Regulation of osteoblast differentiation

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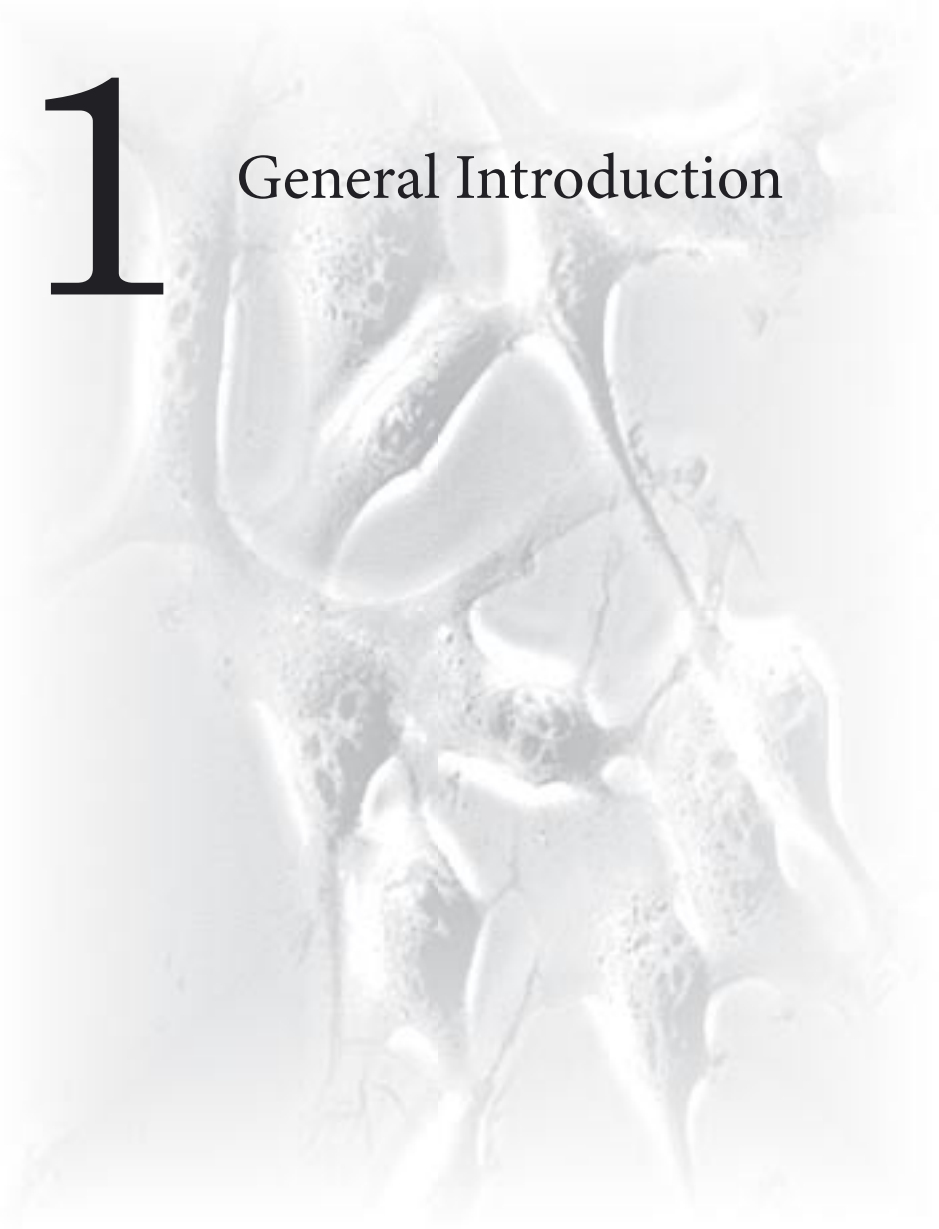
1 General Introduction



Osteoporosis

Bone is a highly mineralized tissue that provides mechanical support and metabolic functions to the skeleton. It can be formed by either intramembraneous ossification (flat bones of skull and clavicle) or endochondral ossification (long bones of axial and appendicular skeleton). In intramembraneous ossification, condensing mesenchymal cells differentiate directly into the bone forming cells, the osteoblasts. In endochondral ossification, the condensing cells first differentiate into chondrocytes. These chondrocytes proliferate and differentiate into mature chondrocytes depositing a cartilaginous matrix. This matrix is subsequently resorbed by the bone resorbing cells, the osteoclasts, and replaced by bone deposited by osteoblasts (reviewed in [1]).

Bone is a dynamic tissue, it constantly undergoes remodeling, a delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts. This physiological process is tightly regulated by local and endocrine factors. In normal bone remodeling, both bone formation and bone resorption are tightly coupled. Osteoclast activation and formation, which result in bone resorption, precede the recruitment, proliferation and differentiation of osteoblasts, which result in bone formation. Disturbance of this process may lead to skeletal diseases such as osteoporosis [2].

Osteoporosis is characterized by reduced bone mass and micro architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture risk. Osteoporosis is a complex multifactorial disease, determined by genetic and environmental factors as well as their interactions.

Current treatment of osteoporosis is mainly based on inhibition of bone resorption by anti-resorptive drugs, such as bisphosphonates and estrogen analogs. For de novo bone formation, anabolic agents are necessary that (1) increase the number and thickness of the trabeculae, (2) restore connectivity between the trabeculae, (3) thicken the cortex, and (4) ultimately reduce facture risk.

One of the bone anabolic agents is parathyroid hormone (PTH). Interestingly, PTH can have both catabolic and anabolic effects on bone: whereas continuous infusion of PTH causes bone loss, intermittent administration of PTH induces bone formation (reviewed in [3,4]). Currently, clinical data show that PTH can be used as an additional treatment for post-menopausal osteoporosis [5-9]. Various mechanisms have been proposed to explain the anabolic effects of PTH: i.e. PTH might have an effect on the proliferation, commitment, differentiation or apoptosis of the osteoblasts.

Hence, to understand the role of PTH in osteoblast biology, as well as for the development of new anabolic drugs, a more complete understanding of these processes is crucial.

In this thesis, we have studied various aspects of osteoblast differentiation, focusing on the role of three major morphogenic signaling pathways, the bone morphogenetic protein (BMP), the Hedgehog (Hh) and the Wingless (Wnt) signaling pathways. In addition, we studied their modulation by PTH and its related peptide (PTHrP). With these studies, a basis can be provided for improved diagnosis of skeletal disease and treatment that is targeted to specific cells in bone tissue.

In the following paragraphs, an overview is given of our current understanding of osteoblast differentiation, followed by the aims and outline of this thesis.

Osteoblastic differentiation

Introduction

Multipotent mesenchymal cells (MSC) are the precursors for the cell types involved in bone formation, such as osteoblasts, osteocytes and chondrocytes, and for other mesenchymal cell lineages, such as myoblasts, and adipocytes [10,11]. Mesenchymal stem cells first have to become committed or determined towards a particular cell lineage. Subsequently, these committed cells proceed through a differentiation process to acquire the cell specific phenotype.

The fate of the uncommitted mesenchymal stem cells depends on interplay between morphogenic proteins, several hormones, growth factors, and cytokines. The integration of these signals eventually activates the expression of a cell-lineage specific set of transcription factors that act as gene expression switches. These master switches induce the expression of lineage specific proteins.

The master switches for osteoblast differentiation include the Runt homology domain protein Runx2 (also known as core binding factor α1 (Cbfα1), osteoblast specific factor 2 (OSF2) or acute myelocytic leukemia 3 (AML3)) and the zinc finger protein osterix (osx) [12-15]. For differentiation into other cell lineages, distinct sets of transcription factors are required, such as Sox9, Sox5 and Sox6 for chondrogenic differentiation, the peroxisome proliferator-activated receptor γ2 (PPARγ2) for adipogenic differentiation and MyoD for myocyte differentiation [16-19].

Osteoblast differentiation is regulated by at least three major morphogenic pathways, the bone morphogenetic protein (BMP), the Hedgehog (Hh), and the Wingless (Wnt) signaling pathway [20-24]. These factors can commit MSC to the osteoblast lineage, and initiate osteoblast differentiation. Activators of various signaling pathways can influence and fine-tune these major pathways, such as fibroblast growth factors (FGFs), members of the growth hormone (GH) / insulin like growth factor (IGF-1) axis, hormones such as vitamin D3 and sex steroids as wells as PTH and PTHrP [6,25-37].

Nevertheless, only parts of the spatio-temporal regulatory mechanisms controlling osteoblastic differentiation are currently known and relationships and possible cross talk between different pathways remain largely unknown. In the following paragraphs, the osteoblastic differentiation process as well as the major signaling pathways involved in this process will be discussed.

Differentiation of osteoblasts

For differentiation towards osteoblasts, multipotent mesenchymal precursors first undergo proliferation, become committed and then differentiate into pre-osteoblasts and subsequently into mature osteoblasts. To examine this process, several osteoblastic cell lines have been used. Available well-characterized cell lines have been derived from a) osteosarcomas such as the rat ROS 17/2.8 and UMR 106 as well as the human Saos-2 and MG-63 and b) from clonal outgrowth from normal cell populations, such as the mouse C2C12, C3H10T1/2, KS483 and MC3T3-E1 cell lines [38-42]. In addition, primary osteoblasts isolated from calvariae, or bone marrow stromal cells can be used to study the differentiation process. In our studies, we have used the C3H10T1/2 and KS483 cell lines, as well as mouse bone marrow cultures. C3H10T1/2 cells are

embryonic multipotent fibroblasts-like cells, which can differentiate towards myocytes, chondrocytes, adipocytes and osteoblasts, although they do not form a mineralized bone matrix in our hands [43-47]. The KS483 cell line is a subclone from the mouse KS4 cell line, which is derived from mouse calvariae. KS483 cells are committed osteoprogenitor cells that still have mesenchymal progenitor cell characteristics since depending upon the appropriate culture conditions, they can differentiate towards mature, mineralizing osteoblasts, lipid droplets containing adipocytes and cartilaginous matrix producing chondrocytes [48-50](this thesis) (figure 1).

