Regulation of osteoblast differentiation

Barendsz-van der Horst, Geertje

3 Hedgehog stimulates only osteoblastic differentiation of undifferentiated KS483 cells

Geertje van der Horst[1], Hetty Farih-Sips[1], Clemens W.G.M. Löwik[1], and Marcel Karperien[1,2]

[1] Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, Leiden
[2] Department of Pediatrics, Leiden University Medical Center, Leiden

Bone 2003 33(6): 899-910

Abstract

The involvement of Hedgehog (Hh) signaling in the initiation of osteoblastic differentiation in the bone collar during endochondral bone formation has been well established. The stages at which Hh acts during osteoblast differentiation as well as its molecular mechanism of action are less well understood. To address these questions, we have made use of the pre-osteoblastic cell line KS483. Firstly, a systematic survey of mRNA expression of osteoblastic differentiation showed expression of IHh and signaling intermediates at all stages. Interestingly, expression of IHh, Gli1 and Ptc1 peaked during the maturation phase. Addition of recombinant human sonic hedgehog (rhSHh) potently increased osteoblastic differentiation of KS483 cells dose-dependently as assayed by a modest increase in alkaline phosphatase activity (ALP), a strong increase in matrix mineralization and increased mRNA expression of established osteoblast marker genes. These effects were blocked by the Hh antagonist cyclopamine, which by itself was ineffective. Addition of rhSHh during early stages was sufficient, while addition to mature osteoblasts had no effect. Furthermore, Hh signaling could be completely blocked by the BMP antagonists soluble truncated BMPR-IA and noggin. In contrast, the BMP-induced differentiation of KS483 cells could only be partly inhibited by high doses of cyclopamine. These data demonstrate that Hh induced osteoblastic differentiation requires functional BMP signaling. In KS483 cells, Hh and BMP synergistically induced ALP activity, only when suboptimal concentrations of BMP were used. This synergy did not occur at the level of immediate early BMP response, but at the level of Hh response as determined by transient transfection studies using either a BMP reporter or a Gli reporter construct. In addition, rhSHh inhibited adipogenesis of KS483 cells cultured in adipogenic culture conditions, suggesting that Hh is involved in directing differentiation of KS483 cells towards osteoblasts at the expense of adipogenesis. Using in situ hybridization, we demonstrated for the first time, IHh mRNA expression *in vivo* in osteoblasts and lining cells in the humerus of developing human skeleton. Our *in vitro* and *in vivo* data indicate a stimulatory role for osteoblast expressed IHh in bone formation in a positive feedback loop. It may recruit progenitor cells in the osteoblastic lineage on the expense of adipocytes and it may stimulate maturation of early osteoblasts.

Introduction

Members of the conserved Hedgehog (Hh) signaling family are involved in many important developmental processes such as patterning and cellular proliferation [15]. Furthermore, they have been shown to play a role in the regulation of skeletal formation in vertebrates (reviewed in [18]). At least three family members have been identified in vertebrates to date: Sonic Hh (SHh), Indian Hh (IHh) and Desert Hh (DHh) [13,12]. IHh has been shown to regulate several aspects of endochondral bone formation. IHh induces expression of parathyroid hormone related peptide (PTHrP) in the chondrocytes at the apical site of the developing long bones, which results in an inhibition of the differentiation of proliferating chondrocytes into mature hypertrophic chondrocytes. Furthermore, Hh stimulates chondrocyte proliferation and maturation [32]. In addition, IHh signaling has been shown to be essential for the initiation of osteoblast formation in the bone collar [32,6]. This is supported by the absence of the bone-specific transcription factor Runx2 (Cbfα1) in the perichondrium of IHh null embryos. Mice in which another Hh member, SHh, has been deleted fail to form vertebrae and display severe defects in distal limb skeletal elements [4]. Furthermore, SHh is able to induce ectopic bone formation *in vivo* when injected in mesenchyme in a manner similar to BMP-2 [20]. These data indicate that Hh signaling is involved in initial steps of osteoblastic differentiation during endochondral bone formation. Whether Hh also plays a role in osteoblastic differentiation during later stages of bone development is less clear.

It has been shown earlier that SHh is able to induce alkaline phosphatase activity (ALP), a marker of osteoblast differentiation in the osteoblast cell line MC3T3-E1 *in vitro* [26]. In addition, when the IHh gene was retrovirally introduced into primary calvarial osteoblast, IHh overexpressing osteoblasts showed much higher ALP activity [18]. In the murine mesenchymal cell line C3H10T1/2, Hh induced osteoblastic differentiation, while simultaneously adipogenesis was inhibited [19,20,26,31,39]. However, it is not known whether Hh affects mineralization of osteoblastic cells. In addition, the mechanism of action of Hh is not very clear yet. Several lines of evidence support a mutual relationship between Hh and BMPs. First, Hh has been shown to promote chondrogenesis by altering the cellular response to BMP signaling [25]. In addition, SHh and BMPs synergistically induced mRNA expression of ALP and osteocalcin (OC) in C3H10T1/2 cells and murine primary osteoblast cells [38]. Furthermore, BMP signaling stimulates mRNA expression of Ihh in chondrocytes and in Drosophila the Hh mediator Gli homologue, ci regulates expression of decapentaplegic, the Drosophila homologue of BMPs [14,1,22,23]. Conversely, Hh induces expression of various BMPs in chondrocytes [22,23]. In contrast, Hh did not alter the mRNA expression levels of BMP-2, -4, -5, -6 and –7 in C3H10T1/2 cells [26]. At the signal transduction level, complexes between truncated Gli3 proteins and intracellular BMP mediators, Smads, have been found in COS-1 and R1B/L17 cells [21]. Still, the precise interactions between Hh and BMP during osteoblast differentiation are not very clear yet.

To address these issues we have used the murine pre-osteoblastic KS483 cell line, which can differentiate into either mature mineralizing osteoblasts or into adipocytes depending on the culture conditions [9,37]. We demonstrate that Hh induces osteoblastic differentiation of undifferentiated osteoblasts at the expense of adipogenesis, in a BMP signaling dependent manner. In addition, we show synergism between Hh and BMPs at the level of Gli reporter activity. Moreover, we identify the osteoblast as a major source of IHh in human long bone development.

