Regulation of osteoblast differentiation

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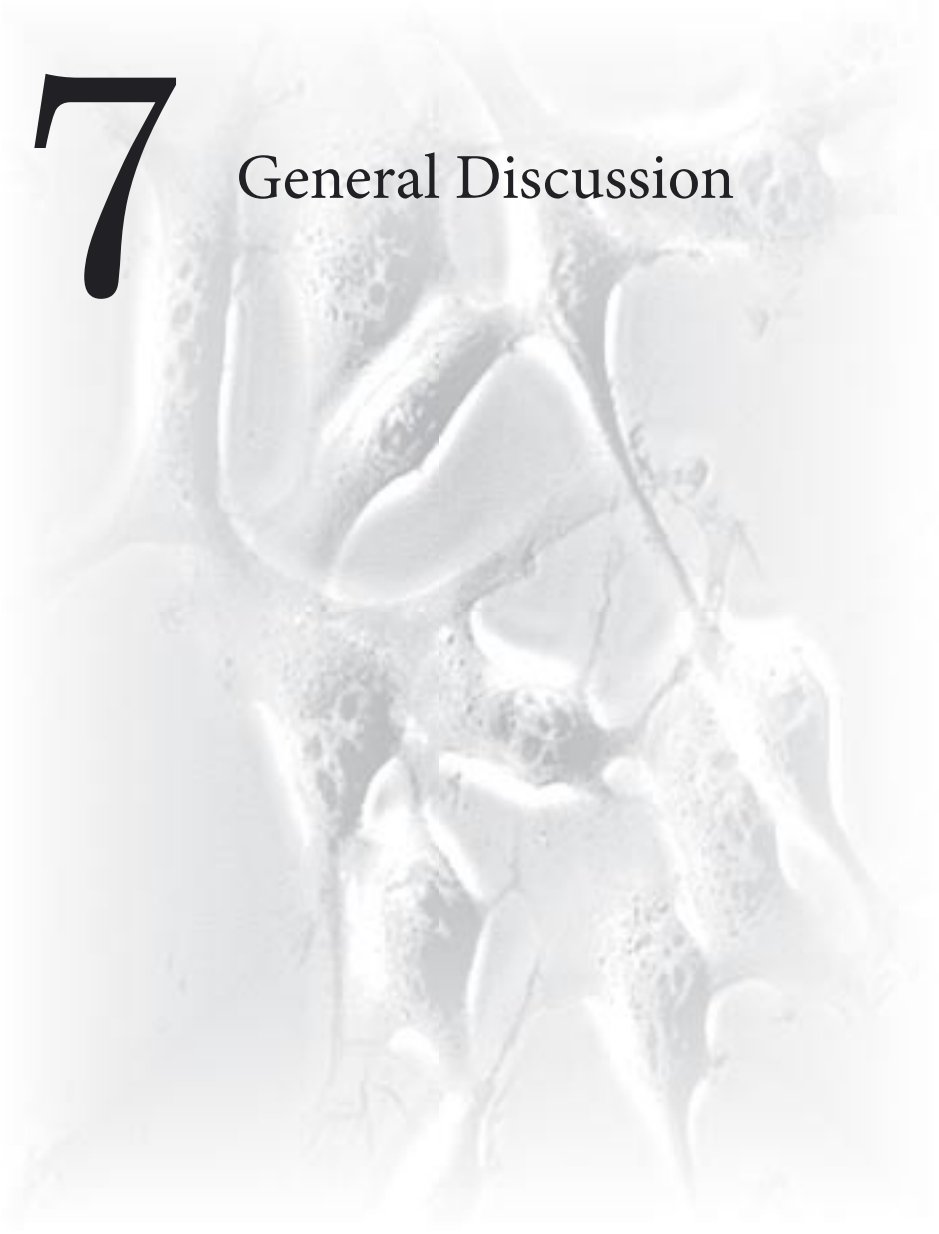
7 General Discussion



General Discussion

For the treatment of osteoporosis, novel anabolic drugs are necessary. One of the prime candidates for this is parathyroid hormone (PTH) and its related peptide (PTHrP). It has been shown that PTH can have both catabolic and anabolic effects on bone depending on administration time and dosage (reviewed in [1,2]). However, the way in which PTHrP mediates its effects on bone is less well studied. In order to study the mechanism by which PTHrP and PTH act, we first need to know more about the regulation and formation of the bone-forming cell, the osteoblast.

In this thesis, we have studied various aspects of osteoblast differentiation, focusing on the role of three major morphogenic signaling pathways and their modulation by PTH and PTHrP. Members of the Bone Morphogenic Protein (BMP), Hedgehog (Hh) or Wnt family of morphogens have been shown to play an important role in the initiation and progression of osteoblast differentiation [3-8]. Downstream targets of the BMP and Hh pathways are the transcription factors RunX2 and osterix (osx); lack of these factors has been shown to impair osteoblast differentiation [9-14]. How these different signal transduction pathways interact and cooperate during osteoblastic differentiation remains an area that needs more exploration. With the results of these studies, a basis could be provided for improved diagnosis of skeletal disease and treatment that is targeted to specific cells in bone tissue.

The major findings of the studies described in this thesis are (1) autocrine BMP signaling is pivotal for all phases of KS483 osteoblastic differentiation, (2) another osteogenic signaling pathway, the Hh pathway, only stimulates early osteoblastic differentiation while mature KS483 cells are unaffected by Hh signals, (3) Wnt/β-catenin signaling inhibits matrix maturation and mineralization and downregulation of this pathway during osteoblast differentiation is, at least partly, mediated by induction of the expression of the secreted Wnt antagonists Dickkopf (Dkk)-1 and Dkk –2, (4) PTHrP and PTH inhibit KS483 osteoblastic differentiation by influencing the major signaling pathways activated by BMP and Hh, downstream of early BMP signaling, but possibly via downregulation of mRNA expression of components of the Hh signaling pathway in undifferentiated cells, (5) besides transcriptional repression of RunX2 and osx also other mechanisms are involved in the inhibition of osteoblast differentiation and matrix mineralization by PTHrP and PTH, these may act in parallel with or downstream of RunX2 and osx.