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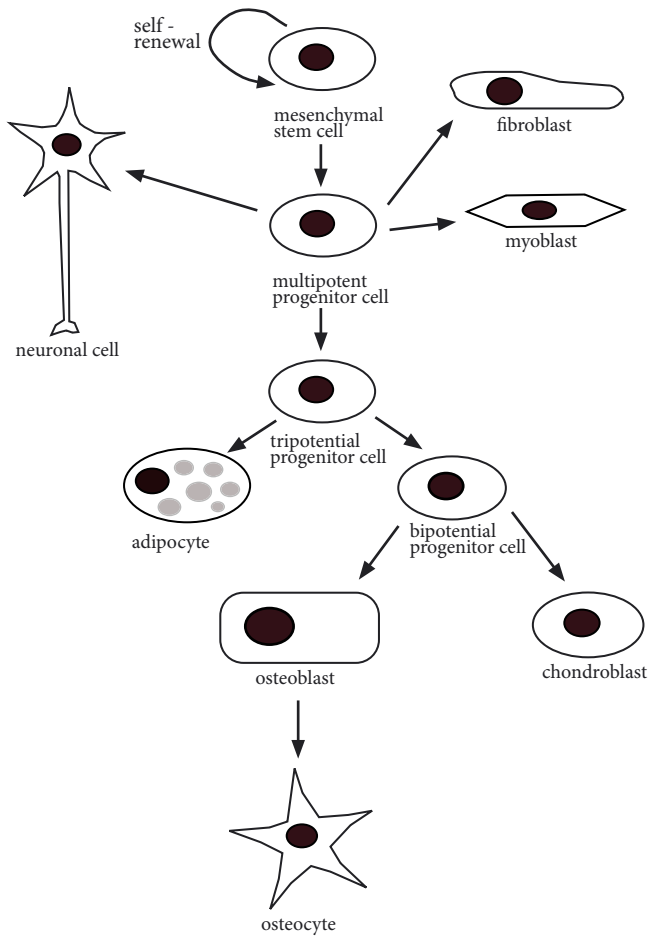

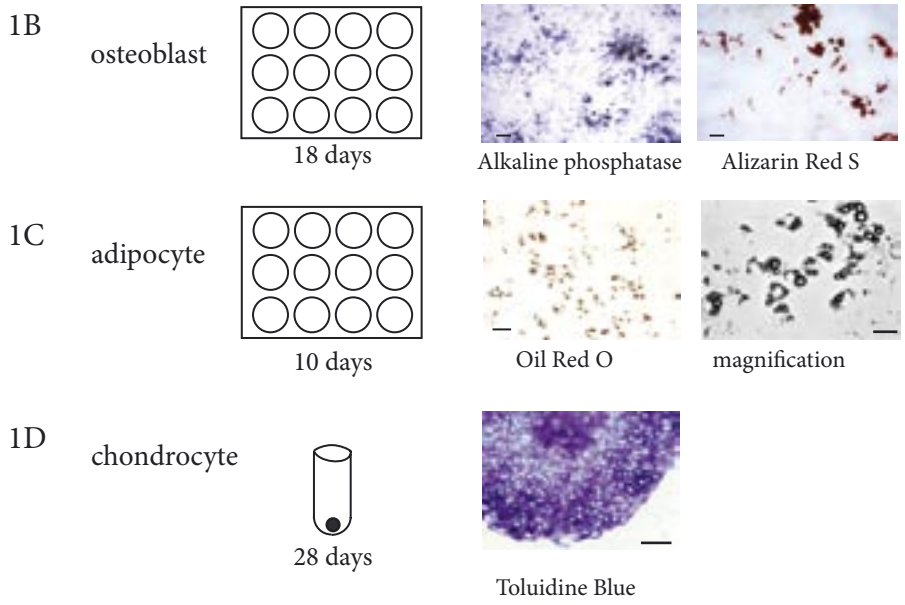

Figure 1 MSC-like KS483 cells can differentiate towards osteoblasts, adipocytes and chondrocytes depending upon the culture conditions.
A) A schematic representation of differentiation of mesenchymal stem cells towards various mesenchymal lineages. B) KS483 cells were differentiated towards mature mineralizing osteoblast in a three-week culture period by culturing in αMEM without phenol red supplemented with 10% FCS and penicillin/streptomycin. From day 4 onwards ascorbic acid and from day 11 β-glycerolphosphate was added. Cells were stained for alkaline phosphatase (ALP) and amount of mineral with Alizarin Red S. Representative images are shown of day 18. C) KS438 cells were differentiated towards lipid droplets-containing adipocytes in a 10-day culture period in αMEM medium supplemented with 10% charcoal treated FCS and penicillin/streptomycin. Cells were stained with Oil Red O and a representative image is shown of day 10 (bar represents 100 μm) with a magnification of the cells, clearly showing the lipid droplets in the cells. (bar represents 10 μmD) KS438 cells were differentiated towards chondrocytes in a 28-days culture period. 200.000 cells were pelleted and cultured in 1 ml high-glucose DMEM supplemented with proline, pyruvate, Insulin Transferin Selenite (ITS) premix, ascorbic acid, dexamethasone and TGFβ-3. Sections were stained with Toluidine Blue and a representative image is shown of day 28. (Bar represents 100 μm)

The osteoblastic differentiation process has been subdivided into several stages, (1) proliferation, (2) extra cellular matrix development (3) matrix maturation and (4) mineralization according to the model proposed by Stein and Lian [51].
To study these phases of osteoblast differentiation, several osteoblastic markers have been used such as alkaline phosphatase (ALP), type I collagen (Coll I), bone sialo protein (BSP), osteopontin (OPN), osteocalcin (OC), and the PTH/PTHrP receptor (PTH1R). Alkaline phosphatase is used as an early marker for osteoblast differentiation, while osteocalcin is used as a marker for terminal osteoblast differentiation. The stages of KS483 osteoblastic differentiation are shown in figure 2.
RunX2 is the key transcription factor required for osteoblast differentiation. This is based on observations in humans, in which heterozygous inactivating mutations in the Runx2 gene lead to cleidocranial dysplasia with delayed ossification and short stature [52,53] as well as in mice, in which knock out of RunX2 results in a total absence of skeletal ossification due to an arrest in osteoblast differentiation [14,15,54,55]. In addition, forced overexpression of RunX2 in non-osteoblastic cells, such as fibroblasts, results in up-regulation of osteoblast specific genes [12]. Transgenic animals overexpressing

a dominant negative form of Runx-2, under the control of the osteoblastic-specific osteocalcin promoter, display decreased bone formation due to decreased function of the osteoblasts, and develop osteopenia postnatally [56]. This indicates that RunX2 regulates the commitment of precursor cells towards osteoblasts, as well as the function of mature osteoblasts. Several isoforms of Runx2 are formed by alternative promoter usage, resulting in different transcripts, of which the MASN splice variant (type II isoform) is bone specific [57-59]. The conserved runt homology domain of RunX2 mediates binding to DNA sequences. This domain heterodimerizes with CBFβ, a partner protein, thereby increasing the affinity of this domain for DNA [60]. A RunX2 binding site has been found in several bone-specific genes, such as type I collagen, OPN, and OC [59,61-63]. The activity of Runx2 is modulated through post-translational modifications by phosphorylation as well as protein-protein interactions [25,64-66]. An example of a protein-protein interaction is the interaction with the transcription factor Twist. Because of a delay in RunX2 expression relative to osteoblast differentiation during mouse development, it was thought that earlier regulatory factors were involved [67-69]. Subsequently, the transcription factor Twist was identified as an inhibitor of RunX2 function via binding with the Twist box (amino acid sequence 186-206 of the Twist proteins 1 and 2), thereby inhibiting osteoblast differentiation [70,71]. For differentiation into osteoblasts, first Twist, which is expressed in proliferating progenitor cells, must be downregulated [72,73], resulting in relief of Twist-mediated inhibition of RunX2 [70,71].

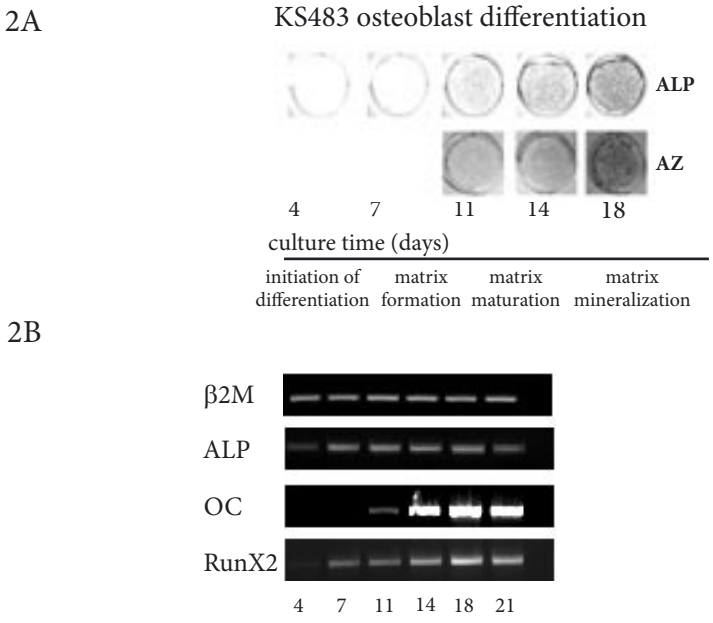

Figure 2 Stages of osteoblast differentiation of KS483.
A) KS483 osteoblastic differentiation is divided in several stages. Initiation of osteoblast differentiation starts at day 4 of culture by induction of ALP expression. Furthermore, deposition of an extracellular matrix begins around day 7. Subsequently, this matrix matures during the following days and mineralizes during the last days of culture. Representative images are shown of ALP and Alizarin Red S stained images. B) During osteoblastic differentiation, mRNA of several osteoblast markers, such as alkaline phosphatase, osteocalcin and the osteoblast specific RunX2 splice variant are expressed in KS483 cells, depending on the differentiation stage of the cells. As a control, expression of the housekeeping gene β2-Microglobulin (β2M) is shown.