Materials and methods

Cell culture and differentiation studies

KS483 cells were cultured routinely as described previously [36]. For differentiation assays, KS483 cells were seeded at a density of 12.000 cells/cm^2. Every 3 to 4 days, the medium was changed. At confluence (from day 4 of culture onwards), ascorbic acid (50 mg/ml, Merck. Inc., NY, USA) and when nodules appeared (from day 11 of culture onwards) β-glycerolphosphate (5 mM, Sigma Chemical Co., St Louis, MO) were added to the culture medium. After three weeks of culture, cells were washed with phosphate buffered saline (PBS), and fixed with 3.7% buffered formaldehyde for 5 minutes. The cultures were analyzed for adipogenesis, osteogenesis and DNA content. First, cultures were stained for Oil red O, which is specific for lipid droplets. Cultures were washed in H_2O, stained with 0.3% Oil red O in 60 % isopropanol for 10 minutes at room temperature and thereafter washed with H_2O. To extract Oil red O from the cells, 300 µl ethanol (100 %) was added. The amount of Oil red O was measured spectrophotometrically at 550 nm with a reference of 650 nm against an Oil red O standard curve. Thereafter, cultures were washed with PBS, stained for alkaline phosphatase (ALP) for 5 minutes with Napthol AS-MX phosphate/Fast blue solution (Sigma). Subsequently, ALP staining was completely washed out of the cell layer with a freshly prepared solution of EtOH supplemented with 10 µl/ml 5M NaOH. After washing with PBS, mineralized nodules were stained with 2% Alizarin Red solution (ICN Biomedicals Inc, OHIO, USA), Alizarin was removed from the cell layer using 10% cetylpiridium CL in 10 mM PO_4 buffer (pH 7.5), and measured at 550 nm. The DNA content of each well was measured as described previously [36]. Pictures of the images were taken and digital imaging was performed as described previously [36].

ALP activity and DNA content

KS483 cells were cultured until confluence and thereafter cultured for 3 days with ascorbic acid in the absence or presence of recombinant human BMP-4 (50-100 ng/ml; R&D systems, Uithoorn, The Netherlands), recombinant human BMP-6 (100 ng/ml; R&D systems), recombinant depalmityolated human sonic hedgehog (rhSHh; 3-300 ng/ml, kindly provided by Dr. A. Esswein, Boehringer Mannheim), cyclopamine (1-10 µM, kindly provided by Dr. W. Gaffield, Western Regional Research Center, Albany, USA), the BMP-antagonists recombinant human BMP receptor IA/Fc chimera (trBMPR-IA, 250 ng/ml, R&D systems) or recombinant noggin (250 ng/ml, R&D systems). At day 11, the supernatants were withdrawn and the cells were washed twice with PBS. ALP activity and DNA content were extracted from the cell layer and measured as described previously [36].

Transfection and assay for luciferase activity

KS483 cells were seeded at a density of 9500/cm^2 in 24-wells plates, and transiently transfected with 1 µg of the reporter construct using Fugene-6 transfection reagent (Roche, Basel, Switzerland), according to the manufacture's protocol. The following reporter constructs were used: the BMP reporter constructs, Msx2-luc and $(BRE)_4$-luc (kindly provided by Dr. P. ten Dijke, Netherlands Cancer Institute, Amsterdam, The Netherlands), and the Gli reporter construct, 8x3'Gli-luc (kindly provided by Dr. Sasaki, Laboratory of Developmental Biology, Osaka University, Osaka, Japan [30]). To correct for transfection efficiency, 100 ng of renilla luciferase (pRL-SV40; Promega, Madison, USA) was co-transfected. 12 hrs after transfection, the medium was changed for medium containing 0.2 % FCS. The cells were kept in this medium for 1 day, and then left either non-stimulated or stimulated for an additional 24 hrs. Luciferase assays were performed with the Dual-Luciferase Reporter assay system (Promega) according to the protocol. 10 µl of cell lysate was first assayed for firefly luciferase and then for Renilla luciferase activity, using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin-Elmer, Boston, USA). Firefly luciferase activity was corrected for renilla luciferase activity.

RT-PCR

RNA isolation was performed by the method described by Chomzynski and Sachi [5] and reverse transcribed into cDNA. cDNA was standardized by competitive PCR using an internal standard (pMUS) as described by van Bezooijen et al [3]. Subsequently, the expression of various genes was examined by semi-quantitative PCR as described previously [36]. For used primersets see table 1.

In situ hybridization

For in situ hybridization, we used the method previously described by our group [11,35]. DIG-labeled probes were made of sequence verified cDNA fragments of human *Ihh*, cloned in PCRtm3. The probes were hydrolyzed to 200 bp prior to hybridization. Sections of normal prenatal humerus were obtained from the department of pathology, where they were routinely used as reference material. A 6th toe was obtained of a 6-month-old baby after surgery informed consent of parents. Images of the sections were taken with the DXM-1200 digital camera (Nikon).

Statistics

Values represent mean ± SEM. Differences were examined by analysis of variance (ANOVA) followed by the post-hoc least significant difference test (LSD). Results were considered significant at $p< 0.05$.