In the following chapter, first the implications of these findings will be discussed in more detail. In addition a system will be described for Flp-mediated, targeted homologous recombination that can be used to either overexpress or silence specific genes in isogeneic lines. When applied to the mesenchymal progenitor cell-like KS483 cell line this system can be used to study signaling pathways important for osteoblast-, adipocyte- and chondrocyte differentiation.

Part I Regulation of osteoblast differentiation

The role of the major morphogenic pathways

Unraveling the pathways involved in osteoblast differentiation and understanding how they interact is likely to provide insights that will enable new therapeutic approaches for anabolic treatment of osteoporosis. In this thesis, we first investigated the effects and mechanisms of action of three major morphogenic pathways, BMP-, Hh- and Wnt signaling, on the various stages of KS483 osteoblastic differentiation. Subsequently, we investigated how PTHrP and PTH signaling might modulate osteoblast differentiation. For these studies mainly the KS483 cell line has been used, which can differentiate *in vitro* towards mature, mineralizing osteoblasts, fat droplets containing adipocytes or cartilaginous matrix producing chondrocytes depending on the culture conditions [15-17]

BMP signaling

In chapter 2, we first addressed the role of the BMP pathway during osteoblast differentiation. The mRNA expression of various components of the BMP pathway during KS483 differentiation into osteoblasts was characterized. Several BMPs, BMP receptors and signaling molecules were found to be expressed throughout KS843 osteoblastic differentiation. Although it has been widely recognized that BMPs are involved in the initiation of osteoblast differentiation, their role during matrix formation and mineralization has remained largely unknown [4,18-20]. The expression of BMP signaling components during later stages of KS483 osteoblastic differentiation suggested that BMP signaling might also be involved during matrix formation and mineralization. These data are in line with the expression of BMP-2 and –4 and BMP receptors during late phases of rat calvarial osteoblast differentiation [21]. In addition, it has already been shown that BMP signaling is involved in apoptosis, suggesting a role in regulation of the life span of osteoblasts [22]. Treatment with BMP antagonists resulted in decreased initiation of osteoblast differentiation in several cell types, such as murine bone marrow cells and KS483 cells [3,23]. In addition, treatment with BMP antagonists decreased the formation of ALP positive and mineralized nodules in KS483 cells in all phases of differentiation [3]. Notably, the BMP antagonists noggin, truncated soluble BMPR-IA or -IB inhibited mineralization of already existing nodules. Furthermore, we showed that addition of BMPs stimulated not only the initiation of differentiation, but also matrix formation and mineralization. Addition of BMPs during late stage osteoblast differentiation still increased mineralization.

Hedgehog signaling

Hedgehog, like BMPs, not only induces the commitment of precursor cells towards the osteoblast lineage, but also stimulates osteoblastic differentiation of uncommitted precursor cells [6,24]. However, the role of Hh during the successive stages of osteoblast differentiation has not been elucidated previously. We studied this in the KS483 cell line (chapter 3). First, we showed that mRNA of members of the IHh signaling pathway were expressed throughout osteoblastic differentiation. We demonstrated that in KS483 cells, IHh, as well as Ptc1 and Gli1 mRNA were expressed more abundantly in mature than in less differentiated osteoblasts. This was in line with findings of Jemtland et al. who

showed that IHh was expressed in UMR106, ROS 17/2.8, and SaOS-2 cells, lines with an mineralizing osteoblast-like phenotype, whereas less differentiated mouse MC3T3-E1 cells did not express IHh [5,24]. Previous studies showed that Hh induced osteoblast differentiation, suggesting that IHh, produced by osteoblasts, stimulates osteoblast differentiation and might recruit precursor cells towards the osteoblast lineage [25-28]. In line with these previous studies, addition of recombinant Hh to KS483 cells significantly increased osteoblast differentiation, as indicated by a modest increase in ALP activity. Moreover, addition of recombinant Hedgehog resulted in a strong increase in matrix mineralization and increased expression of osteoblast markers in KS483 cells. This is in marked contrast with BMP signaling, which potently stimulated both ALP activity and mineralization (chapter 2), suggesting that 1) ALP activity and matrix mineralization can be distinctly regulated, and that 2) Hh and BMP have a different mode of action.

There were also other marked differences between the two pathways; first, BMPs were shown to have an effect at all phases of differentiation (chapter 2), while only undifferentiated KS483 cells responded to Hh. In addition, autocrine Hh signaling did not play a role in osteoblast differentiation, as shown by the lack of effect of the Hh antagonist cyclopamine, while autocrine BMP signaling appeared to be crucial for osteoblast differentiation.

After we had found these differences between the effects of Hh and BMPs at various osteoblast differentiation stages, we wanted to investigate potential crosstalk between the two pathways. Before describing our findings we will discuss interactions between Hh and BMPs, which had previously been demonstrated in chondrocytes. In chondrocytes, BMPs can increase mRNA expression of IHh. Furthermore, *in vivo*, exposure of cartilage to the BMP antagonist noggin has been shown to reduce IHh expression [29,30] and the IHh promoter contains multiple Smad binding sites which can be activated by BMP treatment. Taken together these observations demonstrate that BMPs can directly regulate IHh expression [31]. In its turn, IHh maintains BMP expression in these cells, indicating the existence of a positive feedback loop between BMPs and IHh. This feedback loop might be cell type specific, since our experiments in KS483 cells did not provide evidence for a similar mechanism. In proliferating chondrocytes, overexpression of IHh in murine cartilage caused increased expression of BMPs [29,30]. Furthermore, Gli transcription factors (which are intracellular mediators of Hh signaling) activated BMP-4 and -7 promoter activity [32]. There is even evidence supporting synergy between BMP and IHh signaling. In C3H10T1/2 cells, SHh, increases Smad1-dependent transcriptional activity [6] (Smad1 is a factor generally associated with BMP signal transduction). Moreover, immuno-precipitation studies showed that Smad1 directly associated with truncated forms of Gli3 proteins, suggesting a possible mechanism for the observed synergy between IHh and BMP pathways [33]. However, neither pathway completely mediates the other's functions. For example, BMPs and IHh act independently of each other to regulate chondrocyte proliferation, and IHh regulates PTHrP expression independently of BMP signaling [30].