The second important transcription factor for bone development is Osterix (osx). Osx is specifically expressed in all developing bones. Furthermore, osx-induced expression of osteoblast specific genes, such as osteocalcin and type I collagen in C3H10T1/2 and C2C12 cells [15]. In contrast to Runx-2 null mice that do not form osteoblasts, osx null mice do form cells of the osteoblastic lineage that express Runx-2, but they do not mature [15]. The expression of Runx2 was unaltered in these mice, suggesting that osx might be downstream of Runx2. In addition, osx contains a RunX2 consensus sequence in its 5' regulatory region, suggesting that osx might be a target of RunX2 [74]. These data indicate that RunX2 plays a role in the initial commitment towards osteoblasts and in the function of mature osteoblasts, while osx is involved in the terminal differentiation process.
Furthermore, other transcription factors are involved in osteoblastic differentiation, such as the homeodomain proteins, Msx2 and Dlx-5, members of the transcription factor complex AP-1, such as DFosB and Fra-1 [75-77] and ATF-4 [78,79].

Regulation of differentiation

The BMP, Hh and Wnt pathway are major morphogenic signaling pathways involved in osteoblastic differentiation. In the following paragraphs, the mechanism of action as well as the effect of these pathways on osteoblast differentiation will be discussed.

Bone morphogenetic proteins

Potent inducers of osteoblast differentiation are the bone morphogenetic proteins (BMPs), which are members of the Transforming Growth Factor-β (TGF-β) super family. BMPs were first identified by their ability to induce ectopic bone formation *in vivo*, but subsequently, it has been shown that they are multifunctional regulators of morphogenesis during embryonic development. BMPs have been shown to regulate the differentiation of various cells implicated in cartilage and bone formation during skeletal development and fracture repair [80,81]. Thus far, over 20 BMPs have been identified, which can be divided into subgroups, according to their amino acid similarity (reviewed in [82]). The BMP-2/-4 group consists of BMP-2, -4, and the drosophila homologue decapentaplegic (dpp), the BMP-6 group consists of BMP-5, BMP-6, BMP-7, BMP-8A, and BMP-8B, and the GDF-5 group consists of GDF-5, GDF-6 and GDF-7. The activity of these BMPs is regulated by soluble BMP antagonists, such as noggin, chordin, Cerberus, DAN and Gremlin, which bind to BMPs, thereby preventing the binding of BMPs to their receptor. Sclerostin can also inhibit BMP activity, although it differs from the classical BMP antagonists, by its unique localization in osteocytes and its inability to antagonize several BMP responses in osteoblastic cells [83].

The role of BMPs in bone formation

Various BMPs and their receptors are expressed in skeletal tissue, i.e. in growth plate chondrocytes (BMP-2 through -7, ALK2, and ActR-II), osteoblasts and osteoprogenitors (BMP-2- through -6, ALK2, and ActR-II) and osteoclasts (BMP-2, -4, -5 and, -6) [84,85]. In addition, BMPs (e.g. BMP-2, -4 and -7) have been shown to be osteoinductive in several mouse models [86]. Targeted overexpression of the BMP antagonists noggin and gremlin

in osteoblasts, results in osteopenia and fractures and osteoblasts of these mice have impaired function [87,88]. In contrast, BMP-3 seems to be an exception to the stimulatory role of BMPs on osteogenesis since BMP-3 opposes the osteogenic effects of BMP-2 in stromal cell lines, and BMP-3-null mice show an increase in bone mineral density and in trabecular bone volume [89]. Moreover, several other knock out mice of the BMP pathway demonstrate their role in skeletal development. First, a naturally occurring mutation in the BMP-5 gene (*short-ear*) results in abnormalities in the skull and parts of the axial skeleton, i.e. sternum, ribs and vertebral processes abnormalities [90,91]. In addition, the effects of gene inactivation on skeletal development were investigated for various other BMPs. However, since BMPs are also expressed and active in other tissues, gene inactivation of BMPs often results in significant phenotypic changes outside the skeleton, for example, inactivation of BMP-8A result in infertility due to defects in spermatogenesis [92]. In addition, BMP-2 deficiency is lethal due to defects in amnion/chorion and in cardiac development [93] while the BMP-4 null mutation is lethal between day 6.5 and 9.5 of gestation because of the lack of mesodermal differentiation and patterning defects [94]. BMP-6-null mice were found to have a delay in ossification of the sternum [95] and BMP-7 null mice also develop modest skeletal abnormalities, including fused ribs, and vertebral, skull, and hind limb defects although the main defects were found in the eye and glomerular development, leading to renal failure and neonatal death [96-98]. Taken together, gene targeting of many members of the BMP family revealed that some of the members are expressed only in certain tissues, and exert specific effects. However, other BMPs, such as BMP-2 and BMP-4, play important roles in many processes during early development, and null mutant embryos of these BMPs die at early embryonic stages. In addition, in osteoblasts, functional redundancy might exist between the different BMPs. Conditional gene targeting can be an important tool to understand the roles of the various BMPs in different tissues *in vivo*.

The role of BMPs during osteoblastic differentiation

The role of BMPs in osteoblastic differentiation has been well established in various osteoblastic cell lines, such as C3H10T1/2, C2C12 and MC3T3, as well as in cultures of calvarial osteoblasts. Using these cells, induction of osteoblast differentiation has been demonstrated for BMP-2, -4, -5, -6 and –7, while BMP-3 has been shown to antagonize osteogenic BMPs [89,99-103]. Osteoblastic cell lines express BMP-2, 3, 4, 5, 6, 7, 8A and 8B, but the expression level depends on the cell line studied [99,104-107]. In addition, experiments using kinase-deficient truncated BMP receptors have demonstrated that BMPs play an important role in osteoblastic differentiation [107,108]. Treatment with the BMP antagonist noggin also potently reduced osteoblast differentiation *in vitro* [108-110]. In addition, studies in mice overexpressing noggin show that noggin, expressed in mature osteoblasts, inhibits osteoblast differentiation and bone formation *in vivo* [111], while noggin null mice show severe defects in skeletogenesis, including joint defects, and aberrant craniofacial bone growth [112].

Taken together, these studies showed that the BMP signaling pathway is required during the successive stages of osteoblast differentiation, i.e. commitment, initiation of differentiation, and progression of differentiation.

BMP signaling pathway

BMPs signal by binding to a heterodimeric complex of type I and II serine-threonine membrane receptors (figure 3). In mammals, three type I (Alk2, Alk3 and Alk6) and two type II (ActIIa and IIb) receptors are involved in BMP signaling. BMP type I receptors require ligand binding for their activity, while type II receptors are constitutively active. Upon ligand binding, the type I receptor is phosphorylated by the type II receptor and subsequently intracellular mediators of the Smad family are phosphorylated. For BMP signaling, Smads 1, 5 and 8 are used, which are activated by phosphorylation and form a heterodimeric complex with Smad 4. Smad 4 is a common-regulatory Smad, used for

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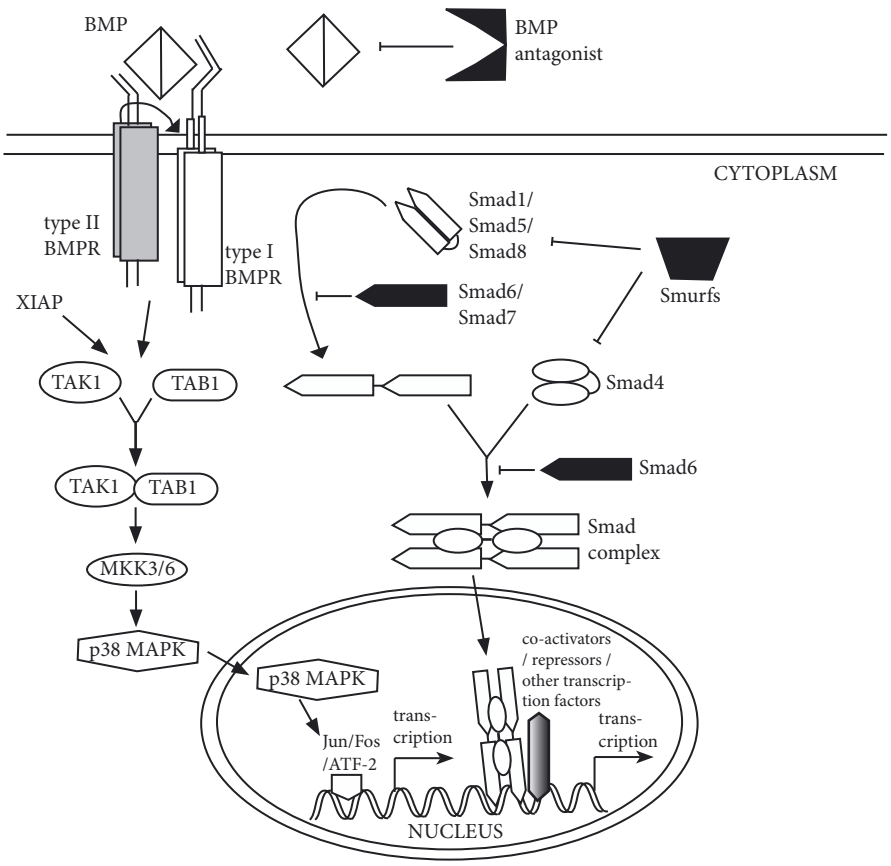

Figure 3 BMP signaling

BMPs bind to BMP type I/type II heteromeric receptor complex, after which the type II receptor phosphorylates the type I receptor. Then Smad 1,5 or 8 are phosphorylated by the activated type I receptor. Activated Smad 1,5 or 8 can form a heteromeric complex with the common mediator Smad 4, which subsequently translocates into the nucleus. There they activate transcription of BMP target genes in combination with other transcription factors. BMP signaling is inhibited by BMP antagonists, which prevent binding of the BMPs to the receptors or by inhibitory Smads 6 and 7,which intracellularly block signaling by Smads. For activation of the p38 MAPK pathway by BMPs, a complex is formed of TAK1 and TAB1, for which XIAP is necessary. The TAK1/TAB1 complex in turn activates p38 MAPK, which translocates to the nucleus and activates the transcription factors Jun/Fos and ATF-2.