Results

Expression of Hh and signaling intermediates during osteoblastic differentiation of KS483 cells

KS483 cells differentiate into mature mineralizing osteoblasts in a three-week culture period. During this process, alkaline phosphatase (ALP) mRNA expression increased from day 4 to 18, decreasing thereafter, whereas expression of the differentiation marker

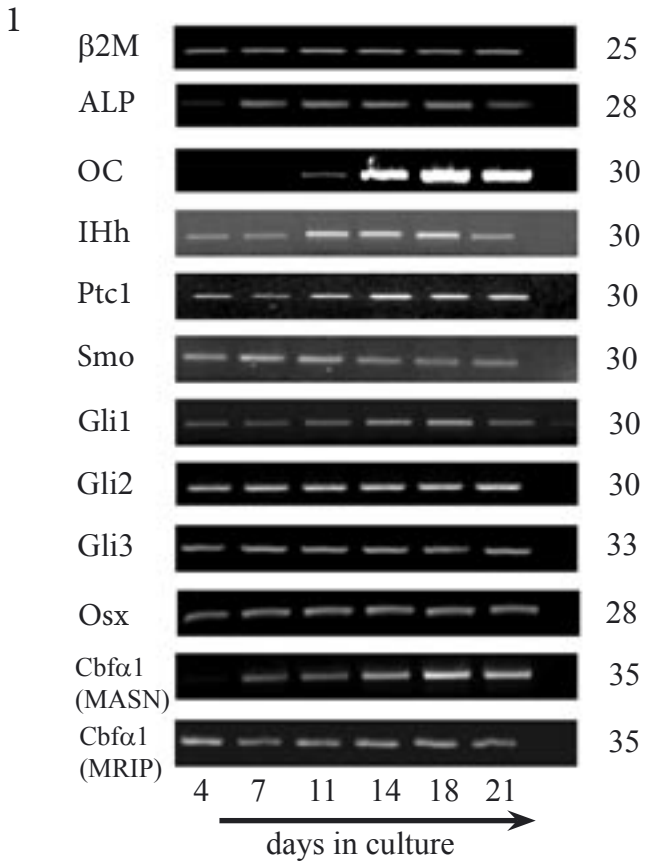


Figure 1 Expression of Hh and Hh signaling intermediates during osteoblastic differentiation of KS483.
At day 4, 7, 11, 14, 18 and 21 of culture, total cellular RNA was isolated and cDNA was prepared and standardized for household gene expression, β2 microglobulin (β2M), by competition PCR. These data are confirmed by semi-quantitative PCR for β2M. Subsequently, semi-quantitative PCR for the indicated genes was performed as described in materials and methods. As a negative control H_2O was used. No bands were seen in negative controls. Lane 1, day 4; lane 2, day 7; lane 3, day 11; lane 4, day 14; lane 5, day 18, lane 6, day 21 of culture. Data are representative for three separate experiments.

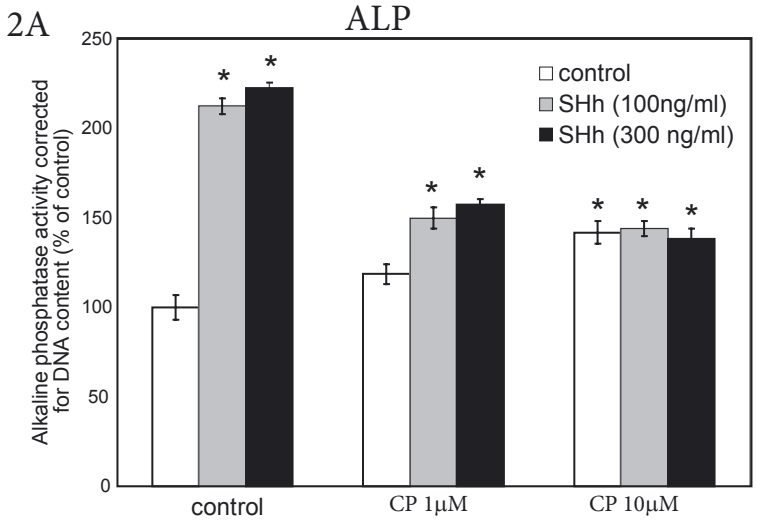
osteocalcin (OC) started at the onset of mineralization (day 11) (figure 1; [10,36]).
KS483 cells also expressed the bone specific transcription factors, osterix (osx) and Runx2 (Cbfα1 MASN splice variant), which are indispensable for osteoblastic differentiation. The expression of the MASN splice variant of Runx2 increased during differentiation. In contrast, the Runx2 MRIP splice variant and osx were expressed at comparable levels at all stages of differentiation (fig. 1).
To investigate the role of Hh signaling in KS483 osteoblastic differentiation, we first surveyed the expression of various Hh-signaling components at characteristic stages of osteoblastic differentiation. KS483 cells expressed Indian Hh (IHh), the Hh receptors patched1 (Ptc1), patched2 (Ptc2) and smoothened (Smo), as well as the transcription factors Gli1, -2 and -3.
Interestingly, IHh mRNA expression peaked during matrix formation and maturation,

and decreased thereafter (fig.1). A similar expression pattern was found for the Hh target genes Gli1 and Ptc1. The expression of Gli2 or –3 mRNA did not change with differentiation. In contrast very low levels of Ptc2 were found throughout differentiation and no Hh interacting protein mRNA (HIP) was detected in KS483 cells (data not shown). Furthermore, we did not detect expression of the Hh family member SHh in KS483 cells (data not shown).

Effect of recombinant Hh on KS483 osteoblastic differentiation

To investigate effects of Hh on osteoblastic differentiation, KS483 cells were cultured for 18 days in the absence or presence of recombinant depalmitolyated sonic hedgehog (rhSHh) and subsequently ALP activity and mineral content of the cultures were measured. To exclude whether the effects of Hh were caused by an effect on cell proliferation, the DNA content of each well was measured. No significant differences were seen in DNA content (data not shown). Continuous addition of rhSHh (dose range of 3-1000 ng/ml) significantly increased osteoblastic differentiation of KS483 cells dose-dependently (data not shown). Continuous treatment with 100 or 300 ng/ml rhSHh resulted in modest effects on ALP activity (2-fold), whereas mineralization was strongly affected (6-fold; fig. 2A and B, respectively). The Hh effect could be completely blocked by the alkaloid cyclopamine, which has previously been demonstrated to inhibit Hh signal transduction in target cells [16,8,34]. Treatment of KS483 cells with 1 μM cyclopamine, slightly induced ALP activity and mineral content, although not significant. Treatment with a high concentration of cyclopamine (10 μM) significantly induced ALP activity, but not mineral content. At this concentration, cyclopamine is known to have Hh independent effects on cholesterol metabolism.

At the mRNA level, continuous treatment with Hh increased the expression of established osteoblastic markers, such as OC, type I PTH/PTHrP receptor (PTH1R), Runx2 (MASN), ALP, BMP-8A and osx.