As described for the chondrocytes, also in KS483 osteoblasts, the effects of IHh on osteoblast differentiation could be completely blocked by BMP antagonists. On the other hand only high doses of cyclopamine inhibited BMP-induced differentiation, suggesting that Hh signaling required functional BMP signaling. However, the Hh pathway did not exclusively function via BMP since Hh and BMP synergistically

induced ALP activity, suggesting that Hh can also act via a BMP independent pathway in KS483 cells. This synergy occurred at the level of Hh response, and not at the level of early BMP response, as shown by transient reporter assays with BRE-luciferase and Gli-luciferase (chapter 3).

Wnt signaling

Recently, the Wnt pathway, a third major morphogenic signaling pathway, was found to be involved in bone development [7]. It was shown that in osteoblast progenitor cells, low levels of Wnt/β-catenin signaling stimulated proliferation and increased the amount of committed osteoblasts [34]. Higher levels were needed for the initiation of osteoblastic differentiation, as determined by ALP activity [34]. However, it turned out, that the effects of this pathway on osteoblast differentiation were very complex and dosage, cell type and differentiation stage dependent. In chapter 5, we described the effects of Wnt/β-catenin signaling on various phases of osteoblastic differentiation of KS483 and murine bone marrow cells *in vitro*. Undifferentiated KS483 cells already expressed sufficient levels of Wnt signaling to induce ALP activity, as indicated by the inability of Wnt3A or LiCl to affect ALP activity. However, both Wnt3A and LiCl did induce nuclear translocation of β-catenin as well as Wnt reporter activation. Furthermore, addition of the Wnt antagonist Dkk-1 dose-dependently decreased basal ALP activity. Interestingly, in long-term experiments, LiCl inhibited bone nodule formation and mineralization dose-dependently in both KS483 and (murine) bone marrow cells. In contrast, treatment with Wnt3A only inhibited nodule formation and mineralization in KS483 cells. In addition, Wnt3A could only inhibit undifferentiated, but not differentiated KS483 cells whereas LiCl inhibited irrespectively of differentiation stage. Interestingly, during late phase osteoblast differentiation, mRNA expression of the Wnt antagonists Dkk-1 and Dkk-2 increased in a specific pattern, while expression of the Frizzled receptors remained unchanged. In stable KS483 cells carrying an FRT insertion site (see below), nodule formation and matrix mineralization was completely blocked by insertion of an RNAi sequence to silence either Dkk-1 or Dkk-2. However, some differences were observed between Dkk-1 and Dkk-2 in the initiation of osteoblast differentiation; Dkk-2_{si} decreased proliferation and initiation of differentiation, while Dkk-1_{si} had no effect. Taken together, these data demonstrated that Wnt/β-catenin signaling needs to be downregulated to enable matrix maturation and mineralization. This downregulation was, at least in part, mediated by increased mRNA expression of the Wnt antagonists Dkk-1 and Dkk-2. In addition, these data suggest that Dkk-1 and Dkk-2 may have distinct functions in osteoblast differentiation. Whereas Dkk-1 may antagonize Wnt(s) induced ALP activity and block the transition of an ALP-positive into a mineralizing osteoblast, Dkk-2 may specifically antagonize Wnt(s) involved in cell proliferation and the initiation of osteoblast differentiation.

Modulation of osteoblast differentiation by PTH/PTHrP signaling

Changes in osteoblast differentiation in PTHrP and PTH1R receptor knockout and transgenic animals indicated that PTHrP signaling also plays a role in osteoblastic bone formation. For example, PTH1R null mice showed decreased trabecular bone, and abnormal bone mineralization [35]. Several groups showed that PTH increased the proliferation of osteoprogenitor cells and osteoblasts *in vitro*, and decreased apoptosis

of pre-osteoblasts and osteoblasts, thereby increasing the number of osteoblasts [36-38]. In addition, in the majority of the osteoblast differentiation studies, PTH inhibited osteoblast differentiation as measured by alkaline phosphatase (ALP) activity and nodule formation in various *in vitro* models [39-41]. Increased osteoblast differentiation has been found in few studies, and current data suggests that the effects of PTH are cell type, differentiation stage, dosage and exposure time dependent [40-43].
In our KS483 model, treatment with PTH or PTHrP resulted in inhibition of osteoblastic differentiation irrespectively of differentiation stage, exposure time and dosage (chapter 6). In figure 1, the possible interactions between PTH(rP) and major morphogenic signaling pathways are depicted and our observations regarding these interactions will be discussed below.

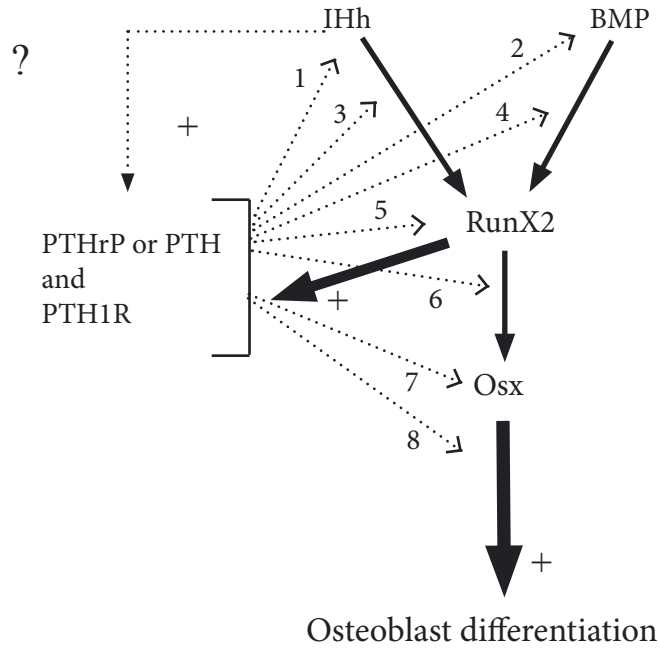


Figure 1 Possible mechanisms by which PTHrP or PTH can interfere with osteoblast differentiation
Inhibition **of osteoblast d**ifferentiation by PTHrP and PTH signaling pathways can occur at the level of 1) IHh expression; 2) expression of BMPs; 3) IHh induced signal transduction; 4) signal transduction 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.