both BMP and TGFβ signaling. Subsequently, the Smad complex translocates to the nucleus where it either acts as a transcription factor, directly binding to DNA (i.e. to the BMP responsive element (BRE) in the Id1 promoter), or binds to existing transcription factors, modulating their activities.
To affect target genes involved in osteoblast differentiation, interactions with the transcription factor Runx2 are required (reviewed in [113]). The role of the BMP receptors and the various Smads has been demonstrated by several studies using overexpression of these components or dominant-negative forms. For example, overexpression of a constitutive active type IA receptor is sufficient for osteo/chondrogenic development of C3H10T1/2 cells, and a constitutive active type IB receptor induces matrix formation in the absence of ligands [114-116]. MC3T3-E1 cells transfected with a truncated BMP receptor (type IA) failed to differentiate into nodule forming osteoblasts [107]. In addition, overexpression of Smad 1 and 5 in C2C12 cells resulted in osteoblastic differentiation, and these effects were enhanced by co-transfection with Smad 4 [117,118].
In addition to the Smad signaling pathway, BMPs can also signal via the p38 Mitogen Activated Protein Kinase (p38 MAPK) pathway (reviewed in [119]). Activation of the BMP receptors then results in the formation of a complex of TGFβ-activated kinase-1 transforming growth factor (TAK-1) and TAK1 binding protein 1 (TAB-1). For the formation of this complex, XIAP (X-chromosome-linked inhibitor of apoptosis protein) is necessary. Subsequently, this complex activates MAPK kinase (MKK)-3 or -6, which phosphorylate p38 MAPK. In the nucleus, the transcription factors Jun, Fos and activating transcription factor ATF2 are activated. Inhibition of this p38 MAPK pathway by synthetic compounds or dominant negative constructs blocks osteoblastic differentiation demonstrating that this pathway is essential for osteoblastic differentiation, although there are some conflicting results [26,27,120,121].

Regulation of the BMP pathway

Many BMPs, receptors and regulatory molecules are expressed in skeletal tissue and play an essential role in the maintenance of osteoblast function and proliferation of uncommitted precursors, but there is a need to temper their activities to maintain coordinated bone (re)modeling. This can be achieved by local feedback mechanisms, binding proteins, and intracellular factors. BMP signaling is controlled by several inhibitory mechanisms such as binding of BMPs to the extracellular BMP antagonists, noggin, Gremlin, chordin or Cerberus or by binding to pseudo-receptors, such as BAMBI. In addition, inhibitory Smads (Smad 6 and 7) or binding to the Smad binding proteins Ski and Tob controls the BMP signaling intracellularly. Moreover, inhibition of the BMP pathway can also occur by degradation of Smads by Smurfs or by inhibition of the p38 MAPK pathway (reviewed in [122]). Often the synthesis of the BMP antagonists is BMP-dependent, demonstrating the need of feedback mechanisms to maintain an ideal balance between BMPs and their antagonists. Taken together, the BMP signaling pathway is composed of a wide array of BMPs, receptors and antagonists, and therefore, complex interaction patterns and regulatory mechanisms exist for the fine-tuning of this pathway.

Hedgehog

The Hedgehog (Hh) pathway is involved in embryonic development, and to date three family members have been identified in vertebrates; Sonic (SHh), Indian (IHh) and Desert hedgehog (DHh) [123]. They display different expression patterns and functions [124,125]. SHh is involved in eye (reviewed in [126]) and neural tube development [127], is one of the main regulators of anterioposterior patterning in the limb bud (reviewed in [128]), and is also involved in differentiation of the somites in the sclerotome [129,130].
Apart from these roles in pattern-forming events, SHh signaling is also active in the adult. For example, SHh is required for proliferation, differentiation and apoptosis of epithelial cells of the stomach and intestine [131,132] and has been implicated in hair follicle growth [133]. The functions of IHh are mainly restricted to the skeleton, where it plays an important role in the development of cartilage and bone [134,135]. IHh is also expressed in the adult kidney and intestine [136]. Finally, DHh is involved in spermatogenesis [137]

The role of the Hedgehog pathway in bone formation

The Hh signaling pathway plays an important role in skeletal development, as underscored by skeletal abnormalities arising from targeted disruptions of components of this pathway in mice, as well as from natural mutations in both mice and human of hedgehog and the intracellular mediators of Hh signaling, the Gli proteins [134,138-143].
The Hh family member IHh is specifically involved in bone formation. IHh null mice demonstrated failure of osteoblast development in endochondral bones as well as markedly reduced chondrocyte proliferation and maturation [134,135].
The role of IHh in cartilage development has been studied extensively. IHh is expressed in early maturing chondrocytes, where it is part of a negative feedback loop with PTHrP, tightly regulating the pace and synchrony of chondrocyte differentiation [144]. In this loop, IHh induces the expression of PTHrP in the chondrocytes, resulting in an inhibition of the differentiation of proliferating chondrocytes into mature hypertrophic chondrocytes [134]. Furthermore, IHh stimulates chondrocyte proliferation and maturation and is indispensable for the initiation of osteoblast formation in the perichondrium [134,135]. The role for Hedgehog in osteoblast differentiation has also been shown by experiments with recombinant N-terminal SHh (N-SHh), which was able to induce ectopic bone formation *in vivo* when injected into mesenchyme, suggesting commitment of precursors to the osteoblast lineage [129,130]. Whether IHh also plays a role in later stages of osteoblast differentiation is less clear.

The role of Hedgehog during osteoblast differentiation

Although IHh is essential for bone formation, most experiments have been done with recombinant N-terminal SHh. However, since the N-terminal part of the Hedgehog molecules is the biological active part, these molecules are believed to be exchangeable [145,146]. *In vitro*, SHh induced alkaline phosphatase in the mouse mesenchymal cell line C3H10T1/2 and the osteoblast cell line MC3T3-E1 [129,146-148]. In addition, recombinant SHh significantly increased the percentage of C3H10T1/2 and ST2 cell lines as well as calvarial cells responding to BMP-2, while adipogenic differentiation of C3H10T1/2 cells was abolished [149]. These data suggest that Hh signaling is involved in the initial steps of osteoblast differentiation and in the commitment of progenitor cells towards

this lineage.

Hedgehog signaling pathway

Hedgehogs are secreted proteins, and upon secretion, the Hh precursor undergoes autocatalytic internal cleavage catalyzed by the C-terminus [145]. The N-terminal domain of hedgehog (Hh-N) has been shown to account for all known signaling activity, while the role for the C-terminal part (Hh-C) is less clear [145,146]. During the autocatalytic cleavage, a cholesterol moiety is attached to the C-terminal part of Hh-N, which is important for regulation of the spatial distribution of the Hedgehog signal [150,151]. Thereafter, the protein is palmitoylated, resulting in 30-fold higher biological activity (figure 4) [152].

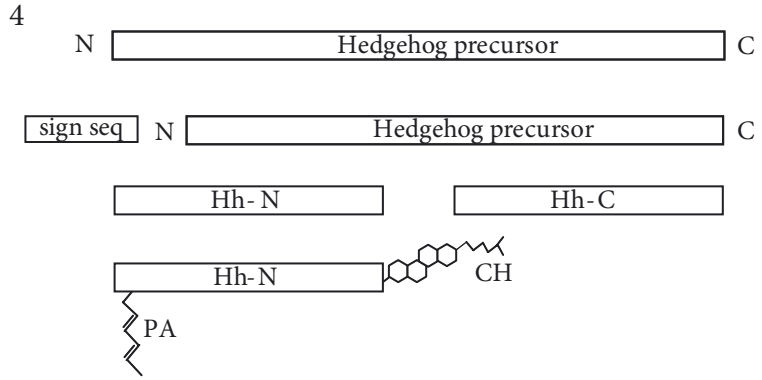

Figure 4 Processing of the Hedgehog protein
First, the signal sequence is cleaved from the Hedgehog precursor. Then autoproteolytic cleavage occurs, resulting in an N-terminal (Hh-N) and C-terminal part (Hh-C). Subsequently, Hh-N obtains a cholesterol moiety (CH) and is palmitoylated (PA).

The 12-transmembrane protein Patched (Ptc) is required for cellular responsiveness to hedgehog [153]. Two different Ptc receptors have been identified in vertebrates [154]. Binding of Hh to Ptc alleviates the inhibitory effect of Ptc on the 7-transmembrane receptor Smoothened (Smo), which then results in the activation of the Hh pathway (figure 5) [155]. In the absence of Hh, Ptc inhibits Smo activity, which could be caused by either direct association with Smo, or by Ptc-mediated transport of an inhibitor across the plasma membrane [156]. The latter is the most likely, since the receptors do not need to bind or co-localize [155].

Signaling events downstream of Smo are poorly understood, but reaches a complex of proteins that inhibit transcription factors from activating Hh targets. This complex consists of the kinase Fused (Fu), the kinesin motor protein Costal2 (Cos2), suppressor of Fused (SUFU) and a zinc finger transcription factor (cubitus interruptus (ci) in Drosophila and Gli in human). In the absence of Hh, this complex cleaves the Gli transcription factor, and the N-terminal fragment of Gli translocates to the nucleus, where it acts as a suppressor of Hh target genes. In the presence of Hh, an activated full-length Gli protein, containing a transcriptional activator domain, binds target genes and upregulates their transcription [157]. In mice, three Gli transcription factors (Gli1, Gli2, and Gli3) have been identified. Gli2 and Gli3 are posttranslationally regulated as described above, while Gli1 is primary regulated at the transcriptional level and is a constitutive activator [158,159]. Analysis of null mutants for each gene has indicated that