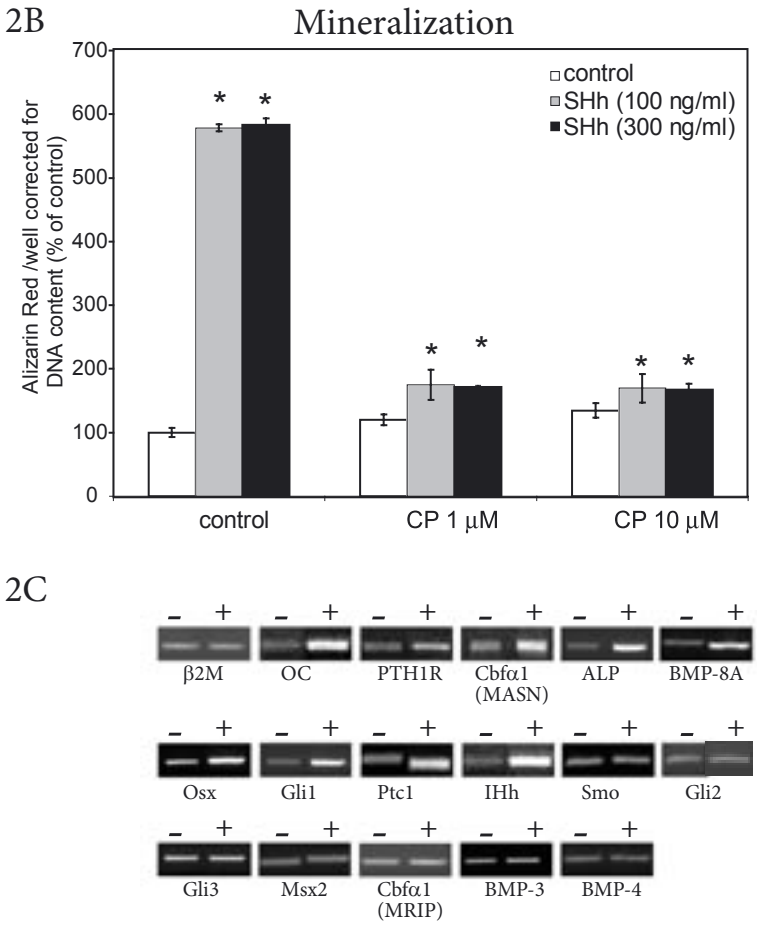
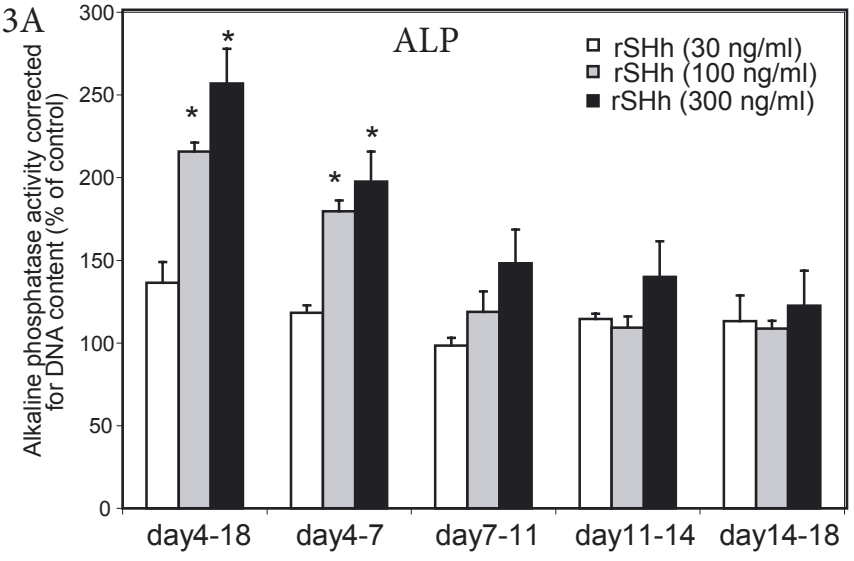


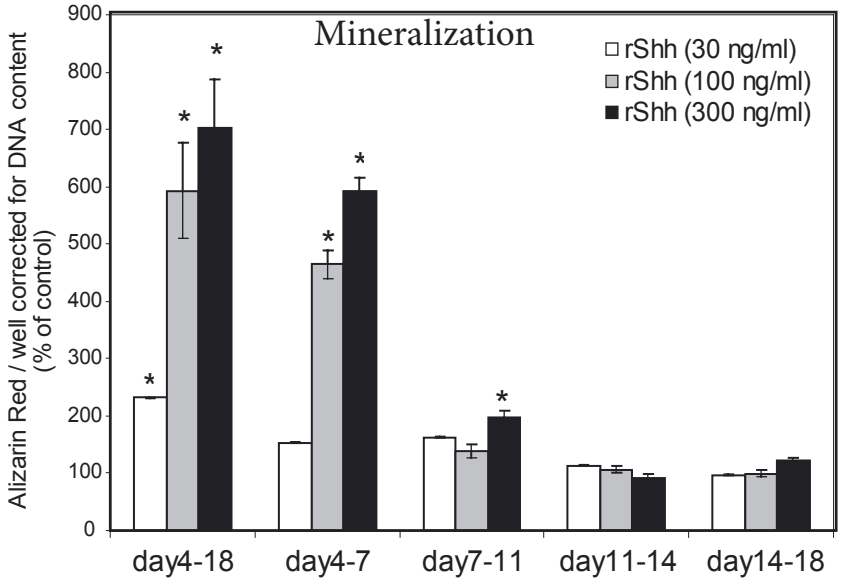
Figure 2 Effects of recombinant SHh on KS483 osteoblastic differentiation.
KS483 cells were cultured until confluence and thereafter treated until day 18 with rhSHh (100 or 300 ng/ml) in the absence or presence of the Hh antagonist cyclopamine (1 or 10 μM). Cells were either lysed and ALP activity and DNA content were measured (A) or cells were fixed with 3.7 % buffered formaline and stained for Alizarin and measured for DNA content. (B). Values represent the average ± SEM of 3 independent duplicate experiments corrected for DNA content and are expressed as percentage of control. * Significant vs. control ($p<0.05$). For effects of Hh on mRNA expression, KS483 cells were cultured until confluence, and thereafter continuously treated with rhSHh (100 ng/ml). At day 18, total cellular RNA of control and Hh-treated cells was isolated and cDNA was prepared and standardized for housekeeping gene expression (β2M) by competition PCR. Subsequently, semi-quantitative PCR for the indicated genes was performed as described in materials and methods. As a negative control H_2O was used. No bands were seen in negative controls. Data are representative for three separate experiments.

