frame 175	-1.65	213357_at
FAM33A	family with sequence similarity 33, member A	-1.65	225686_at
DDT	D-dopachrome tautomerase	-1.65	202929_s_at
PSEN1	presenilin 1 (Alzheimer disease 3)	-1.66	207782_s_at
PLA2G12A	phospholipase A2, group XIIA	-1.66	223373_s_at
LOC339231	hypothetical protein LOC339231	-1.66	234972_at
HSPC039	HSPC039 protein	-1.66	211406_at
LOC55954	hypothetical protein LOC55954	-1.66	218752_at
ATP5C1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1.66	208870_x_at
MFN2	mitofusin 2	-1.66	216205_s_at
NS	nucleostemin	-1.66	217850_at
YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	-1.66	210317_s_at
MAX	MAX protein	-1.66	210734_x_at
EFG1	mitochondrial elongation factor G1	-1.66	232296_s_at
KLHL12	kelch-like 12 (Drosophila)	-1.67	219931_s_at

HIST1H2AC	histone 1, H2ac	-1.67	215071_s_at
CDK2	cyclin-dependent kinase 2	-1.67	211804_s_at
LOC151194	similar to hepatocellular carcinoma-associated antigen HCA557b	-1.67	1553743_at
NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	-1.67	210048_at
MAGOH	mago-nashi homolog, proliferation-associated (Drosophila)	-1.67	210092_at
CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	-1.67	1558214_s_at
BCL2L1	BCL2-like 1	-1.68	215037_s_at
ZNF277	zinc finger protein (C2H2 type) 277	-1.68	1555193_a_at
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	-1.68	212105_s_at
OASL	2'-5'-oligoadenylate synthetase-like	-1.68	210797_s_at
VPS29	vacuolar protein sorting 29 (yeast)	-1.69	223026_s_at
FHL1	four and a half LIM domains 1	-1.69	201539_s_at
CGRRF1	cell growth regulator with ring finger domain 1	-1.69	204605_at
KIAA0276	KIAA0276 protein	-1.69	212853_at
C14orf92	chromosome 14 open reading frame 92	-1.69	201683_x_at
GGA2	golgi associated, gamma adaptin ear containing, ARF binding protein 2	-1.69	214190_x_at, 208915_s_at
MAP2K1IP1	mitogen-activated protein kinase kinase 1 interacting protein 1	-1.70	217971_at
AUP1	ancient ubiquitous protein 1	-1.70	220525_s_at
SAMHD1	SAM domain and HD domain 1	-1.70	1559883_s_at
G1P2	interferon, alpha-inducible protein (clone IFI-15K)	-1.70	205483_s_at
RPL36AL	ribosomal protein L36a-like	-1.70	201406_at
ZCCHC7	zinc finger, CCHC domain containing 7	-1.70	1555562_a_at
CCNDBP1	cyclin D-type binding-protein 1	-1.70	223084_s_at
PTK9	PTK9 protein tyrosine kinase 9	-1.71	214007_s_at
RNP24	coated vesicle membrane protein	-1.71	204426_at, 204427_s_at
P14	pre-mRNA branch site protein p14	-1.71	223416_at
TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa	-1.71	203893_at
HLA-C	major histocompatibility complex, class I, C	-1.71	210514_x_at
C2orf6	chromosome 2 open reading frame 6	-1.71	201299_s_at
PRKAB1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	-1.71	201835_s_at
NUDT2	nudix (nucleoside diphosphate linked moiety X)-type motif 2	-1.71	218609_s_at
RPL23	ribosomal protein L23	-1.72	200888_s_at
SFRS3	splicing factor, arginine/serine-rich 3	-1.72	202899_s_at
LGALS8	lectin, galactoside-binding, soluble, 8 (galectin 8)	-1.72	210732_s_at
TXNDC	thioredoxin domain containing	-1.72	208097_s_at
ATP6V1E1	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E isoform 1	-1.72	208678_at
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	-1.72	205071_x_at
MGC45594	hypothetical protein MGC45594	-1.73	1554239_s_at
CKS1B	CDC28 protein kinase regulatory subunit 1B	-1.73	201897_s_at
CWF19L1	CWF19-like 1, cell cycle control (S. pombe)	-1.73	233568_x_at
KBTBD2	kelch repeat and BTB (POZ) domain containing 2	-1.73	223584_s_at
EPRS	glutamyl-prolyl-tRNA synthetase	-1.74	200841_s_at
e(y)2	e(y)2 protein	-1.74	218482_at
BCAP29	B-cell receptor-associated protein 29	-1.74	205084_at
LOC200916	similar to ribosomal protein L22	-1.74	225541_at
ILF3	interleukin enhancer binding factor 3, 90kDa	-1.74	208930_s_at