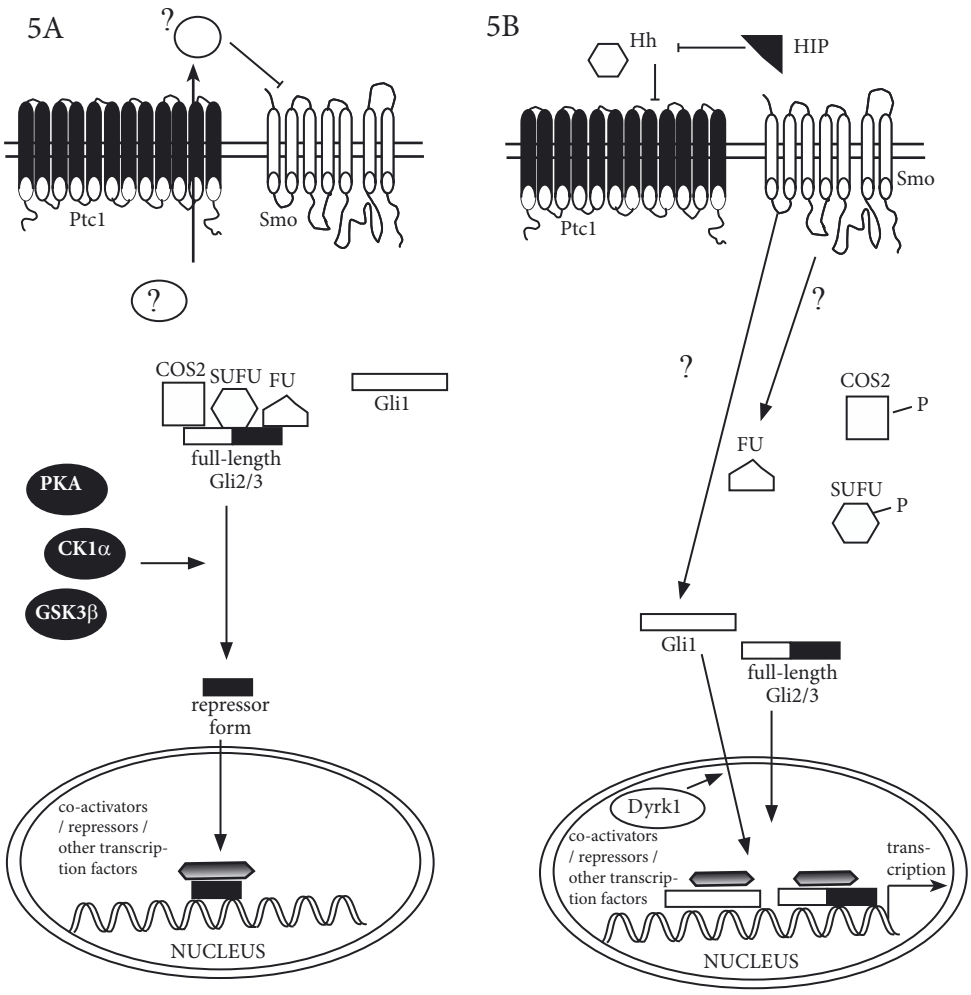

Figure 5 Hedgehog signaling
A) In the absence of Hedgehog, Patched 1 (Ptc1) inhibits Smoothened (Smo) activity, which is probably caused by Ptc1-mediated transport of an inhibitor across the plasma membrane. In vertebrates, an intracellular complex is found, consisting of Fused (Fu), Costal2 (Cos2), suppressor of Fused (SUFU) and a zinc finger transcription factor Gli (Gli2 or Gli3). The kinases Glycogen Synthase Kinase 3b (GSK3b), Casein Kinase 1a (CK1a) and protein kinase A (PKA) phosphorylate SUFU, which together with Cos2 cleaves the Gli 2 or 3 full-length precursors, resulting in the N-terminal form lacking transactivation factors. This N-terminal form of Gli2 or 3 translocates to the nucleus, where it acts as a repressor of Hh target genes. B) Binding of Hh to Ptc1 alleviates the inhibitory effect of Ptc on Smo, which then results in dissociation of the complex, thereby releasing full-length Gli2 or 3. This activated full-length Gli binds Hh target genes and upregulates their transcription. In addition, binding of Hh activates Gli1, which translocates to the nucleus. In the nucleus Gli1 can activate Hh target genes, which is stimulated by the kinase Dyrk1.

the Gli proteins have different functions. Furthermore, it has been shown that each Gli preferentially activates a distinct set of Hh target genes [160].
Gli1 null mice appear to be normal and viable, but they show defects in SHh signaling in combination with a Gli2 mutation suggesting that the function of Gli1 is redundant [161]. Overexpression of Gli1 in cultured cells or transgenic embryos can induce transcription of Hh target genes in the absence of Hh activity [162]. Gli2 mutants display defects in

neural tube development [163], and Gli3 null mice have polydactyly and defects in the central nervous system associated with ectopic SHh expression. These data indicated that Gli3 plays a role in repressing SHh signaling [161]. Gli3 plays an important role in the development of limb bud, in the regulation of digit number and identity [140,164]. Which Gli is involved in chondrogenic and osteoblastic differentiation is not known yet, but it is likely that a combination of the three Gli's is important.

Regulation of the hedgehog pathway

Intracellularly, the activity of the Hh signaling pathway is regulated by kinases, including Glycogen Synthase Kinase 3β (GSK3β), Casein Kinase 1α (CK1α) and protein kinase A (PKA), which oppose activation of Hh. These kinases phosphorylate SUFU, which together with Cos2 cleaves the Gli full-length precursor, resulting in the repressor form [165,166]. Another kinase, called Dyrk1 stimulates Gli1 activation of target gene transcription [167,168] (figure 5).

In contrast to the BMP and Wnt pathway, the Hh pathway consists of very few family members, and only one Hh antagonist has been identified until now, the Hedgehog interacting protein (HIP). HIP is a membrane glycoprotein that binds to all three mammalian Hedgehog proteins. Overexpression of HIP in cartilage, where Indian hedgehog (IHh) controls growth, leads to a short skeleton, a finding resembling that seen when IHh function is lost [134] HIP is a target of Hh signaling, constituting a negative feedback loop for the control of Hh signaling [169].

Wnt

Wnts are highly conserved secreted glycoproteins involved in a wide range of processes, such as embryogenesis, morphogenesis, organogenesis, and axis specification [170]. In addition, they are also involved in renewal of the cells of colon, skin and hair follicles in adults [171,172].

The role of Wnt signaling in bone formation

Recently, a role for the canonical Wnt/β-catenin signaling pathway in bone formation has been revealed by mutations in the Wnt co-receptor LRP5. Loss of function of LRP5 results in decreased bone mass accrual during growth and cause the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome (OPPG) in humans and mice predominantly by decreasing osteoblast proliferation [23,23,173,174,174]. Moreover, it was demonstrated that these effects on bone formation are independent of Runx2. In contrast, mutations resulting in an LRP5 resistant to inactivation by Dickkopf (Dkk) cause an autosomal-dominant high bone mass trait [175-178]. Other missense mutations in the LRP5 gene also resulted in altered bone mass [178]. These data suggested that LRP5 might contribute to the regulation of osteoblast proliferation, activity and life-span of the osteoblasts and plays a central role in the control of bone mass. However, LRP5 null mice do contain osteoblasts and mineralized skeletons, indicating that LRP5 is not essential for the osteoblastic differentiation from mesenchymal stem cells.

Mice with truncated forms of the other Wnt co-receptor, LRP6 die at birth and display a truncated axial skeleton, mid and hind brain defects and limb patterning defects [179]. In addition, LRP6+/- mice have a lower total and trabecular bone mineral density

[180,181]. In contrast to LRP5, no human diseases associated with the LRP6 gene have been identified.
Other clues for the involvement of the Wnt signaling pathway in bone formation were shown in mice with a targeted loss of β-catenin, which is a central, intracellular mediator of Wnt signaling. Loss of function of β-catenin in osteoblasts by specific deletion using a collagen I Cre transgenic mouse resulted in low bone mass [182], whereas osteoblast-specific overexpression of a constitutively active β-catenin increased bone mass, though this new-formed bone is woven [183].
Which Wnts are involved in the regulation of bone formation is largely unknown, although a role for Wnt10b has already been described. Overexpression of Wnt10b driven by the adipocyte-specific FABP promoter display increased bone volume and more trabecular bone, while amount of fatty tissue decreased [184]. Furthermore, several knock outs of Wnt antagonists have been described, which have a bone phenotype and will be discussed below.

The role of the Wnt/β-catenin pathway during osteoblast differentiation

The role of specific Wnt pathway components during osteoblastic differentiation is currently subject of extensive research. The stimulatory role of the Wnt pathway in the commitment towards the osteoblast lineage, as well as in the initiation of osteoblast differentiation has been shown in several multipotent cell lines. Some Wnts (e.g. Wnt1, 2 and 3A, but not 4 and 5) induced ALP activity and proliferation of these cell lines. No effects were observed on the expression of RunX2 and the osteoblast markers osteocalcin and collagen type I [173,185]. The role of this pathway and its regulatory molecules in matrix formation and mineralization is not clear yet. Low doses of LiCl, which is an activator of the Wnt pathway, or recombinant Wnt3A stimulated proliferation of human bone marrow derived MSC, while high doses of LiCl and Wnt3A inhibited proliferation and initiated osteoblast differentiation [186]. Furthermore, continuous presence of LiCl or Wnt3A during osteoblastic differentiation of these MSCs respectively inhibited and completely blocked matrix mineralization [187]. In addition, in primary osteoblasts from both wild type and LRP5 null mice, expression of Frizzled-2 and -6 decreased after 10 days of culture in conditions favoring mineralization, suggesting that osteoblasts may downregulate certain Wnt receptors during differentiation [23].

Wnt /β-catenin signaling pathway

As currently understood, Wnts activate at least three signaling pathways, the canonical Wnt / β-catenin pathway, the Wnt / Ca^{2+} and the Wnt planar polarity pathway (reviewed in 188). In human and mouse, 19 Wnt genes have been identified, which are divided into functional classes; members of the Wnt1 class activate the Wnt / β-catenin pathway (e.g. Wnt1, 2, 3, 3A, 8, 8b and 10b), while Wnts belonging to the Wnt5A class activate the other pathways (e.g. Wnt4, 5A, 5B, 6, 7A and 11). In the canonical Wnt / β-catenin pathway (figure 6), Wnt signaling is transduced by 7-transmembrane receptors of the Frizzled family and single-pass membrane co-receptors, the low-density lipoprotein receptor-related proteins (LRP5 and 6) [189]. Until now, 9 members of the Frizzled family have been found in mice and 10 in human.
In the absence of Wnt, a multiprotein complex is formed consisting of GSK3 β,

Adenomatous Polyposis Coli (APC), Disheveled (Dsh) and Axin. This complex phosphorylates β-catenin, targeting it for destruction by the ubiquitin-proteasome pathway [190,191]. Binding of Wnt to the receptors, results in phosphorylation of LRP5 by an unknown kinase, thereby creating a docking site for Axin [192]. Then GSK3 β is excluded from the multi-protein complex, which results in stabilization of β-catenin and its accumulation in the cytoplasm. Subsequently, β-catenin enters the nucleus, where it forms a complex with transcription factors of the T-cell factor (TCF) / Leukocyte Enhancer Factor (LEF) family to activate transcription of Wnt target genes [170,193,193]. Later studies indicated that LEF/TCFs not only activate gene transcription in the presence of Wnt signals, but also act as potent repressors in the absence of Wnt [171,194]. To this end, LEF/TCFs interact with various co repressor molecules such as Groucho (reviewed in [195]).