BMP and PTH/PTHrP signaling

Since autocrine BMP signaling is important during all phases of KS483 osteoblastic differentiation [3,44], we proposed that PTHrP and PTH might inhibit differentiation by interfering with the BMP signaling pathway. Indeed, in KS483 cells, BMP signaling could be inhibited by PTHrP or PTH and vice versa (chapter 6). These data are in line with observations in chondrocytes, where BMP signaling stimulated chondrocyte maturation and could partially overcome the inhibitory effects of PTHrP on chondrocyte maturation [45]. Further studies showed that, in KS483 cells, PTHrP or PTH-mediated inhibition was downstream of early, Smad-dependent BMP signaling as well as of BMP-induced p38 MAPK signaling, suggesting that the PTHrP or PTH-induced inhibition on BMP signaling is an indirect response. Prolonged exposure to 10^{-7} M hPTHrP (1-34) decreased mRNA expression of BMP-8A, but not of BMP-4 and BMP-7 (chapter 6). However, as BMP-8A is a differentiation marker, its inhibition could also be due to the inhibitory effect of PTHrP or PTH on osteoblast differentiation.

Hedgehog and PTH/PTHrP signaling

PTHrP and PTH could also affect the Hh pathway. Interactions between the Hh and PTHrP have already been described in the growth plate, where they interact in a negative feedback loop.

In this feedback loop, IHh induces the expression of PTHrP at the peri-articular cartilage and PTHrP acts, through the PTH1R throughout the growth plate, and in particular, in the pre-hypertrophic chondrocytes, to prevent hypertrophic chondrogenic differentiation and decreases IHh expression [46]. Addition of N-terminal SHh, which contains the biological active part of Hh, can also increase the expression of PTHrP in osteoblasts-like cells and primary calvarial osteoblasts *in vitro* [24]. The SHh-induced expression of PTHrP might be part of a negative feedback loop counteracting Hh induced differentiation as has been described in chondrocytes [46].

In KS483 cells, Hh-induced ALP activity as well as nodule formation and mineralization could be inhibited by PTHrP or PTH and vice versa. In undifferentiated KS483 cells, PTHrP inhibited expression of IHh. In addition, mRNA expression of the Hh target genes Ptc1 and Gli1 was also decreased after PTHrP treatment, suggesting that IHh signaling was also inhibited, either directly or indirectly via inhibition of Hh expression.

The mechanism by which Hh acts during osteoblast differentiation strongly resembles its action in CFK-2 chondrocytic cells where IHh induces chondrogenic differentiation, and PTHrP antagonizes Hh-mediated differentiation through a PKA-dependent mechanism [47].

Wnt and PTH/PTHrP signaling

Preliminary studies have been done regarding the interactions between PTHrP and Wnt signaling, the third morphogenic pathway. In KS483 cells neither PTH nor PTHrP showed any effect on nuclear β-catenin translocation in undifferentiated KS483 cells and furthermore, neither altered basal or Wnt3A-induced Wnt reporter activation (unpublished observations). This is in line with data from osteoblast progenitor cells which showed that PTHrP did not regulate β-catenin expression [48]. However, it remains possible that PTH and/or PTHrP interact with Wnt signaling in mature differentiated

osteoblasts. One publication showed that hPTH treatment dramatically induced a Wnt reporter in chondrocytes at the centre of the rudiments [49]. Further studies would be required to address the interactions between Wnt and PTHrP signaling in differentiated osteoblasts. In addition, it would be interesting to investigate whether PTH or PTHrP treatment could affect nuclear β-catenin translocation *in vivo*.

RunX2 and PTH/PTHrP signaling

One of the downstream targets of the Hh and BMP pathway is the MASN splice variant of RunX2. It is known that Runx2 can stimulate transcription of osteoblast related genes. In addition, Runx2, at least in part, regulates the response of pre-osteoblasts to extracellular matrix signals [50,51]. Several pathways can modulate the expression levels of RunX2, as well as its activity posttranslationally. Moreover, Runx2 has been shown to interact with co-regulatory proteins for gene activation and repression, such as AP-1, Lef-1, Smad complexes, and Groucho [52-55]. For example, BMPs can increase Runx2 expression, but can in addition, also activate other signaling pathways that, together with Runx2, stimulate osteoblastic gene expression [11,12]. The Hh pathway, has also been shown to be involved in regulation of RunX2 [13,14] and recently, the Wnt transcription factor Lef-1 has been shown to inhibit RunX2-dependent activation of the osteocalcin promoter [53].

The relation between RunX2 and PTH/PTHrP signaling is more complex. It has been shown that PTHrP inhibits RunX2 expression both at the mRNA and protein level in chondrocytes [56]. However, in the osteosarcoma cell line UMR 106, no changes were found in RunX2 mRNA or protein expression levels after PTH treatment.

In addition, PTHrP and PTH could regulate RunX2 activity by posttranslational modifications [56,57]. PTH can phosphorylate RunX2 in a PKA and MAPK dependent manner. These events are, however, associated with activation of the transactivation functions of RunX2. It seems, therefore, unlikely that these actions are involved in the inhibitory actions of PTHrP or PTH on osteoblast differentiation of KS483 cells [57,58].