Furthermore, mRNA expression of the known Hh target-genes, Gli1 and Ptc1 was up regulated as well as the expression of IHh itself. In contrast, mRNA expression of Smo, Gli2, Gli3 was not affected. Interestingly, the expression of the homeobox protein Msx2, Runx2 (MRIP), BMP-3 and BMP-4 was not altered (fig. 2C).

3A


ALP
rSHh (30 ng/ml)
rSHh (100 ng/ml)
rSHh (300 ng/ml)
Alkaline phosphatase activity corrected for DNA content (% of control)
300
250
200
150
100
50
0
day4-18
day4-7
day7-11
day11-14
day14-18

3B


Mineralization
rShh (30 ng/ml)
rShh (100 ng/ml)
rShh (300 ng/ml)
Alizarin Red / well corrected for DNA content (% of control)
900
800
700
600
500
400
300
200
100
0
day4-18
day4-7
day7-11
day11-14
day14-18

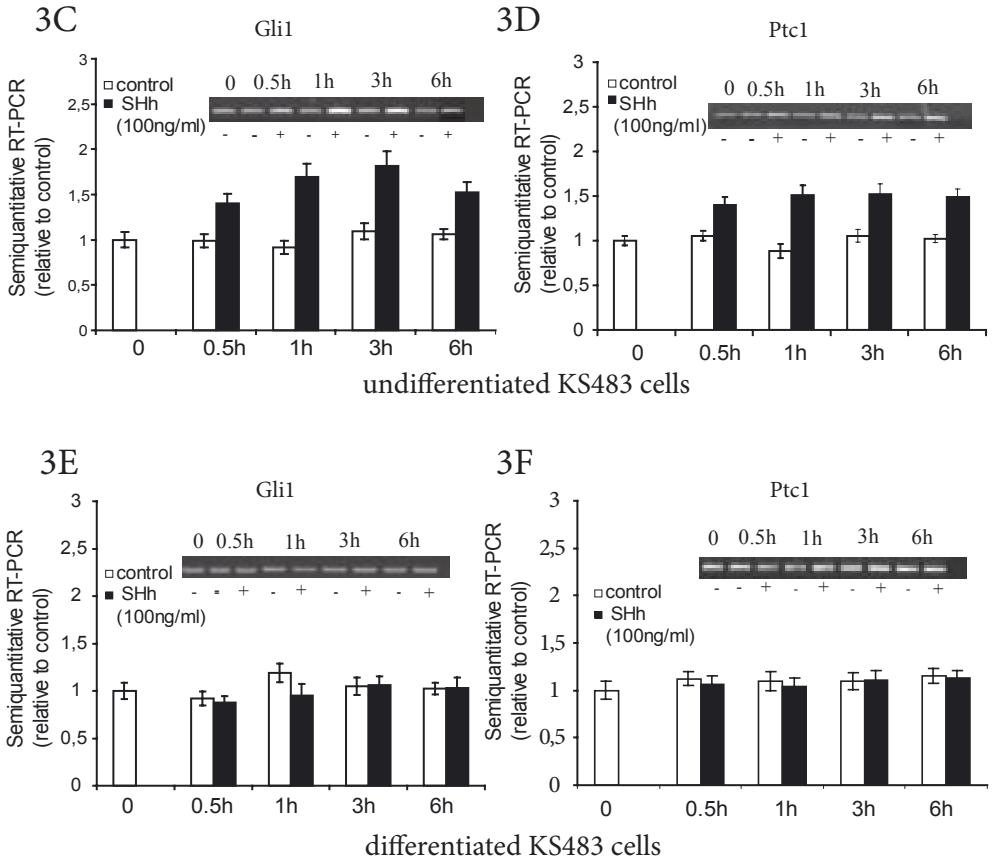


Figure 3 Time window of Hh action.
KS483 cells were cultured until confluence and thereafter cultured until day 18 for the indicated time periods with either 30, 100 or 300 ng/ml rhSHh. ALP activity (A) and amount of Alizarin Red/well (B) were measured. Values were corrected for DNA content and represent the average ± SEM of 2 independent triplicate experiments corrected for DNA content and are expressed as percentage of control. * Significant vs. control ($p<0.05$). Effect of rhSHh on Hh target gene expression in non-differentiated and differentiated cells. KS483 cells were cultured until day 4 or 11, respectively for undifferentiated (C, D) and differentiated cells (E, F). Cells were stimulated with rhSHh (100 ng/ml) for the indicated time periods. Total cellular RNA of control and Hh-treated cells was isolated and cDNA was prepared and standardized for housekeeping gene expression (β2M) by competition PCR. Subsequently, semi-quantitative PCR for Ptc1 and Gli1 was performed as described in materials and methods. As a negative control H_2O was used. No bands were seen in negative controls. The intensity of the bands was quantified using Scion Image. Data are representative for three separate experiments (values were corrected for β2M expression and represent average ± SEM). (G) KS483 cells were cultured until confluence and thereafter treated for 72 hrs with rhSHh (100 ng/ml). RNA was isolated and cDNA was prepared and standardized for housekeeping gene expression (b2M) by competition PCR. Subsequently, semi-quantitative PCR for the indicated genes was performed. No bands were seen in negative controls.