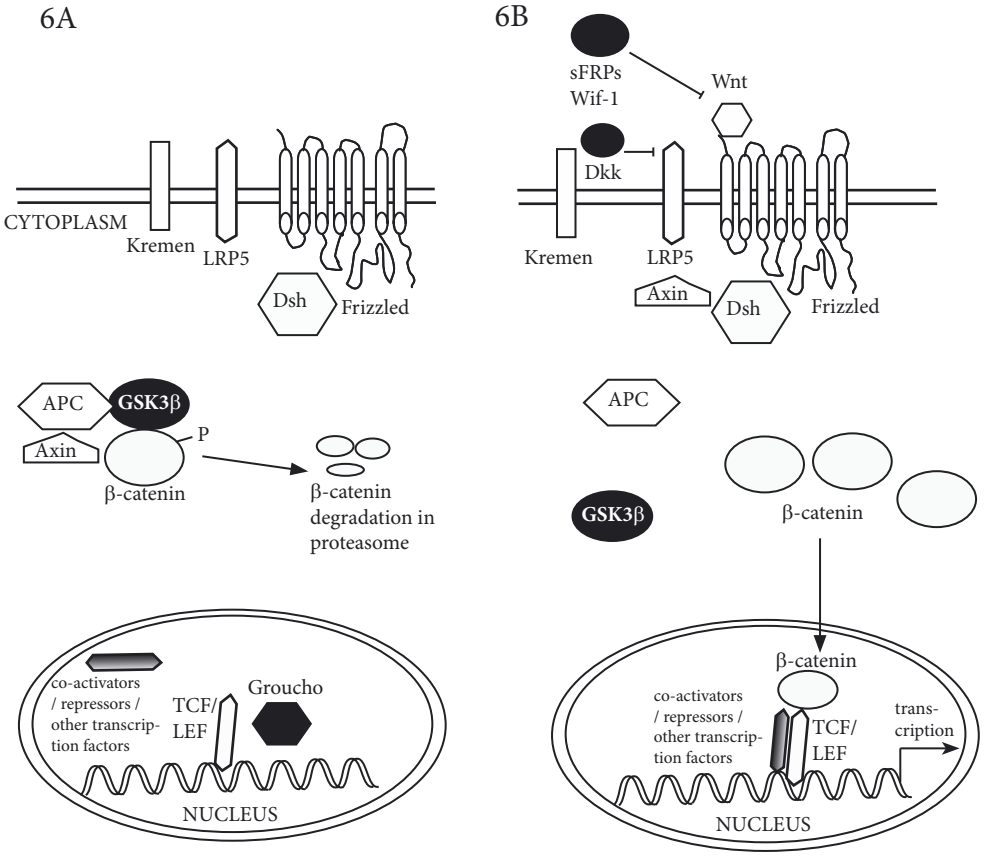

Figure 6 Canonical Wnt / b-catenin signaling
A) In the absence of Wnt protein, β-catenin is found in the cytoplasm, in a complex with several other molecules, including APC, Axin and GSK3 kinase. In this complex, GSK3 phosphorylates β-catenin leading to subsequent degradation of this molecule via the ubiquitin/proteasome pathway. B) Upon binding of Wnt to the receptors Frizzled and LRP5, dishevelled (Dsh) is activated. Axin translocates to LRP5, thereby releasing β-catenin from the complex and phosphorylation. Subsequently, β-catenin translocates to the nucleus, where it activates transcription factors of TCF/LEF family. The activation by Wnt is also regulated by Wnt antagonists including the soluble Frizzled related proteins (sFRPs) and Wnt inhibitory factor (Wif-1) which bind directly to Wnt and the Dickkopfs (Dkk), which bind to LRP5 and the co-receptor Kremen.

Regulation of the Wnt/β-catenin pathway

The Wnt pathway is a complex pathway, consisting of several different Wnts and Frizzled receptors and intracellular components as described above. For the regulation of this pathway, several intracellular and extracellular proteins exist. Intracellularly, the Wnt antagonists are APC, Axin, GSK3β and negative transcription factors. Extracellularly, two classes of secreted Wnt antagonist are found. First, secreted Frizzled related proteins (sFRPs), Cerberus and Wnt-inhibitory factor-1 (Wif-1) are Wnt antagonists which bind to Wnts and thereby interfere in the binding between Wnt and the Frizzled receptors. Second, Dickkopf proteins are Wnt antagonists that compete for the LRP receptor, thereby preventing binding of the co-receptor to Frizzled and Wnts.

Various studies have implicated a role for Wnt antagonists in bone formation. Wif-1 was detected in ossification centers in mouse embryos and was identified as a new marker of osteoblast differentiation after BMP-2 treatment [196]. In addition, expression of several sFRPs was found in skeletal tissues. sFRP3 was found in the cartilage [197], while sFRP2 was detected in primary human osteoblasts [198]. Targeted disruption of sFRP1 increased bone density in trabeculae, but not in cortical bone and prevented apoptosis of osteoblast cell lines *in vitro* and *in vivo* [199].

Dickkopf proteins inhibit Wnt signaling by binding to LRP5 and the membrane receptor Kremen1 or 2 (Krm)[200]. As described above, a mutation in the Dkk-1 binding domain of LRP5 resulted in high bone mass trait. Expression of Dkk-1 in multiple myeloma patients resulted in inhibition of bone formation [177,201]. These data suggest that Dkk-1 plays an inhibitory role in bone formation. Dkk-1 expression has been found in osteocytes and the osteosarcoma cell lines MG63 and SAOS [202,203]. Other members of the Dickkopf family are Dkk-2, -3 and –4, but their role in bone formation is less clear. Interestingly, Dkk-2 can both activate and inhibit Wnt signaling, which is probably dependent upon the expression of Kremen 2 [200]. In the presence of Krm2, a complex is formed between Krm2, Dkk-2 and LRP6, and subsequently internalization occurs by endocytosis, resulting in depletion of LRP6 from the plasma membrane and a blockade of canonical Wnt signaling. In the absence of Krm2, Dkk-2 functions as an agonist of Wnt signaling [204]. Dkk-3 has no known effect on Wnt signaling. Until now no effects of Dkk-2, Dkk-3 or Dkk-4 on bone formation have been found.

Taken together, Wnts and their antagonists may affect bone density in a variety of ways depending upon the environment and differentiation status of the cells.

Interplay between the three morphogenic pathways

The recent finding that the Wnt pathway is another important pathway involved in osteoblast differentiation, emphasizes how little we actually know about the regulation of osteoblastic differentiation. How this pathway and other key signal transduction pathways, like BMP and Hedgehog are integrated and the how these pathways interact are currently subject of many studies.

During embryogenesis, many examples of crosstalk between these pathways are found [205,206]. For example the Wnt/β-catenin and BMP pathways interact during the formation of the apical ectodermal ridge (AER) and the dorsal ventral axis in limb development [207,208]. During limb development, distinct signaling centers are responsible for the axis specification. The AER controls proximal-distal elongation, the zone of polarizing activity (ZPA) is responsible for anterior-posterior patterning, while the ectoderm of the

limb directs the dorsal-ventral axis. Using several conditional knock-out mice, including BMP receptor IA and β-catenin, it has been shown that β-catenin acts downstream of the BMP receptor IA during the formation of the AER, while it acts upstream of, or in parallel with the BMP receptor IA during dorsal-ventral axis formation [207]. In addition, during cartilage development interactions are found between the IHh and BMP pathways, BMPs expressed in the perichondrium changed the expression of IHh in adjacent chondrocytes and vice versa [209]. Furthermore, it has been shown that Wnt signaling has both positive and negative effects on Gli2 and Gli3 expression during somite formation, and the expression of members of the Wnt family is regulated by Gli proteins [210,211].

However, how the different signal transduction pathways integrate and cooperate during osteoblastic differentiation remains an area that needs more exploration. It has been suggested that the effects of BMP-2 on extracellular matrix mineralization by osteoblasts are mediated, at least in part, by the induction of a Wnt autocrine/paracrine loop and that the capacity of BMP-2 and SHh to induce ALP relies on Wnt expression and the Wnt/LRP5 signaling cascade [185]. In addition, β-catenin signaling was induced by BMP2 in C3H10T1/2 cells. However, the effects of β-catenin and BMP2 were not completely overlapping, suggesting that only part of BMP2-induced differentiation depends on β-catenin signaling. Vice versa, β-catenin also participates in non-BMP2-dependent differentiation [212].

Moreover, the SHh and BMP pathway cooperate during osteoblast differentiation. This has been shown by studies in several cell lines. SHh and BMP-2 synergistically induced ALP activity and expression of osteocalcin mRNA in C3H10T1/2 cells, while additive effects of SHh and BMP-2 were observed in mouse primary osteoblastic cells. Pretreatment with SHh enhanced the response to BMP-2 in C3H10T1/2 and MC3T3-E1 cells. This synergistic effect was at least partly mediated, by a positive effect on BMP-induced gene transcription via Smads and was independent of Gli1 [149,213]. Other interactions between Smad and Gli have been shown, such as association of Smad1 with the truncated C-terminal form of Gli3 repressor [214].

In addition, the Hedgehog mediator Gli activated BMP promoter activity, whereas a BMP response element was found in the IHh promoter [215,216]. Furthermore, the three pathways affect each other's gene expression pattern, thereby altering these pathways [185,217-219].

In addition to crosstalk between the three morphogenic pathways, members of various other signaling families including pathways activated by FGF's, hormones such as vitamin D3 and PTH, and PTHrP also regulate osteoblastic differentiation. However, in contrast to the morphogenic pathways, these compounds cannot induce osteoblastic differentiation from precursor cells. Their effects on osteoblast differentiation might be explained by their effects on influencing and fine-tuning the three major morphogenic pathways.