KIF22	kinesin family member 22	-1.74	216969_s_at
MLSTD1	male sterility domain containing 1	-1.75	220615_s_at
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	-1.75	204285_s_at
GGCX	gamma-glutamyl carboxylase	-1.75	214006_s_at
NDUFA11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11, 14.7kDa	-1.76	225304_s_at
Dlc2	dynein light chain 2	-1.76	229106_at
C20orf111	chromosome 20 open reading frame 111	-1.76	209020_at
CTSL	cathepsin L	-1.77	1563445_x_at
PPIE	peptidylprolyl isomerase E (cyclophilin E)	-1.77	210502_s_at
RPL38	ribosomal protein L38	-1.77	221943_x_at
ZNF32	zinc finger protein 32 (KOX 30)	-1.78	209538_at
NDUFA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7, 14.5kDa	-1.78	202785_at
NUSAP1	nucleolar and spindle associated protein 1	-1.78	219978_s_at
LSM8	LSM8 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.78	219119_at
LOC93081	hypothetical protein BC015148	-1.78	213346_at
FLJ21908	hypothetical protein FLJ21908	-1.78	1557984_s_at
MGC16028	MGC16028 similar to RIKEN cDNA 1700019E19 gene	-1.79	226195_at
LMAN1	lectin, mannose-binding, 1	-1.79	203293_s_at
SPC18	signal peptidase complex (18kD)	-1.79	216274_s_at
COX17	COX17 homolog, cytochrome c oxidase assembly protein (yeast)	-1.79	203880_at
FXR1	fragile X mental retardation, autosomal homolog 1	-1.79	201635_s_at
SCAMP1	secretory carrier membrane protein 1	-1.79	206668_s_at, 1552978_a_at
PSPC1	paraspeckle component 1	-1.80	222611_s_at
RBX1	ring-box 1	-1.80	218117_at
FLOT1	flotillin 1	-1.82	208748_s_at
MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1	-1.82	210018_x_at
ADA	adenosine deaminase	-1.83	216705_s_at
PSME3	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	-1.83	209853_s_at, 200987_x_at
C21orf4	chromosome 21 open reading frame 4	-1.83	219600_s_at
AP4S1	adaptor-related protein complex 4, sigma 1 subunit	-1.83	210278_s_at
MED6	mediator of RNA polymerase II transcription, subunit 6 homolog (yeast)	-1.84	207079_s_at
MCP	membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)	-1.84	211574_s_at
FLJ20516	timeless-interacting protein	-1.84	219258_at
PC4	activated RNA polymerase II transcription cofactor 4	-1.84	214512_s_at
SERF1A	small EDRK-rich factor 1A (telomeric)	-1.85	223539_s_at
TIM14	homolog of yeast TIM14	-1.85	225359_at
SRPR	signal recognition particle receptor ('docking protein')	-1.87	200917_s_at
CASP1	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	-1.87	211367_s_at
VPS35	vacuolar protein sorting 35 (yeast)	-1.87	222387_s_at
C6orf125	chromosome 6 open reading frame 125	-1.87	224448_s_at
ZNF626	zinc finger protein 626	-1.88	1552643_at
ARPC4	actin related protein 2/3 complex, subunit 4, 20kDa	-1.89	217818_s_at, 211672_s_at