Time period of the Hh effect

KS483 osteoblastic differentiation can be divided into various periods, according to the model of Stein and Lian [33]. To determine during which phases Hh was able to stimulate osteoblastic differentiation, KS483 cells were treated with rhSHh during consecutive time windows and the effect on ALP activity and mineral content was measured after

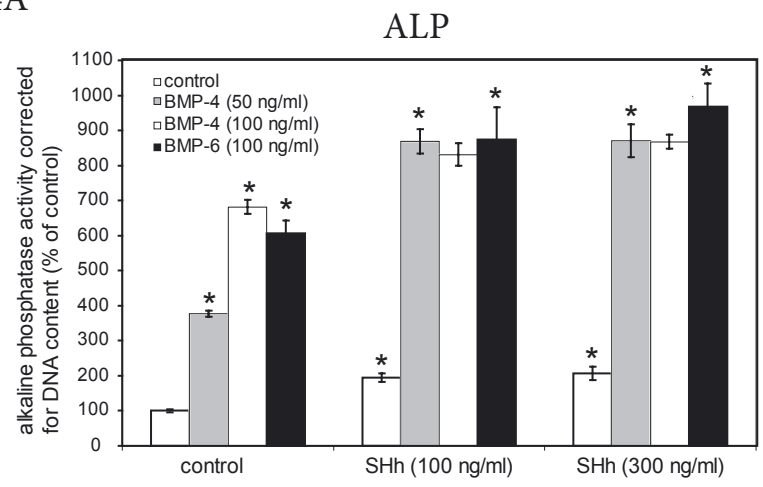
a three-week culture period. Continuous treatment with rhSHh dose-dependently induced ALP activity and mineral content (fig. 3A and B). Treatment with rhSHh during the first 3 days after confluence (day 4-7) is sufficient to induce ALP activity, whereas addition during day 7-11 was less effective. Addition of rhSHh during later stages of differentiation did not affect differentiation.
These data suggest that Hh predominantly affects less differentiated osteoblasts. To further investigate this, we treated undifferentiated and differentiated KS483 cells (respectively cultured until day 4 and 11) with 100 ng/ml rhSHh and examined the mRNA expression patterns of Hh responsive genes after 0.5 - 1 - 3 and 6 hrs. As shown in figure 3, the expression of Gli1 and Ptc1 is up regulated by rhSHh in undifferentiated cells only (fig. 3C-F). Furthermore, we examined the effect of Hh on gene expression of undifferentiated KS483 cells. Therefore, we treated confluent KS483 cells (day 4) for 72 hrs with rhSHh (100 ng/ml) and performed semi-quantitative RT-PCR. rhSHh treatment resulted in increased mRNA expression of the target genes Gli1, Ptc1 as well as Ihh (fig. 3G). ALP mRNA expression is slightly induced, as confirmed by the modest increase in ALP activity. In addition, no effect was seen on mRNA expression of the Hh receptor Smo, BMP-4, Osx and both splice variants of Runx2 (fig. 3G).

Interactions between Hh and BMPs

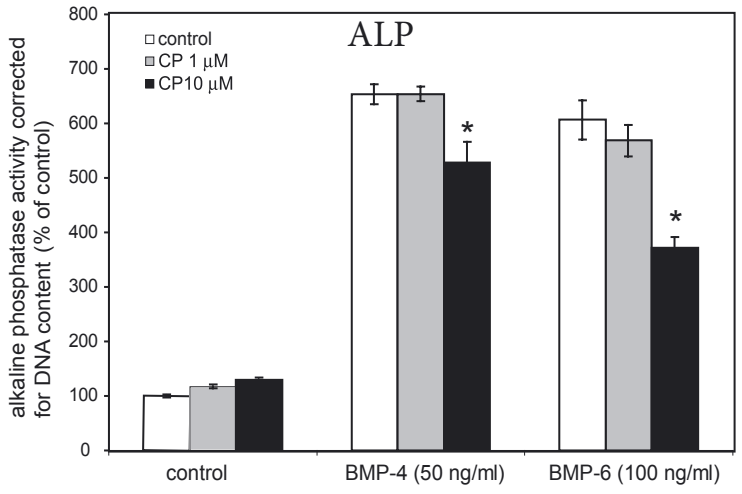
Previously, we have shown that autocrine BMP signaling is crucial for osteoblastic differentiation of KS483 cells. In addition, in various model systems, it has been shown that Hh exerts its effect via BMPs. This prompted us to investigate the relationship between Hh and BMPs more thoroughly. Therefore, we manipulated KS483 osteoblastic differentiation by treating the cells either with BMP-4, BMP-6 and SHh as activators and with noggin, soluble truncated BMPR-IA and cyclopamine as inhibitors of respectively the BMP and IHh signaling systems. ALP activity was measured and corrected for the amount of DNA. As shown in figure 4A, co-stimulation of BMP-4 (50 ng/ml or 100 ng/ml) or –6 (100 ng/ml) with rhSHh (100 or 300 ng/ml) synergistically enhanced ALP activity. However, synergism was only found when suboptimal concentrations of BMPs were used.
BMP-4 and –6 induced ALP activity could be partially inhibited only by high doses of the hedgehog antagonist cyclopamine (10 μM, fig. 4B). This concentration is known to have side effects on cholesterol metabolism. In contrast, Shh-induced ALP activity was completely blocked by the BMP antagonists noggin and soluble truncated BMPR-IA (fig. 4C). Similar effects were seen using the BMP antagonist trBMPR-1B (data not shown).
To investigate at which level Hh and BMP interaction occurs, transient transfection experiments were performed with either BMP reporter constructs or Gli reporter constructs. First, KS483 cells were transiently transfected with the SMAD dependent promoter-reporter construct Msx2 coupled to luciferase. BMP-4 and –6 induced luciferase activity of the Msx2 promoter dose-dependently (fig. 4D; dose range BMP-6: 50-200 ng/ml, data not shown).
In contrast, rhSHh itself did not induce BMP-responsive promoters nor did co-stimulation enhance reporter activity (fig. 4D). Similar results were found using the $(BRE)_4$-luc reporter (data not shown). As expected, the BMP antagonist noggin could block the BMP-induced luciferase expression completely. However, only high

concentrations of the Hh antagonist cyclopamine could slightly inhibit BMP-induced luciferase, in parallel to the effects on ALP activity (fig. 4E).
We further investigated interactions between BMP and Hh signaling by using a Gli reporter. This reporter can be used as a SHh responsive element [29]. Recombinant SHh induced expression of the Gli reporter dose-dependently (2 to 3-fold; fig. 4F). The stimulatory effects of Hh were specifically blocked by the antagonist cyclopamine, which by itself had no effect on luciferase expression. Interestingly, the Gli reporter was also activated by BMP-4 (2-fold) and this effect could only be blocked by high concentrations of cyclopamine (10 μM). Furthermore, the effect of rhSHh on the Gli reporter could be synergistically enhanced with BMP-4 (8-fold; fig. 4F).