More promising in this respect is the observation that PTH can also increase proteasomal proteolysis of Runx2 via the E3 ligase (ubiquitin-protein isopeptide ligase) (Smurf1) that targets Runx2 for degradation [59]. In addition, PTHrP or PTH might also indirectly affect RunX2 activity via the major morphogenic pathways BMP, Hh or Wnt.

In our studies, we first checked whether PTHrP interfered with RunX2 transcription in KS483 cells. In chapter 6 we show that treatment with hPTHrP (1-34) for up to 24h had no significant effect on mRNA expression of RunX2. In contrast, treatment of undifferentiated KS483 cells with hPTHrP (1-34) for 72 hours prevented the increase in RunX2 mRNA and protein expression normally observed during osteoblast differentiation. This suggested that transcriptional regulation of RunX2 was indirectly involved in the inhibitory effects of PTHrP or PTH on osteoblast differentiation. Remarkably, overexpression of RunX2 under control of the EF1α promoter could not reverse the inhibitory effects of PTHrP or PTH on osteoblast differentiation. PTHrP and PTH inhibited ALP activity to a similar extent in control and RunX2 overexpressing clones. This could be due to the PTHrP or PTH-induced modest decrease in RunX2 mRNA and protein expression. However, matrix mineralization was almost completely blocked in the RunX2 overexpressing clone, suggesting that other mechanisms may be involved, such as posttranslational modification of RunX2, mechanisms downstream

of RunX2 or RunX2 independent mechanisms. Since we did not observe dissociation between RunX2 mRNA and protein expression, it seemed unlikely that proteasomal degradation played an important role in the PTHrP or PTH-mediated inhibition of osteoblast differentiation. Another way in which PTHrP might also decrease RunX2 activity could be by inducing an antagonist of RunX2, such as Twist [60,61]. Interestingly, the Twist promoter contains a putative CRE near its transcription start site (our unpublished observation [62,63]). Whether PTHrP-mediated inhibition of RunX2 activity occurs via induction of Twist expression will be subject of further studies.

Osterix and PTH/PTHrP signaling

PTHrP and PTH could also regulate the expression or activity of other genes involved in matrix mineralization acting downstream of RunX2, such as osterix (osx).

Osx has been shown to be indispensable for osteoblast differentiation and bone formation [10] and is expressed in cells associated with trabecular bone of growing mice and with early osteoblast markers, such as type I collagen and bone sialoprotein. Osx expression *in vitro* has been found in differentiating fetal rat calvarial cells stimulated with dexamethasone. Osx mRNA expression appeared at day 11, increased up to day 15 and decreased thereafter. RunX2 expression in these cells appeared earlier, at day 5, and followed a similar pattern for the later time points [64]. In contrast, during osteoblastic differentiation of KS483 cells, no significant changes were found in mRNA levels of osx (chapter 6). Overexpression of osx cells resulted in increased osteocalcin mRNA expression in C2C12 and C3H10T1/2 cells but only increased collagen I expression in C2C12 cells 10. In addition, the formation of mineralized bone nodules increased when osx was overexpressed in murine embryonic stem cells [65].

The relationship between osx and PTHrP is not very clear yet. In mice, treated intermittently with PTH, no significant changes were observed in RunX2 mRNA expression, whereas PTH increased osx as well as osteocalcin expression [66]. Whether PTHrP or PTH affects osx activity has not been described.

In chapter 6, we show that, *in vitro*, PTHrP can inhibit osx mRNA expression in differentiated KS483 cells already after 6 hours treatment, suggesting that this is an intermediate-early response. In undifferentiated KS483 cells, PTHrP did not affect osx mRNA expression. This could be due to low levels of PTH1R mRNA expression and the low cAMP response after PTHrP treatment in undifferentiated KS483 cells (chapter 6). In our system, overexpression of osx predominantly stimulated late stage osteoblast differentiation, as shown by increased matrix mineralization (chapter 6), although to a lesser extent than RunX2 overexpression. Early osteoblast differentiation, as assayed by ALP activity, was only borderline increased in the osx overexpressing clone.

Forced overexpression of osx under control of the EF1α promoter could not rescue the inhibition of ALP activity caused by PTHrP or PTH treatment, in line with the absence of an effect on osx on early osteoblast differentiation. In addition, osx overexpression could not reverse the PTHrP or PTH-induced inhibition of matrix mineralization. These data suggest that several mechanisms probably acting simultaneously or in parallel are responsible for the inhibition of matrix mineralization by PTHrP or PTH. Furthermore, we cannot exclude that PTHrP and PTH represses osx at the posttranslational level by inhibiting its transactivation functions.

The inhibitory effects of PTHrP and PTH on matrix mineralization, in which inhibition

of osx expression may play a role, might be a relevant finding with respect to the anabolic actions of PTHrP and PTH on bone. In particular it should be noted that PTHrP and PTH potently inhibited matrix mineralization in mouse bone marrow cultures without affecting ALP-activity in long-term cultures, indicating that this action of PTHrP and PTH is not restricted to KS483 cells.
This observation may be clinically relevant as anabolic treatment regimes using PTH induce new bone formation which is relatively under-mineralized [67].

Interaction between PTH/PTHrP signaling and major morphogenic pathways.

In our system the modulation of osteoblast differentiation by PTHrP and PTH occurred downstream of early BMP signaling and prolonged exposure to PTHrP or PTH had no effect on the mRNA expression of BMP-4 and –7. However, whether PTHrP or PTH treatment can affect the mRNA expression of other BMPs still has to be examined. In addition, our data showed that in undifferentiated cells, PTHrP and PTH might act via downregulation of mRNA expression of components of the Hh signaling pathway (fig. 2, arrow 1) and that PTHrP and PTH had an inhibitory effect on Hh signaling either directly or indirectly by downregulation of Hh expression (arrow 3). Moreover, we showed that PTHrP and PTH had no direct effect on RunX2 mRNA expression. PTHrP and PTH might therefore instead inhibit RunX2 activity via posttranslational modification (arrow 6) or might act downstream of RunX2 or even via RunX2 independent mechanisms. On the other hand PTHrP did downregulate the mRNA expression of the downstream transcription factor osx, in differentiated, but not in undifferentiated KS483 cells (arrow 7). In addition to its effects on osx expression, PTHrP or PTH might also affect osx activity itself (arrow 8).
Taken together, these data show that PTHrP or PTH-mediated inhibition of osteoblast differentiation can occur via multiple mechanisms. Besides direct effects on the expression of osx and the function of RunX2 also other mechanisms are involved that act in parallel with or downstream from these transcription factors. As all these mechanisms are involved in the inhibition of osteoblast differentiation and matrix mineralization by PTHrP and PTH these issues require further study. Finally, although we showed that the Wnt pathway was not affected by PTH/PTHrP signaling in undifferentiated KS483 cells, the effect of PTHrP and PTH on Wnt signaling in differentiated KS483 cells was not studied and still requires further investigation.