CDC40	cell division cycle 40 homolog (yeast)	-1.89	203377_s_at
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-1.89	202086_at
COX7C	cytochrome c oxidase subunit VIIc	-1.90	201134_x_at, 217491_x_at
C9orf83	chromosome 9 open reading frame 83	-1.90	219356_s_at, 218085_at
MRP63	mitochondrial ribosomal protein 63	-1.90	221995_s_at
COX7B	cytochrome c oxidase subunit VIIb	-1.90	202110_at
ANAPC10	anaphase promoting complex subunit 10	-1.91	207845_s_at
CHCHD7	coiled-coil-helix-coiled-coil-helix domain containing 7	-1.91	222701_s_at
COMMD6	COMM domain containing 6	-1.91	225312_at
PRIM2A	primase, polypeptide 2A, 58kDa	-1.92	1554885_a_at
CALD1	caldesmon 1	-1.94	201615_x_at
FLJ11193	hypothetical protein FLJ11193	-1.94	1552660_a_at
CAPZA1	capping protein (actin filament) muscle Z-line, alpha 1	-1.96	227364_at
RAB23	RAB23, member RAS oncogene family	-1.96	220955_x_at
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3	-1.97	201532_at
SNRPE	small nuclear ribonucleoprotein polypeptide E	-2.00	215450_at
MGC40157	hypothetical protein MGC40157	-2.00	225065_x_at
IFI44	interferon-induced protein 44	-2.01	214453_s_at
ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	-2.05	1554878_a_at
STAT1	signal transducer and activator of transcription 1, 91kDa	-2.06	AFFX-HUMISGF3A/M97935_5_at
HLA-F	major histocompatibility complex, class I, F	-2.07	229420_at
C11orf13	chromosome 11 open reading frame 13	-2.08	204927_at
LSM3	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-2.08	202209_at
SLC16A1	solute carrier family 16 (monocarboxylic acid transporters), member 1	-2.10	1557918_s_at
RP42	RP42 homolog	-2.12	222678_s_at
UQCRB	ubiquinol-cytochrome c reductase binding protein	-2.17	209066_x_at, 209065_at, 205849_s_at
NCKAP1	NCK-associated protein 1	-2.18	217465_at
SFRS10	splicing factor, arginine/serine-rich 10 (transformer 2 homolog, Drosophila)	-2.22	210180_s_at
OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	-2.30	205552_s_at

Table S2: Genes being regulated by 1,25D3 during the pre-mineralization period (days 3 and 7).

Gene Symbol	Gene Title	Fold change	Probe Set ID
FLVCR	feline leukemia virus subgroup C cellular receptor	1.82	222906_at
RPS6KA4	ribosomal protein S6 kinase, 90kDa, polypeptide 4	1.77	204632_at
DLEU2	deleted in lymphocytic leukemia, 2	1.71	1556821_x_at
VRK3	vaccinia related kinase 3	1.66	218022_at
RBPMS2	RNA-binding protein with multiple splicing 2	1.63	228802_at
CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	1.59	203794_at
TFCP2	Alpha-globin transcription factor CP2	1.59	227637_at
ZNF791	zinc finger protein 791	1.58	1553704_x_at
LOC283508	hypothetical protein LOC283508	1.58	227415_at
AP1GBP1	AP1 gamma subunit binding protein 1	1.56	221937_at
GBA2	glucosidase, beta (bile acid) 2	1.56	223921_s_at
MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha	1.55	224378_x_at
OSBPL5	oxysterol binding protein-like 5	1.55	233734_s_at
SAC3D1	SAC3 domain containing 1	1.54	205449_at
NUDT1	nudix (nucleoside diphosphate linked moiety X)-type motif 1	1.54	228231_at
TXN2	thioredoxin 2	1.54	209077_at
FLOT2	flotillin 2	1.53	201350_at
CENPV	centromere protein V	1.51	226610_at
EPB41L4B	erythrocyte membrane protein band 4.1 like 4B	1.51	220161_s_at
STX17	syntaxin 17	1.51	226662_at
PPP6C	protein phosphatase 6, catalytic subunit	1.50	225429_at
MAGI1	BAI1-associated protein 1	-1.52	225474_at
MGC33371	hypothetical protein MGC33371	-1.52	1553749_at
KIAA1524	KIAA1524 protein	-1.52	1553810_a_at
UBA6	ubiquitin-like modifier activating enzyme 6	-1.52	222602_at
TTC14	tetratricopeptide repeat domain 14	-1.53	225180_at
SFRS18	splicing factor, arginine/serine-rich 18	-1.53	212177_at
SAT	spermidine/spermine N1-acetyltransferase	-1.53	230333_at
NPIPL2	Nuclear pore complex-interacting protein-like 2 precursor	-1.53	221992_at
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	-1.54	202269_x_at
C9orf150	chromosome 9 open reading frame 150	-1.54	227443_at
RAB23	RAB23, member RAS oncogene family	-1.54	220955_x_at
TRPM7	transient receptor potential cation channel, subfamily M, member 7	-1.54	223324_s_at
ACTR8	ARP8 actin-related protein 8 homolog (yeast)	-1.54	218658_s_at
SIVA	CD27-binding (Siva) protein	-1.56	222030_at
TAF1B	TATA box binding protein (TBP)-associated factor, RNA polymerase I, B, 63kDa	-1.56	214690_at
CHD9	chromodomain helicase DNA binding protein 9	-1.56	212615_at
PARP3	poly (ADP-ribose) polymerase family, member 3	-1.56	209940_at
ALS2CR4	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4	-1.57	228255_at
HERC4	hect domain and RLD 4	-1.57	208055_s_at
KIAA1468	KIAA1468	-1.57	225506_at
UGCG	UDP-glucose ceramide glucosyltransferase	-1.58	204881_s_at
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	-1.58	206765_at
BRCA2	breast cancer 2, early onset	-1.59	214727_at