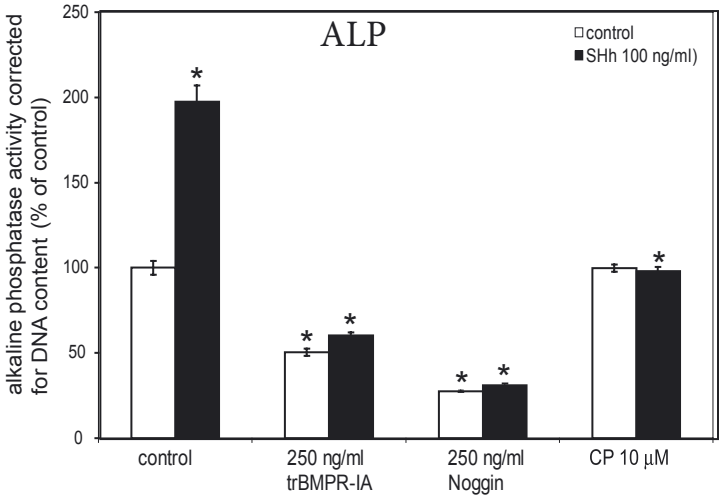
4A



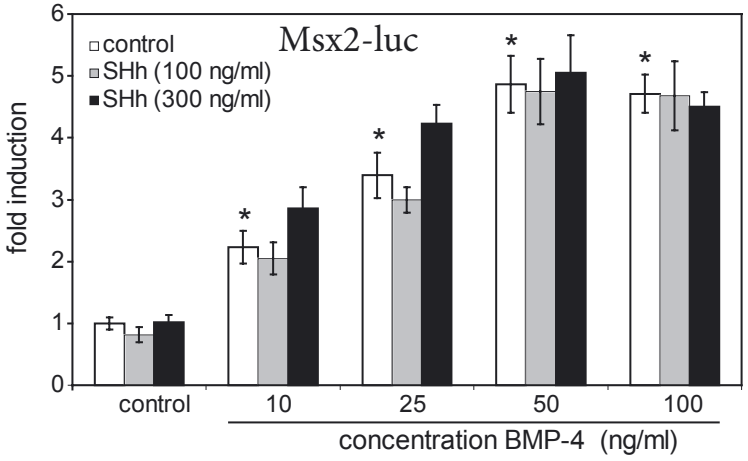
4B



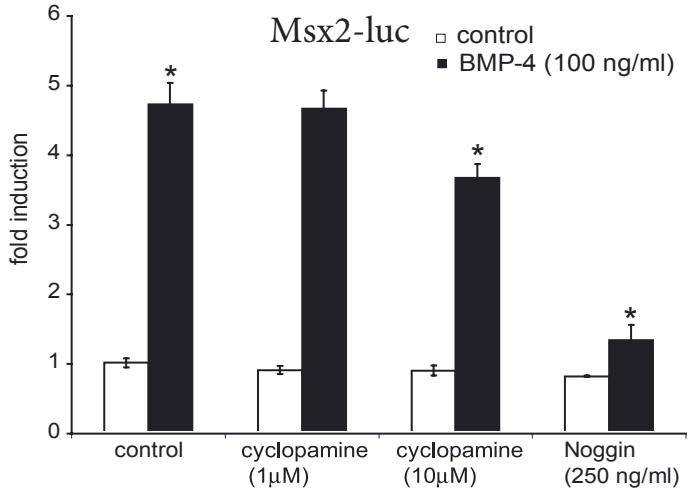
4C


ALP
control
SHh 100 ng/ml)
alkaline phosphatase activity corrected for DNA content (% of control)
250
200
150
100
50
0
control
250 ng/ml trBMPR-IA
250 ng/ml Noggin
CP 10 μM

4D


Msx2-luc
control
SHh (100 ng/ml)
SHh (300 ng/ml)
fold induction
6
5
4
3
2
1
0
control
10
25
50
100
concentration BMP-4 (ng/ml)

4E


Msx2-luc
control
BMP-4 (100 ng/ml)
fold induction
6
5
4
3
2
1
0
control
cyclopamine (1μM)
cyclopamine (10μM)
Noggin (250 ng/ml)