Time window of action of the morphogenic pathways.

We demonstrated that the BMP signaling pathway is important during all phases of KS483 osteoblastic differentiation. On the other hand, the Hh pathway affects only early stages of osteoblast differentiation, while terminal differentiation remains unaffected. Our data and previous literature suggest that the Wnt signaling pathway stimulates early osteoblast differentiation, whereas for terminal differentiation and mineralization Wnt signaling needs to be downregulated. The time window of action of each of these pathways is shown in figure 2.
Taken together, the three major osteogenic signaling pathways discussed, differ in their effect on osteoblast differentiation, in their time window of action (fig. 2) and in their

interaction with PTHrP or PTH (fig. 3).
How these data can be extrapolated to the *in vivo* situation still needs to be studied. After continuous addition of PTHrP or PTH both bone formation and resorption increase, although the increase in bone resorption is higher than the increase in bone formation, leading to bone loss [1]. The formation of mature osteoblasts is repressed after continuous treatment.
In recent randomized controlled clinical trials of intermittent PTH treatment, PTH decreased incidence of vertebral and non-vertebral fractures in postmenopausal women [68]. In addition, intermittent treatment resulted in a marked increase in BMD at trabecular bone sites, an increase in cortical thickness and enhanced bone as shown by and early rise of bone formation markers followed by significant increases in bone resorption. The increase in bone formation is higher than the increase in resorption resulting in new bone tissue, which is initially under-mineralized. However, osteoblast maturation is not completely blocked by PTH. PTHrP might keep osteoblastic cells longer in a proliferative competent state (reviewed in [67]).
In the end, when the anabolic and catabolic mechanisms of PTH or PTHrP are elucidated, both mechanistically and in molecular terms, it might be possible to develop PTH analogues that are more anabolic.

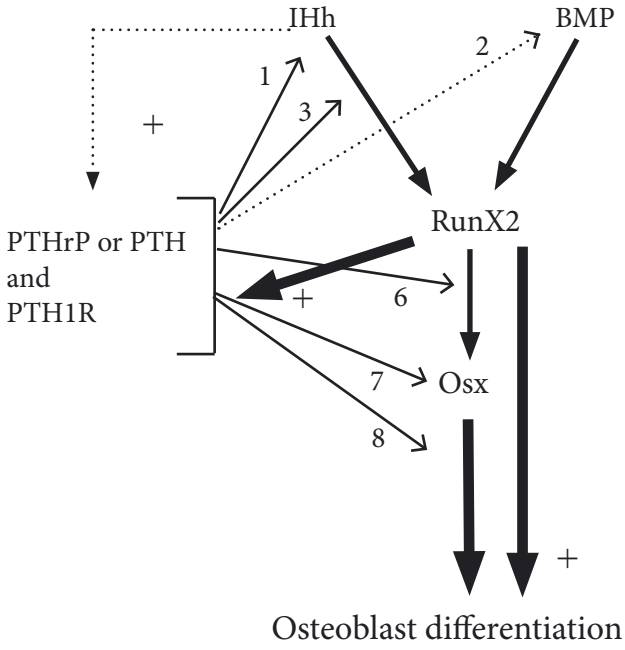


Figure 2 Mechanisms by which PTHrP and PTH can interfere with osteoblast differentiation
Interaction of PTHrP and osteogenic signaling pathways can occur at the level of Hh expression and signaling in undifferentiated KS483 cells, since PTH or PTHrP downregulated mRNA expression of components of the Hh signaling pathway (arrow 1) and PTHrP or PTH affect early Hedgehog signaling, since PTHrP treatment downregulated Hh target gene expression (arrow 3). PTHrP had no direct effect on RunX2 mRNA expression, but probably acts posttranslationally (arrow 6). PTHrP downregulated Osx mRNA expression in differentiated, but not in undifferentiated KS483 cells (arrow 7). Furthermore, the inhibitory effects of PTHrP could not be overcome by overexpression of either RunX2 or Osterix, suggesting that PTHrP or PTH act on posttranslational modification of these transcription factors or downstream of these factors (arrows 6 and 8).