CLCA2	chloride channel, calcium activated, family member 2	-1.59	206165_s_at
WDR48	WD repeat domain 48	-1.59	221735_at
C4orf16	hypothetical protein PRO0971	-1.59	219023_at
E2F3	E2F transcription factor 3	-1.59	203692_s_at
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	-1.60	201447_at
ZC3H8	zinc finger CCCH type domain containing 8	-1.60	223506_at
SAMHD1	SAM domain and HD domain 1	-1.61	204502_at
ANKRD12	ankyrin repeat domain 12	-1.63	212286_at
SLC6A15	solute carrier family 6 (neurotransmitter transporter), member 15	-1.65	206376_at
RP11-248N6.3	lectin-like NK cell receptor	-1.65	216387_x_at
TRIM23	tripartite motif-containing 23	-1.65	210995_s_at
PPHLN1	periphilin 1	-1.66	227025_at
CCDC52	coiled-coil domain containing 52	-1.67	234995_at
LIN9	lin-9 homolog (C. elegans)	-1.67	235039_x_at
C1orf71	chromosome 1 open reading frame 71	-1.68	225551_at
CENPE	centromere protein E, 312kDa	-1.68	205046_at
SUMO1	SMT3 suppressor of mif two 3 homolog 1 (yeast)	-1.68	216556_x_at
RICTOR	RPTOR independent companion of MTOR, complex 2	-1.69	228248_at
MBNL2	muscleblind-like 2 (Drosophila)	-1.70	205018_s_at
KIAA1009	chromosome 6 open reading frame 84	-1.72	206005_s_at
WDR81	WD repeat domain 81	-1.72	226738_at
CEP290	centrosomal protein 290kDa	-1.73	205250_s_at
TIMP3	TIMP metallopeptidase inhibitor 3	-1.75	201149_s_at
ZBTB6	zinc finger and BTB domain containing 6	-1.78	206098_at
CHST15	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	-1.81	203066_at
SAMD9	sterile alpha motif domain containing 9	-1.82	219691_at
C20orf19	chromosome 20 open reading frame 19	-1.83	219961_s_at
BPAG1	bullous pemphigoid antigen 1, 230/240kDa	-1.88	212253_x_at
ARHGAP18	Rho GTPase activating protein 18	-1.89	225173_at
GLIPR1	GLI pathogenesis-related 1 (glioma)	-2.26	204221_x_at

Table S3: Genes being regulated by 1,25D3 during the pre-mineralization (early) and mineralization (late) period.

Gene Symbol	Gene Title	fold change early	fold change late	Probe Set ID
C9orf150	chromosome 9 open reading frame 150	-1.54	-1.60	227443_at
RAB23	RAB23, member RAS oncogene family	-1.54	-1.96	220955_x_at
SAMHD1	SAM domain and HD domain 1	-1.61	-1.70	204502_at

Table S4: Genes regulated by 1,25D3 in the mineralization period and being involved in the GO-term "RNA splicing" (GO:0008380).