Parathyroid hormone and its related peptide PTHrP

As described earlier, PTH and PTHrP have been shown to display both catabolic and anabolic actions on bone. In this paragraph, first the role of this pathway in bone development is described, then the proposed role of PTH and PTHrP during osteoblast differentiation, followed by the signal transduction pathway induced by PTH or PTHrP. Subsequently, several hypotheses are given how PTH(rP) may exerts its anabolic function in bone. Finally, a model is proposed by which PTH(rP) signaling might control the pace of osteoblast differentiation.

The role of PTH and PTHrP in bone formation

The critical role of PTH(rP) in bone development is underscored by several natural and targeted knockouts of these genes and their receptor in human and mice.

Loss of function of the PTH1R in mice, show growth plate abnormalities due to premature, accelerated hypertrophic chondrocyte maturation [220]. It has been shown that the PTH1R plays a crucial role in a negative feedback loop in the growth plate. Indian hedgehog (IHh), produced by pre-hypertrophic and hypertrophic chondrocytes, stimulates production of PTHrP by perichondral cells at the distal ends of the long bones. PTHrP then binds to the PTH1R and maintains chondrocytes in a proliferative, less differentiated state. This less differentiated state delays the production of IHh. PTHrP and IHh constitute a negative feedback loop that synchronizes and determines the pace of differentiation of chondrocytes in the growth plate (reviewed in [221]).

A similar loss of function of the PTH1R occurs in humans with Blomstrand lethal osteochondrodysplasia [222]. They also show advanced skeletal maturation and premature ossification of the skeleton. In addition to the obvious cartilage phenotype, PTH1R null mice also show decreased trabecular bone, and abnormal bone mineralization [223].

The converse findings, i.e. skeletal abnormalities due to decelerated chondrocyte maturation, were observed in human Jansen's metaphyseal chondrodysplasia displaying severe growth plate abnormalities, which lead to short-limbed dwarfism and hypercalcemia [224]. In mice, targeted mutation of an activating mutation in the PTH1R to chondrocytes resulted in delayed differentiation of hypertrophic chondrocytes and mineralization [225].

The most important ligand for the PTH1R in bone is PTHrP. This is demonstrated by the strikingly similar phenotype of mice with a homozygous loss of the *pthrp* gene. Deletion of PTHrP results in embryonic lethality perinatally, likely from respiratory failure due to abnormalities in endochondral bone development. The mice display severe abnormalities in cartilage and bone development, in particular short limbs, mandible and small rib cages, due to premature terminal differentiation and mineralization of chondrocytes [226,227]. Of note is the observation that the PTH1R deficient mice display a delay in vascular invasion of the early cartilage, while this is not seen in the PTHrP null mice. In addition, ablation of the PTH1R gene affects osteoblastic gene expression more than ablation of the PTHrP gene, indicating that the PTH1R mediates the action of both PTH and PTHrP [228]. Transgenic mice, which overexpress PTHrP in tissues expressing the collagen type II promoter, show shortened limbs due to delayed mineralization and chondrocyte maturation in the growth plate, in line with the inhibitory effect of PTHrP on chondrocyte differentiation [229].

Finally, PTHrP does not only play a role in the development of endochondral bone, but also during adult stages of bone remodeling, since mice heterozygous for PTHrP deletion develop osteopenia when reaching adulthood (reviewed in [230]) [231].
A less striking bone phenotype is found in PTH deficient mice. These mice were viable and displayed a slightly expanded hypertrophic zone probably due to reduced resorption of terminal differentiated chondrocytes. Furthermore, they show reduced amount of osteoblasts and trabecular bone [232]. In addition, they suffered hypocalcemia, hyperphosphatemia, and low circulating 1,25-dihydroxyvitamin D3 levels, which is consistent with primary hypoparathyroidism [233].

The role of PTH and PTHrP during osteoblastic differentiation

A number of *in vitro* studies have been performed to investigate the mechanism of action of PTH or PTHrP on proliferation and differentiation of osteoblasts using several *in vitro* culture systems, but the results obtained are inconsistent.
PTHrP stimulated proliferation of primary bone marrow cells from rat, of fetal rat osteoblasts, and of the ROS 17/2.8 osteoblastic cell line [234-236]. PTH has been shown to stimulate proliferation of primary osteoblastic cells isolated from human trabeculae and from rat or chick calvariae, and the rat osteoblastic cell line UMR-106 [237-241]. In another study, it was shown that PTH increased proliferation in UMR-106 cells [242].
Moreover, PTH and PTHrP have been shown to have positive as well as negative effects on osteoblastic differentiation. Both PTH and PTHrP inhibited osteoblastic differentiation and terminal differentiation as measured by alkaline phosphatase activity and nodule formation in various *in vitro* systems, such as the ROS-17/2.8 cell line, primary osteoblastic cells isolated from calvariae of rat embryos, and human osteoblast-like cells [243-245]. In contrast, PTH and PTHrP had stimulatory effects on osteoblastic differentiation of the mouse osteoblastic cell line MC3T3-E1, although these results could not be confirmed by other groups [42,246].
Several groups reported that PTH exerted diverse effects on osteoblast differentiation depending on dosage, differentiation stage and administration time [34,247-249]. For example, when osteoblastic cells of rat calvariae were continuously exposed to PTH the hormone always inhibited osteoblast differentiation, but when cells were treated intermittently it exerted an anabolic effect depending on the exposure time [34]. In addition, in mice with targeted overexpression of a constitutively active form of the PTH1R in osteoblast, osteoblast function increased in trabecular bone, and at the endosteal surface of the cortical bone [250]. In contrast, the osteoblastic activity in the periosteum was inhibited. The amount of mature osteoblasts, as well as the pool of pre-osteoblasts was increased in the trabecular bone environment due to increased proliferation and decreased apoptosis [250].
Taken together, these observations demonstrate that the action of PTH on the osteoblasts varies with differentiation stage, dosage of PTH or PTHrP, exposure time, and/or environmental conditions.

The PTH and PTHrP signaling pathway

Parathyroid hormone (PTH) is a major regulator of calcium homeostasis. As a result of actions of PTH on bone, the kidney, and indirectly the intestine, the blood calcium

concentration rises (reviewed in [251]). PTHrP is structurally related to PTH, they share 8 of the 13 amino acids of the N-terminus and bind to the seven transmembrane G protein coupled cell surface receptor (PTH1R) with equal affinity [252].

Intracellularly, binding of PTH(rP) to the receptor can result in activation of several signaling pathways, such as the cyclic 3',5'-adenosine monophosphate (cAMP) / Protein kinase A (PKA) pathway and the Protein kinase C (PKC) pathway, of which the cAMP pathway is the most used (figure 7). Binding of PTH to the receptor, results in activation of adenylate cyclase through the Gαs, which in turn generates cAMP. Subsequently, cAMP binds to the regulatory subunit of PKA, which releases the active catalytic subunits of the enzyme. The catalytic form of PKA phosphorylates proteins on serine residues, which often causes changes in the target proteins' structure and function.

PTH has also been demonstrated to activate phospholipase C (PLC) by Gαq leading to the formation of diacylglycerol (DAG) which activates protein kinase C (PKC) and 1,4,5-inositol trisphosphate (IP_3) [253,254], resulting in increased intracellular free Ca^{2+} [255]. PTH may also stimulate non-PLC mediated activation of the PKC pathway [256]. Additionally, PTH can stimulate extracellular influx of Ca^{2+} through cAMP-dependent and cAMP-independent regulation or PKC-dependent regulation of calcium channels [257,258].

In kidney cells, other major target cells of PTH and PTHrP, activation of either the cAMP or PKC pathways is influenced by the expression of an adaptor protein called NHERF (Na/H exchange regulatory factor 1 or also called ezrin-binding protein 50). NHERF binds to the PTH1R through a PDZ domain interaction. This domain mediates protein-protein interactions, and was originally identified in the proteins post-synaptic density-95 (PSD-95), and zona occludens-1 (ZO-1) as a 90 amino acids repeat, containing the conserved motif Gly-Leu-Gly-Phe. PTH treatment of cells that express NHERF2 which formed a complex with the PTH1R, noticeably activated PLC and inhibited adenylate cyclase via stimulation of inhibitory G proteins [259]. A major function of NHERF is bringing the PTH1R in close proximity with intracellular signal transducers. This regulatory mechanism has however only been described in kidney cells. Whether regulation of PTH signaling by NHERF also plays a role in osteoblasts has to be elucidated.

PTH treatment via either pathway results in changes in activity of transcription factors and target gene expression [260]. PTH regulates mainly the activity of transcription factors such as cAMP response element binding protein (CREB) and activator protein-1 (AP-1). In bone, a relevant target is RunX2, which can be phosphorylated by PKA and MAPK (reviewed in [261]). Activation of CREB by PKA by phosphorylation on serine 133, is required for the PTH mediated stimulation of c-fos transcription [262-264]. In osteoblasts, PTH also increases expression of c-jun [265-267]. When Fos heterodimerizes with a member of the Jun family it forms an active complex called AP-1, that can bind DNA and regulate transcription of many genes with an AP-1 site in their promoter, such as collagen I and osteocalcin. At the transcriptional level, PTH causes the decreased expression of type I collagen, alkaline phosphatase, osteonectin, osteopontin while increasing the expression of collagenase-3 [268-271]. The effect of PTH on the expression of these factors can be mimicked by cAMP analogs or other activators of PKA, and is independent of PKC activity [66,263].