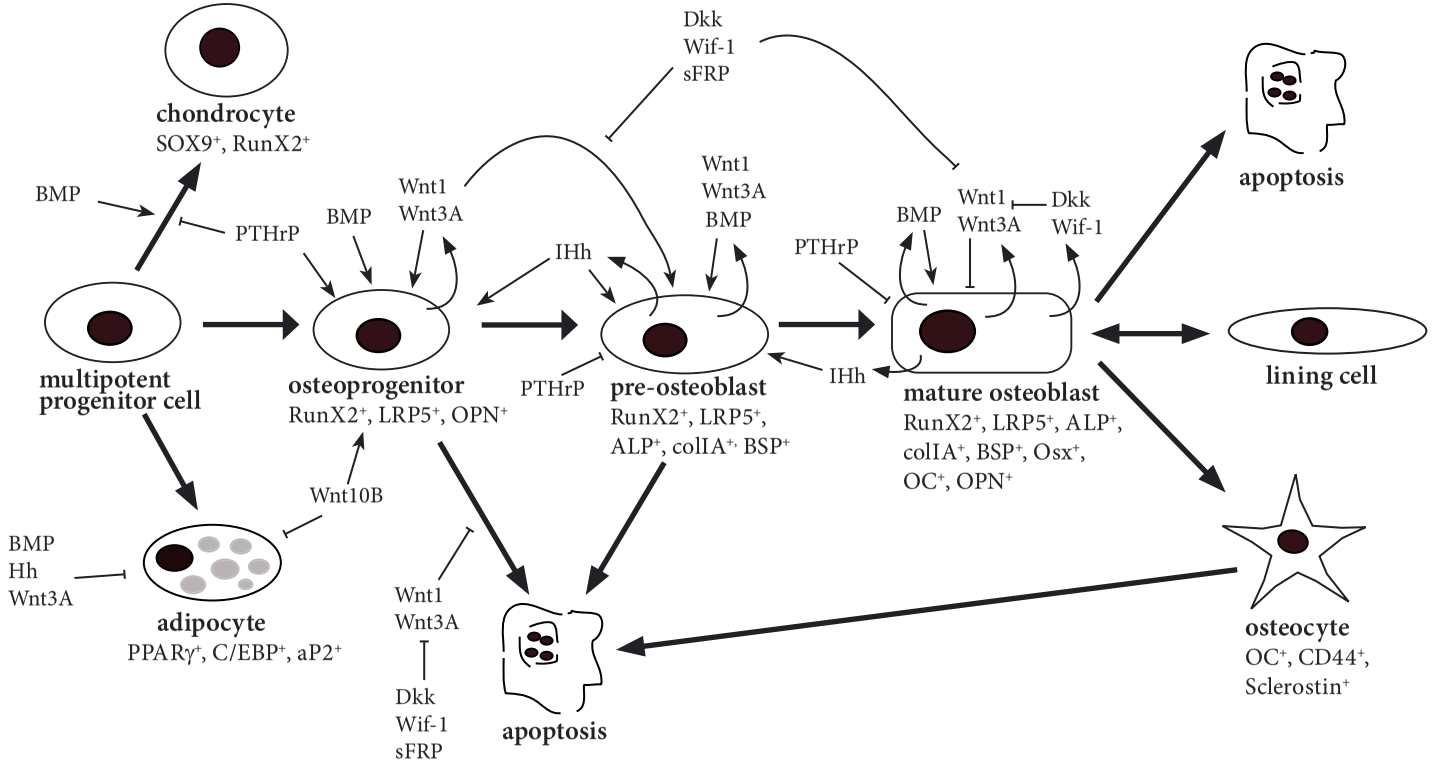


Figure 3 Effects and time window of action of major signaling pathways during differentiation
For the differentiation of multipotent mesenchymal cells towards specialized cells, first the cells proliferate (self renewal), then commit towards the adipocyte, chondrocyte or osteoblast lineage and finally differentiate. These processes are regulated by several pathways and essential transcription factors (expression of several pathways and transcription factors is depicted under the cells). The BMP, Hh and Wnt signaling pathways inhibit adipogenic differentiation. Chondrogenic differentiation is inhibited by PTHrP and stimulated by BMPs, whereas different Wnts have opposite effects. For osteoblastic differentiation, osteoprogenitors first differentiate towards pre-osteoblasts, which then differentiate into mature osteoblasts. During this process a matrix is formed, which eventually becomes mineralized. The BMP, Hh, Wnt and PTHrP pathways signal differentially during these subsequent phases. Mature osteoblasts can eventually differentiate towards osteocytes, become lining cells, or undergo apoptosis.

Part II KSFrt model for the generation of stable cell lines using Flp-mediated homologous recombination

Flp-mediated homologous recombination

To investigate the role of major signaling pathways in osteoblast differentiation and potential crosstalk between these pathways, pharmacologic experiments involving e.g. recombinant growth factors or specific inhibitors can be used. However, there are several limitations to these methods, such as the lack of available recombinant proteins or the limited specificity of the inhibitors [69]. In addition, transient transfections can be used to insert vectors in the cell for overexpression studies or gene knock down using RNAi or dominant negative constructs. However, using transient transfections, not all cells are transfected, thereby blunting the effect on markers such as ALP activity. Moreover, for these experiments only undifferentiated, proliferating cells can be used.

To study effects of gene overexpression or knock down during the whole differentiation period, stable cell lines need to be generated. However, stable transformants often show extreme variability in expression of the introduced transgene due to the highly variable number of copies integrated into the genome and to positional effects on gene expression. Furthermore, integration can occur at a genomic locus involved in the regulation of differentiation or other cellular functions, thereby interfering with or even disrupting the function of these genes independently of the transgene function. All this compromises direct comparison of different expression constructs in clones obtained from the same parental cell line.

Some drawbacks in generation of stable cell lines could be circumvented by site-specific integration of DNA at a pre-selected site in the genome such as used in Flp-mediated homologous recombination. This method requires the introduction of a unique FRT site in the genome of the cell since these sites are not present in the mammalian genome [70]. First a single FRT site is introduced in the cell's genome as an integral part of the open reading frame of a blasticidin resistance gene, thus creating an Frt host cell line. This site can subsequently be used for targeted insertion of a transgene by Flp-mediated recombination. The sequence specificity mediates the in frame fusion of the genomic FRT site with a homologous site in a crippled hygromycin resistance gene present in the transgene construct thereby reconstituting a functional enzyme. This allows positive selection for stable integrants and simultaneously negative selection for random integrants in a single step.