Gene Symbol	Gene Title	Probe Set ID
CDC40	cell division cycle 40 homolog (S. cerevisiae)	203377_s_at
CUGBP1	CUG triplet repeat, RNA binding protein 1	1555467_a_at
HNRNPC	heterogeneous nuclear ribonucleoprotein C (C1/C2)	200751_s_at
LSM3	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)	202209_at
MAGOH	mago-nashi homolog, proliferation-associated (Drosophila)	210092_at
NAA38	LSM8 homolog, U6 small nuclear RNA associated (S. cerevisiae)	219119_at
PPIE	peptidylprolyl isomerase E (cyclophilin E)	210502_s_at
SF3A1	splicing factor 3a, subunit 1, 120kDa	201356_at, 216457_s_at, 227516_at
SF3B14	splicing factor 3B, 14 kDa subunit	223416_at
SFRS3	splicing factor, arginine/serine-rich 3	202899_s_at
SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	202690_s_at
SNRPG	small nuclear ribonucleoprotein polypeptide G	205644_s_at
SNRPN	small nuclear ribonucleoprotein polypeptide N	206042_x_at
SRRM1	serine/arginine repetitive matrix 1	201224_s_at
SRRM2	serine/arginine repetitive matrix 2	207435_s_at
TRA2B	transformer 2 beta homolog (Drosophila)	210180_s_at
ZMAT5	zinc finger, matrin type 5	218752_at

Table S5: Genes regulated by 1,25D3 in the mineralization period and being involved in the GO-term “cell cycle phase” (GO:0022403).

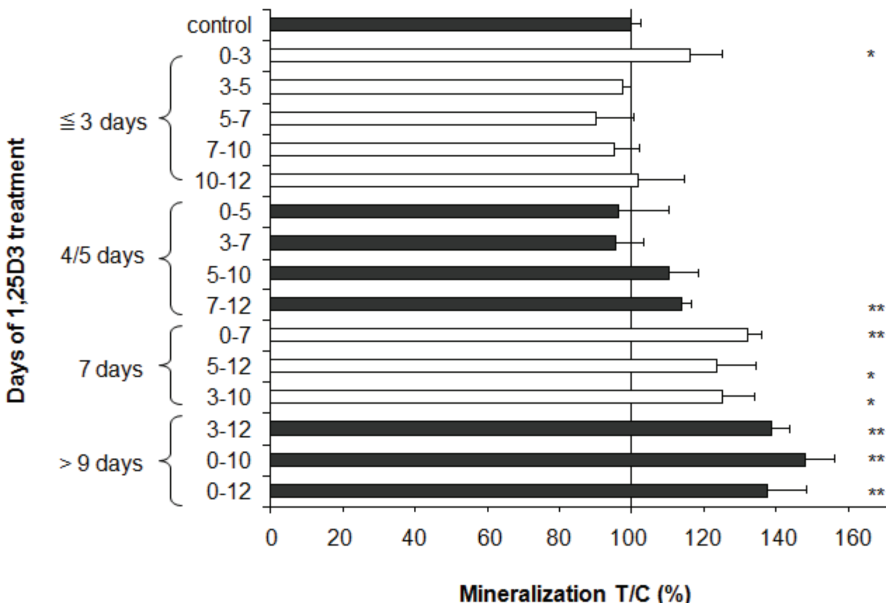
Gene Symbol	Gene Title	Probe Set ID
ANAPC10	anaphase promoting complex subunit 10	207845_s_at
CCNK	cyclin K	225824_at
CDK2	cyclin-dependent kinase 2	211804_s_at
CETN3	centrin, EF-hand protein, 3 (CDC31 homolog, yeast)	209662_at
DNM2	dynamin 2	202253_s_at
GSPT1	G1 to S phase transition 1	225276_at
GTSE1	G-2 and S-phase expressed 1	204315_s_at
ILF3	interleukin enhancer binding factor 3, 90kDa	208930_s_at
KIF22	kinesin family member 22	216969_s_at
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	1554768_a_at
MARK4	MAP/microtubule affinity-regulating kinase 4	55065_at
NEK2	NIMA (never in mitosis gene a)-related kinase 2	211080_s_at
NUSAP1	nucleolar and spindle associated protein 1	219978_s_at
PML	promyelocytic leukemia	211013_x_at
PURA	purine-rich element binding protein A	204021_s_at
SKA2	spindle and kinetochore associated complex subunit 2	225686_at
SKP2	S-phase kinase-associated protein 2 (p45)	210567_s_at, 203626_s_at
STAG1	stromal antigen 1	202294_at
TGFB1	transforming growth factor, beta 1	203085_s_at
TIPIN	TIMELESS interacting protein	219258_at
TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)	210052_s_at

Table S6: Genes regulated by 1,25D3 in the mineralization period and being involved in the GO-term "translation" (GO:0006412).