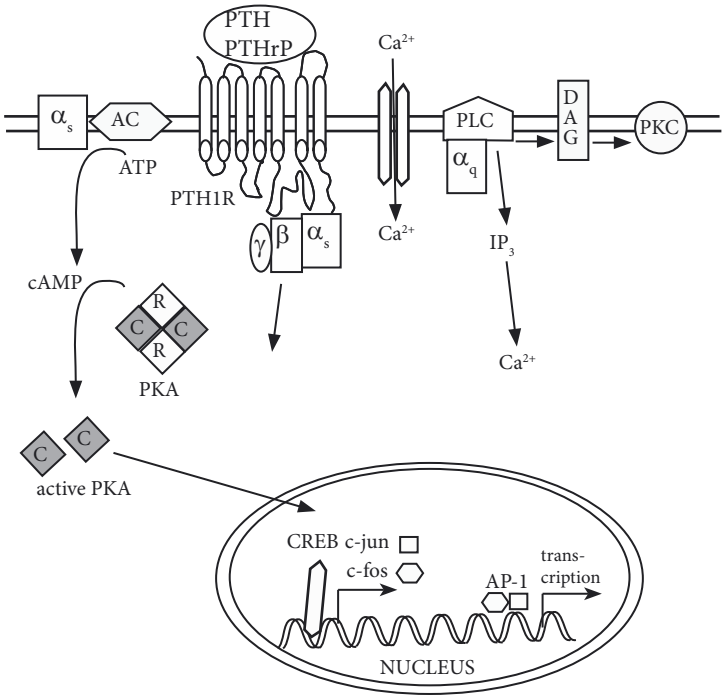

Figure7 PTH(rP) signaling
Binding of PTH or PTHrP to the PTH1R, results in activation of adenylate cyclase through the G_{as}, which in turn activates cAMP. cAMP stimulates PKA. PKA then phosphorylates proteins at serine residues, such as CREB, which is required for the expression of c-fos and c-jun. They form a complex called AP-1, which activates many PTH(rP) target genes. Binding of PTH(rP) can also activate PLC through the G_{aq}, which stimulates the formation of DAG and IP_3. Subsequently, PKC is activated by PLC and intracellular Ca^{2+} ions are released from the endoplasmatic reticulum by IP_3.

Mechanism of action of PTH and PTHrP

In bone remodeling, the activities of the osteoblasts and the osteoclasts must be tightly regulated in order to maintain skeletal integrity. PTH plays an important role in both bone formation and resorption. Although the osteoblasts likely mediate both the anabolic and catabolic actions of PTH, the molecular mechanism is not completely understood. Several mechanisms of actions for the anabolic effect of PTH have been proposed. For the anabolic effect, the amount or function of the osteoblasts needs to be increased. This could be accomplished by either increased proliferation of osteoblasts and/or osteoprogenitors, increased commitment of mesenchymal precursor cells towards the osteoblast lineage, increased conversion of resting lining cells towards functional mature osteoblasts, increased matrix production by mature osteoblasts or by decreased apoptosis of the osteoblasts.

Several studies indicated that PTH and PTHrP could increase osteoblast and osteoprogenitor proliferation both *in vitro* and *in vivo* [33,237,239,272]. However, the anabolic responses of PTH could not be solely dependent upon increased proliferation, since PTH could still induce an equal anabolic response in normal mice and SAMP6 mice with defective osteoblastogenesis [273] Furthermore, in another study, it was shown that PTH-induced bone formation was independent of osteoblast proliferation, and most

likely due to activation of preexisting bone lining cells to active bone matrix producing osteoblasts [274]. However, in mice treated intermittently with PTH, no changes in morphology of the lining cells were found [273].
The rate of commitment of mesenchymal precursor cells towards the osteoblast lineage, also contributes to the pool of osteoblastic cells, and intermittent administration of PTH has been shown to increase this rate [33].
The effects of PTH and PTHrP on osteoblast differentiation and terminal differentiation are controversial, both positive and negative effects have been found, and the effects appear dosage, differentiation stage and exposure time-dependent, as well as dependent upon the surrounding bone microenvironment [34,36,42,244,245,247,249,250]. In cultured cells, PTH decreased the production of type I collagen [268], and could enhance the synthesis of non-collagenous proteins, including osteocalcin [275,276]
Finally, PTH has been shown to inhibit osteoblast apoptosis, thereby increasing the life-span of the osteoblast. Since apoptosis is the most important fate of the osteoblast, changes in apoptosis could alter the rate of bone formation [273,277]. However, these data do not prove that PTH-induced decrease in osteoblast apoptosis is the only factor involved in the anabolic effect of PTH.

PTH(rP) signaling determines the pace of osteoblast differentiation

In preliminary experiments, it was shown that PTH inhibited osteoblastic differentiation of the KS483 cell line. Similar effects were found in chondrocytes, where PTHrP and PTH signaling inhibits chondrocyte differentiation. It has been shown that in the growth plate, PTHrP determines the synchronization and pace of chondrocyte differentiation [278-280]. Since the chondrocyte and the osteoblast are both derived from the same lineage and the same morphogenic pathways, such as BMPs, Hh and Wnts are involved in the differentiation process, we hypothesized that PTHrP determines the pace of osteoblast differentiation via a similar mechanism as has been shown in chondrocytes [217,278-287]. In addition, it was shown that the PTH1R and its ligands were linked with RunX2, an essential transcription factor for the initiation and progression of osteoblast differentiation, in a negative feedback loop. In this feedback loop osteogenic signals generated by Hh and BMPs converging on RunX2 as a common target are antagonized by signals derived from the PTH/PTHrP-receptor system. We propose the following model: PTHrP signaling might control the pace of osteoblast differentiation by counteracting IHh and BMP signaling, converging on RunX2 at various levels (Figure 8): at the level of IHh expression (arrow 1), IHh induced signal transduction (arrow 3), BMP expression (arrow 2) or signal transduction mediated by BMPs (arrow 4). In addition, PTHrP might affect the expression or posttranslational modification of the transcription factors RunX2 or osx (respectively arrows 5 and 6 for RunX2 and 7 an 8 for osx). In turn, IHh may induce PTHrP expression by an as yet unresolved mechanism, and RunX2 may be involved in the regulation of PTH1R, constituting a negative feedback loop. In this model, increased levels of PTHrP will decrease osteoblast differentiation, analogous to the role of PTHrP in chondrocyte differentiation [278-280].

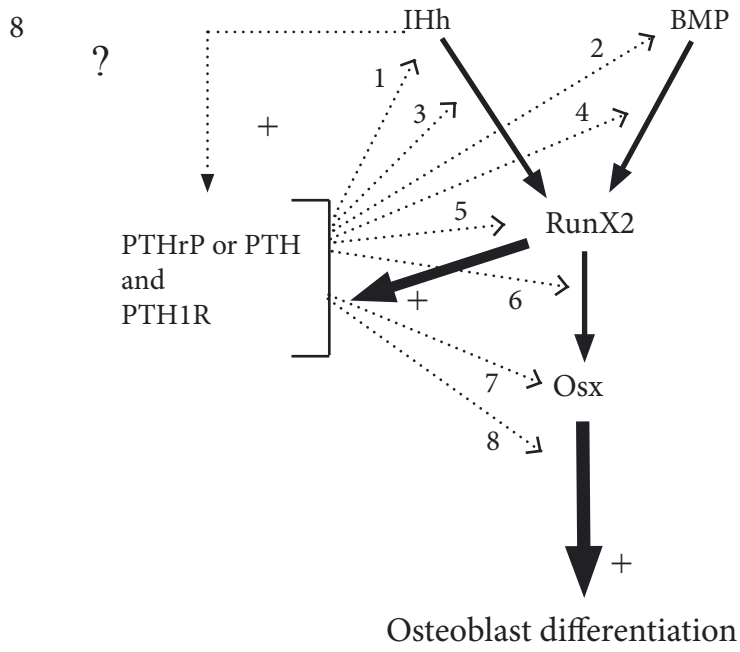

Figure 8 Possible mechanisms by which PTHrP or PTH can interfere with osteoblast differentiation
Inhibition of osteoblast differentiation by PTHrP and PTH signaling pathways can occur at the level of 1) IHh expression; 2) expression of BMPs; 3) IHh induced signal transduction; 4) signal transduction mediated by BMPs, 5) expression of RunX2, 6) posttranslational modification of RunX2, 7) expression of osterix or 8) posttranslational modification of Osterix. In turn, IHh may induce PTHrP expression by an as yet unresolved mechanism, and RunX2 may be involved in the regulation of PTH1R, constituting a negative feedback loop.

Outline and Aims of this thesis

The regulation of the differentiation process of mesenchymal stem cells towards mature osteoblasts by Hh and BMPs has not been fully understood yet. Furthermore, the Wnt/β-catenin pathway has recently been described to be involved in bone formation. Hence, for the development of novel anabolic treatments we first need to know more about the regulation of osteoblast differentiation. Therefore, the effects on differentiation and time windows of action of the BMP, Hh and Wnt signaling pathways were studied in more detail in this thesis. In addition, the molecular mechanism by which the PTH1R controls osteoblast differentiation by interference with the above-described osteogenic pathways was studied at various levels. For these studies, mainly the KS483 cell line has been used for *in vitro* differentiation assays.

In **chapter 2** of this thesis, the expression of components of the BMP pathway in KS483 cells as well as the role and time window of action of BMP signaling in KS483 osteoblastic differentiation was studied.

In **chapter 3** the role of Hedgehog signaling in various phases of osteoblastic differentiation as well as in adipogenic differentiation of KS483 cells is addressed. Furthermore, the localization of IHh in the human developing skeleton was studied.

Subsequently, in **chapter 4**, a model is described, which can be used for targeted Flp-mediated recombination in KS483 cells for either overexpression or specific knock down of genes using RNA interference. This model can be used to easily generate stable isogenic cell lines (KSFrt cells), which can be used for studying the effects of signaling cascades in all phases of mesenchymal differentiation by introducing or downregulating various components of these pathways. This was previously not possible, since transient transfection methods are unachievable in differentiated cells and conventional generation of stable cell lines introduces clonal variation.
In addition, it was demonstrated that KS483 cells display mesenchymal progenitor cell like characteristics, since they cannot only differentiate towards osteoblasts, and adipocytes, but also towards cartilaginous matrix producing chondrocytes.
This model was used in **chapter 5** to analyze Wnt signaling in various phases of osteoblast differentiation and revealed the crucial role of the Wnt antagonists Dkk-1 and Dkk-2. Data suggested that Dkk-1 plays an important role in the repression of ALP activity, and in the transition of an ALP positive towards mineralized osteoblast, while Dkk-2 acts on proliferation and the initiation of osteoblast differentiation.
Furthermore, in **chapter 6**, the mechanisms by which PTH(rP) signaling might inhibit osteoblast differentiation are described. Finally, general conclusions and discussion are located in **chapter 7**.

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