KSFrt model

In chapter 4, we generated various KSFrt host clones. As expected, likely due to the random nature of insertion as well as clonal variability, the host clones displayed considerable variation in basal and BMP-induced ALP activity as well as in the formation of a mineralized bone matrix when cultured in osteogenic conditions. The 3B2 clone was indistinguishable from the parental KS483 cell line in osteoblastic and adipogenic culture conditions, whereas the amount of cartilaginous matrix production was relatively increased. In the second clone, 4C3, the basal amount of ALP-positive and mineralized

nodules was lower than that of the parental cells, although BMP-induced differentiation was augmented. Furthermore, a relative increase in adipocytes was found under both basal and indomethacin-stimulated conditions, whereas chondrogenic differentiation was similar to the parental KS483 cell line. The third clone, 4D3, displayed increased basal osteoblast differentiation, which could still be further increased by BMPs. Basal adipogenic properties were similar to parental KS483 cells, whereas indomethacin-induced adipogenesis was modestly increased. Finally, the amount of cartilaginous matrix was relatively increased in the 4D3 clone compared to the parental line.
We showed that these different KSFrt host clones could be used for overexpression or knock down of any gene of interest. In addition, the KSFrt clones can be used for promoter-reporter studies and *in vivo* studies. In the following paragraphs, this will be discussed using some examples.

Overexpression studies

Overexpression of a luciferase reporter gene using the EF1α promoter showed that in the KSFrt host cell lines the FRT site was integrated in a genomic region which was transcriptionally active and relatively insensitive to positional effects on EF1a-promoter driven transgene expression. Proof of principle for gene function studies by overexpressing a more relevant transgene was obtained in KSFrt clones overexpressing RunX2. When a single copy of a RunX2 gene was inserted in the unique FRT site of either KSFrt host cell line, in all three cases this was sufficient to cause increased osteogenic and accelerated hypertrophic chondrocyte differentiation, while adipogenic differentiation was blocked. These effects are fully in line with the described effects of RunX2 on differentiation of mesenchymal cell lineages (reviewed in [71,72]). In addition, overexpression of osx, another important transcription factor for osteoblast differentiation, also resulted in increased osteoblast differentiation, although to a lesser extent than RunX2 overexpressing KSFrt cells (chapter 6).

Knock down studies using RNA interference

Human RNAse H1 and mouse U6 promoters driving short hairpin oligo's have been used previously for successful knock-down of gene expression [73,74]. Use of this RNAse H1 promoter in KSFrt clones, resulted in an RNAi response in transiently transfected cells, but not in stably transfected cells, indicating that one copy of this vector was not sufficient to elicit a biological response.
Since the presence of an upstream enhancer is required for transcriptional regulation of the "natural" RNAse H1 promoter, we added extra copies of this enhancer, and thus generated the p5HI vector [75]. Stable integration of this vector containing the human RNAseH1 promoter and the enhancers efficiently silenced the expression of endogenously expressed genes in a sequence-specific manner. The increased efficiency is most likely explained by increased transcription of the hairpin oligonucleotides. Proof of principle was obtained in isogenic stable cell lines expressing an RNAi construct targeting RunX2 ($RunX2_{si}$) or a mutant construct ($mtRunX2_{si}$). Osteoblast differentiation was impaired in the $RunX2_{si}$ cell lines, while adipocyte differentiation was unaffected. These data are in line with data from RunX2 deficient mice, of which the calvarial cells express very low levels of early osteoblast markers (ALP/osteonectin), and no osteocalcin and osteopontin, which are markers for mature osteoblasts [9,76,77].

Although delivery of siRNA cassettes into mammalian cells has been shown earlier using adenovirus, retrovirus or lentivirus, these systems appeared to require larger copy numbers of siRNA per genome, whereas our data clearly demonstrated that one copy of an RNAi vector is sufficient for silencing endogenously expressed genes [78-81].

Using the KSFrt system to study the Wnt signaling pathway

An example how the KSFrt model could be used is shown in chapter 5, where we used the KSFrt system to silence the Wnt antagonists Dkk-1 and Dkk-2. Our data showed that these Wnt antagonists play an important role during osteoblastic differentiation. It would be interesting to further define the role of the respective Dkk proteins during differentiation. Moreover, it would be interesting to investigate whether other Wnt antagonists also play an important role in this process, such as the soluble Frizzled related proteins (sFRPs) or the Wnt inhibitory factor-1 (Wif-1), which bind directly to Wnts [82,83]. In a pilot experiment KSFrt cells were generated stably expressing an RNAi construct targeting either the Wnt antagonist Wif-1 ($Wif\text{-}1_{si}$) or the Adenomatous Polyposis Coli gene (APC_{si}), which is part of an intracellular complex involved in Wnt signaling. Preliminary data demonstrated that silencing Wif-1 results in extremely inhibited proliferation and cell death likely due to apoptosis since they phenotypically showed rounded cells that contain membrane blebbing (unpublished data). In the APC_{si} line, the proliferation rate as well as the initiation of osteoblast differentiation were significantly decreased compared to controls. These data are in line with the absence of bone in teratocarcinomas of $APC^{-/-}$ embryonic stem cells [84]. Further studies are needed to define the role of APC and Wif-1 during osteoblastic differentiation.

Other applications of the KSFrt model

We have demonstrated that KSFrt cells can be used for lineage-specific promoter studies. Luciferase activity driven by an adipocyte-specific promoter fragment (aP2) was increased during adipogenic differentiation and could be induced by stimulators of adipogenesis such as indomethacin, whereas the promoter-less construct had no luciferase activity. We are currently generating osteocalcin (OC)-luciferase and Collagen X-luciferase reporter lines to monitor respectively osteoblast and chondrocyte differentiation. Other promoter-reporter cell lines might also be generated, such as reporter lines coupling luciferase with the BMP responsive element (BRE) to study BMP signaling, the CREB binding responsive element (CRE) to study PTHrP signaling, the TCF/LEF binding element (TBE) to study Wnt signaling or the Hh responsive element to study Hh signaling [85-88].

In addition to the *in vitro* studies, these reporter lines and other genetically modified KSFrt cells can be used for *in vivo* studies, as has been shown by the intra-osseous injection of KSFrt cells overexpressing luciferase.

Taken together, with this model, individual genes and their role in the differentiation process towards different mesenchymal lineages can be studied in a fast and reproducible way using either overexpression or RNAi knock down. This model can be used for instance to study the function of novel genes that were identified by microarray analysis or to further examine cooperation between different morphogenic pathways.

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