Gene Symbol	Gene Title	Probe Set ID
BCL9L	B-cell CLL/lymphoma 9-like	228065_at
EIF2B3	eukaryotic translation initiation factor 2B, subunit 3 gamma, 58kDa	218488_at
EIF2C2	eukaryotic translation initiation factor 2C, 2	225827_at
EIF4E	eukaryotic translation initiation factor 4E	201435_s_at
EIF4G1	eukaryotic translation initiation factor 4 gamma, 1	208625_s_at
EIF4G3	eukaryotic translation initiation factor 4 gamma, 3	201936_s_at
EPRS	glutamyl-prolyl-tRNA synthetase	200841_s_at
GFM1	G elongation factor, mitochondrial 1	232296_s_at
GSPT1	G1 to S phase transition 1	225276_at
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	227377_at
IMP3	IMP3, U3 small nucleolar ribonucleoprotein, homolog (yeast)	221688_s_at
MRPL1	mitochondrial ribosomal protein L1	223154_at
MRPL48	mitochondrial ribosomal protein L48	218281_at
MRPL51	mitochondrial ribosomal protein L51	223086_x_at
MRPS10	mitochondrial ribosomal protein S10	224247_s_at, 222522_x_at
MRPS18B	mitochondrial ribosomal protein S18B	208907_s_at
NCOA5	nuclear receptor coactivator 5	225145_at
OXSM	3-oxoacyl-ACP synthase, mitochondrial	219133_at
PAIP2	poly(A) binding protein interacting protein 2	222983_s_at
RARS2	arginyl-tRNA synthetase 2, mitochondrial	232902_s_at
RPL22L1	ribosomal protein L22-like 1	225541_at
RPL23	ribosomal protein L23	200888_s_at
RPL31	ribosomal protein L31	200963_x_at
RPL36A	ribosomal protein L36a	201406_at
RPS27L	ribosomal protein S27-like	218007_s_at
RPS7	ribosomal protein S7	200082_s_at
RPS9	ribosomal protein S9	214317_x_at
RRBP1	ribosome binding protein 1 homolog 180kDa (dog)	201204_s_at
RSL24D1	ribosomal L24 domain containing 1	222465_at
RSL24D1	ribosomal L24 domain containing 1	217915_s_at
SARNP	SAP domain containing ribonucleoprotein	224914_s_at
TPR	translocated promoter region (to activated MET oncogene)	201730_s_at

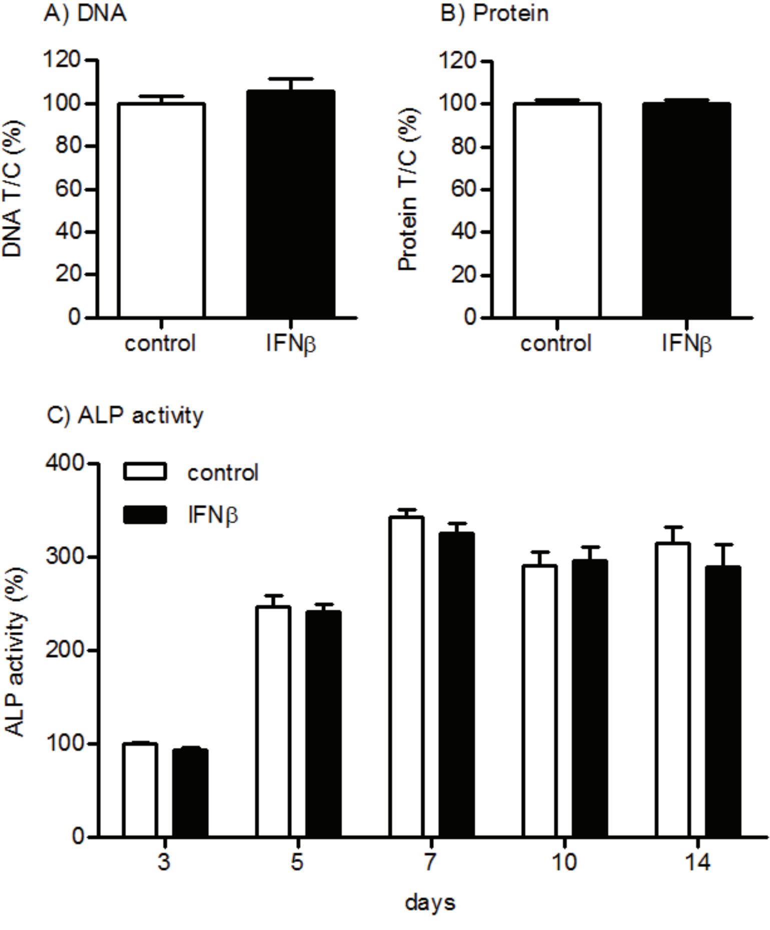
Table S7: Genes regulated by 1,25D3 in the mineralization period and being involved in the GO-term “cell death” (GO:0008219).

Gene Symbol	Gene Title	Probe Set ID
ACTN1	actinin, alpha 1	211160_x_at
ATXN2	ataxin 2	202622_s_at
BAG1	BCL2-associated athanogene	211475_s_at
BAG3	BCL2-associated athanogene 3	217911_s_at
BAG4	BCL2-associated athanogene 4	219624_at
BCAP29	B-cell receptor-associated protein 29	205084_at
BCL2L1	BCL2-like 1	215037_s_at
BID	BH3 interacting domain death agonist	227143_s_at
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	211367_s_at
CASP2	caspase 2, apoptosis-related cysteine peptidase	209811_at
CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	1558214_s_at
DNM2	dynamin 2	202253_s_at
EP300	E1A binding protein p300	202221_s_at
FXR1	fragile X mental retardation, autosomal homolog 1	201635_s_at
HIP1	huntingtin interacting protein 1	226364_at
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	203332_s_at
ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	205176_s_at
MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1	210018_x_at
MARK4	MAP/microtubule affinity-regulating kinase 4	55065_at
MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	202086_at
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	202647_s_at
NUDT2	nudix (nucleoside diphosphate linked moiety X)-type motif 2	218609_s_at
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	204285_s_at
PML	promyelocytic leukemia	211013_x_at
PPP1R13L	protein phosphatase 1, regulatory (inhibitor) subunit 13 like	218849_s_at
PSEN1	presenilin 1	207782_s_at
PTEN	phosphatase and tensin homolog	228006_at
PURA	purine-rich element binding protein A	204021_s_at
PURB	purine-rich element binding protein B	226762_at
SKI	v-ski sarcoma viral oncogene homolog (avian)	229265_at
SON	SON DNA binding protein	226465_s_at
SRA1	steroid receptor RNA activator 1	224130_s_at
SRGN	serglycin	201858_s_at
STAT1	signal transducer and activator of transcription 1, 91kDa	AFFX-HUMISGF3A/M97935_5_at
STK17B	serine/threonine kinase 17b	205214_at
TGFB1	transforming growth factor, beta 1	203085_s_at
TMX1	thioredoxin-related transmembrane protein 1	208097_s_at
XIAP	X-linked inhibitor of apoptosis	225859_at, 225858_s_at
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	200641_s_at



Supplementary Figure S1. Identifying important phases of 1,25D3 treatment within the pre-mineralization period. During the pre-mineralization period (days 0-12) SV-HFO cells were treated with 1,25D3 at different stages and for different durations. SV-HFO cells were harvested at the onset of mineralization and mineralization rate was measured. Control condition was set to 100%. * p-value < 0.05, ** p-value < 0.01.

Chapter 2



Supplementary Figure S1. IFNb effects on cell growth, osteoblast differentiation and mineralization. SV-HFO cells were cultured until the onset of mineralization (day 14), harvested and concentrations of A) DNA and B) protein were measured as cell growth parameters. C) ALP activity reflecting osteoblast differentiation was determined at days 3, 5, 7, 10 and 14.

Curriculum Vitae

Viola Jasmin Wöckel is born on 28th December, 1981 in Peine (Germany). In 2001 she graduated from Gymnasium Groß Ilsede (Germany) and started her studies in Biology at the Technical University Carolo-Wilhelmina in Braunschweig (Germany) with majors in Genetics, Cell Biology and Plant Biology. In 2004, Viola did a 3 month internship at Dr. Jean Vance's lab in the Group of Molecular and Cell Biology of Lipids, University of Alberta, Edmonton (Canada). During her internship she studied the transport of lipoproteins in neurons of a mouse Niemann-Pick-C disease model. With the diploma thesis "Establishing lentiviral vectors for stable and inducible IRF-1 expression", which was performed in the department of Gene Regulation and Differentiation of Dr. Hans-Jörg Hauser, Helmholtz Center for Infection Research, Braunschweig (Germany), Viola received the grade of "Diplom Biologist" in 2006. In 2007 Viola joined the Marie-Curie-funded European Union Training Network "NucSys". Her PhD project was performed in the Department of Internal Medicine, Erasmus MC (The Netherlands) under the supervision of Prof. dr. Hans van Leeuwen and was focused on inhibitors of bone formation in $1\alpha,25(OH)_2D_3$ environment. In 2010 Viola left academics and joined the Clinical Research Organization "Quintiles B.V." working as in-house Clinical Research Associate on multiple clinical studies.

PhD Portfolio

Name:	Viola Jasmin Wöckel
Erasmus MC Department:	Internal Medicine
European Training Network:	Systems biology of Nuclear Receptors (NucSys)
Research School:	Erasmus Postgraduate School Molecular Medicine (MolMed)
PhD Period:	January 2007 – June 2010
Promotor:	Prof. dr. J.P.T.M van Leeuwen
Co-promotor:	Dr.ing. B.C.J. van der Eerden

PhD training activities

General courses:

Presentation Skills (NucSys)	2007
Biomedical English Writing and Communication (MolMed)	2008
Project Management (NucSys)	2009
Grant writing (NucSys)	2009

Specific courses:

Biomedical Research Techniques (MolMed)	2007
Molecular Medicine or Mechanisms of gene expression (NucSys)	2007
Mouse embryonic stem cell culture training course (NucSys)	2007
Workshop: Browsing Genes and Genomes (MolMed)	2007
Neuro-Immuno-Endocrinology (MolMed)	2008
Annual Course of Molecular Medicine (MolMed)	2008
PhD training course on bone metabolism (European Calcified Tissue Society)	2008
System biology - from molecules to life (Federation of the Societies of Biochemistry and Molecular Biology)	2009

Awards

2010 – 1st Poster Award of the Molecular Medicine Day, Erasmus MC, Rotterdam

2010 – 2nd Poster Award of Internal Medicine, Erasmus MC, Rotterdam

2009 – 2nd Poster Award of Internal Medicine, Erasmus MC, Rotterdam

Presentations at (inter)national conferences

17th Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (NVCB) (oral presentation), Zeist, The Netherlands (2007)

Molecular Medicine Day (Poster), Rotterdam, The Netherlands (2009)

European Symposium on Calcified Tissues (Poster), Vienna, Austria (2009)

Vitamin D Workshop (Poster), Brugge, Belgium (2009)

Attended conferences

Osteoimmunology, Rhodes, Greece (2008)

European Symposium on Calcified Tissues, Barcelona, Spain (2008)

31st Annual Meeting of American Society for Bone and Mineral Research, Denver, USA (2009)

International Symposium on Stem Cells, Development and Regulation, Amsterdam, The Netherlands (2009)

Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (2007-2009)

Molecular Medicine Day (2007-2008)

Science Days Internal Medicine (2007-2010)

NucSys Meetings (2007 – 2009)

Erasmus MC – Internal Medicine Seminars

Endocrinology Lectures (monthly)

Internal Medicine Departmental Seminar (weekly)

Bone and Calcium Research Literature Discussion (weekly)

Bone and Calcium Research Work Discussion (weekly)

List of publications

1α,25-$(OH)_2D_3$ Acts in the Early Phase of Osteoblast Differentiation to Enhance Mineralization Via Accelerated Production of Mature Matrix Vesicles

V.J. Woeckel, R.D.A.M. Alves, S.M.A. Swagemakers, M. Eijken, H. Chiba, B.C.J. van der Eerden, J.P.T.M. van Leeuwen

J Cell Physiol. 2010 Nov;225(2):593-600.

IFNβ Impairs Extracellular Matrix Formation Leading to Inhibition of Mineralization by Effects in the Early Stage of Human Osteoblast Differentiation

V.J. Woeckel, M. Eijken, J. van de Peppel, H. Chiba, B.C.J. van der Eerden, J.P.T.M. van Leeuwen

J Cell Physiol. 2012 Jun;227(6):2668-76

Evidence of Vitamin D and interferon-β cross-talk in human osteoblasts with 1α,25-dihydroxyvitamin D(3) being dominant over interferon-β in stimulating mineralization.

V.J. Woeckel, M. Koedam, J. van de Peppel, H. Chiba, B.C.J. van der Eerden, J.P.T.M van Leeuwen

J Cell Physiol. 2012 Sep;227(9):3258-66.