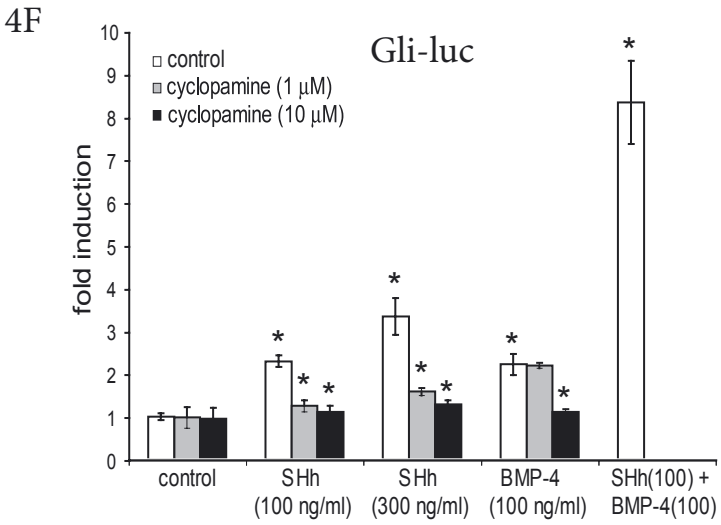


Figure 4 Interactions between Hh and BMP.
KS483 cells were cultured until confluence and thereafter treated with (A) BMP-4 (50 or 100 ng/ml) or BMP-6 (100 ng/ml) in combination with rhSHh (100 or 300 ng/ml). (B) BMP-4 (50 ng/ml) or BMP-6 (100 ng/ml) in combination with cyclopamine (1 or 10 μM). (C) rhSHh (100 ng/ml) in combination with either soluble truncated BMPR-IA (250 ng/ml), soluble noggin (250 ng/ml) or cyclopamine (10 μM). Cultures were stopped at day 11 and ALP activity and DNA content was measured. Values represent the average ± SEM of three independent triplicate experiments corrected for DNA content and are expressed as percentage of control. * Significant vs. control ($p<0.05$). KS483 cells were transiently transfected with Msx2-luc and (D) stimulated with BMP-4 (10, 25, 50 or 100 ng/ml) in combination with either 100 ng/ml or 300 ng/ml rhSHh or (E) with BMP-4 (100 ng/ml) in combination with either cyclopamine (10 μM) or noggin (250 ng/ml). (F) KS483 cells were transiently transfected with a Gli-reporter construct and were stimulated with either rhSHh (100 or 300 ng/ml) or BMP-4 (100 ng/ml) in combination with cyclopamine (10 μM). Values represent the average ± SEM of 3 independent triplicate experiments corrected for transfection efficiency and are expressed as fold induction. * Significant vs. control ($p<0.05$).

Effect of hedgehog on adipogenic differentiation of KS483 cells.

Recently we have shown that the bipotential precursor cell line KS483 can differentiate into either osteoblasts or adipocytes depending on the culture conditions [9]. To examine the effect of Hh on adipogenic differentiation, KS483 cells were cultured in 10% charcoal stripped serum and treated with different concentrations of rhSHh from day 4 onwards. At day 7, both osteoblast and adipocytes were found in the cultures and nodules appeared at day 11 of culture. Like under normal serum conditions, Hh increased both ALP activity (data not shown) and the amount of mineral after 18 days of culture (fig. 5B). In addition, treatment with rhSHh decreased adipogenesis as assayed by the number of Oil Red O positive adipocytes (fig. 5A), as well as the amount of Oil Red O staining (data not shown).

To further investigate the effects of Hh on adipogenesis, we treated KS483 cells with the adipogenic stimulator indomethacin for 18 days in stripped serum. Indomethacin increased the amount of adipocytes dose-dependently and reciprocally inhibited osteoblastic differentiation as assayed by ALP activity (data not shown) and amount of mineral (respectively fig. 5A and B). These effects could be reversed by rhSHh, suggesting that Hh is involved in directing differentiation of mesenchymal precursor cells towards osteoblasts.

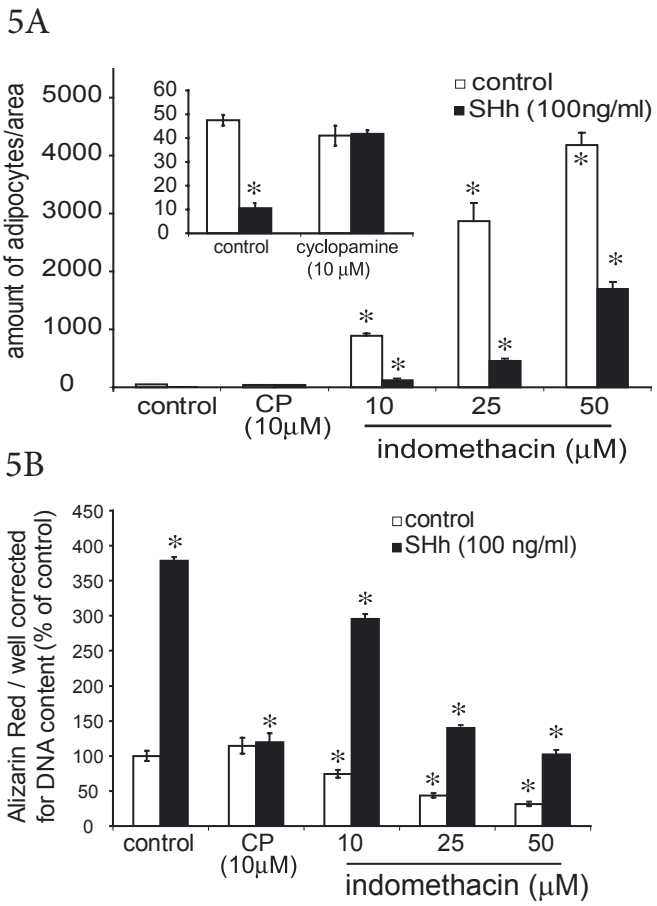


Figure 5 Effect of Hh on KS483 adipogenic differentiation.
KS483 cells were cultured in charcoal stripped serum until confluence and thereafter either non-treated or treated with rhSHh (100 ng/ml), cyclopamine (10 μM) or indomethacin (10, 25 or 50 μM). Cultures were fixed at day 18 with 3.7 % buffered formaline and stained for adipocytes and amount of mineral . The amount of adipocytes / area (A; average ± SEM of 2 independent counts) and amount of Alizarin Red (B) were measured. Data are expressed as percentage of control and are representative for two independent duplicate experiments (mean ± SEM) * significant vs. control ($p<0.05$).

Localization of IHh mRNA in human bone

It has been shown earlier that Hh is important for the formation of the bone collar during initial stages of endochondral bone formation. Whether Hh is also involved in later stages of osteoblastic differentiation is less clear. Therefore, we examined IHh expression in a 22-weeks-old prenatal human embryonic humerus and a 6-months-old postnatal toe. As expected, IHh mRNA was detected in the hypertrophic chondrocytes of the growth plate (fig. 6A). Interestingly, Ihh mRNA was also found in osteoblasts and lining cells in prenatal human humerus (fig. 6B). In addition, IHh mRNA was found in hypertrophic chondrocytes and osteoblasts in a 6-months-old postnatal toe (fig. 6C). No signals were obtained using sense IHh probes (fig. D).

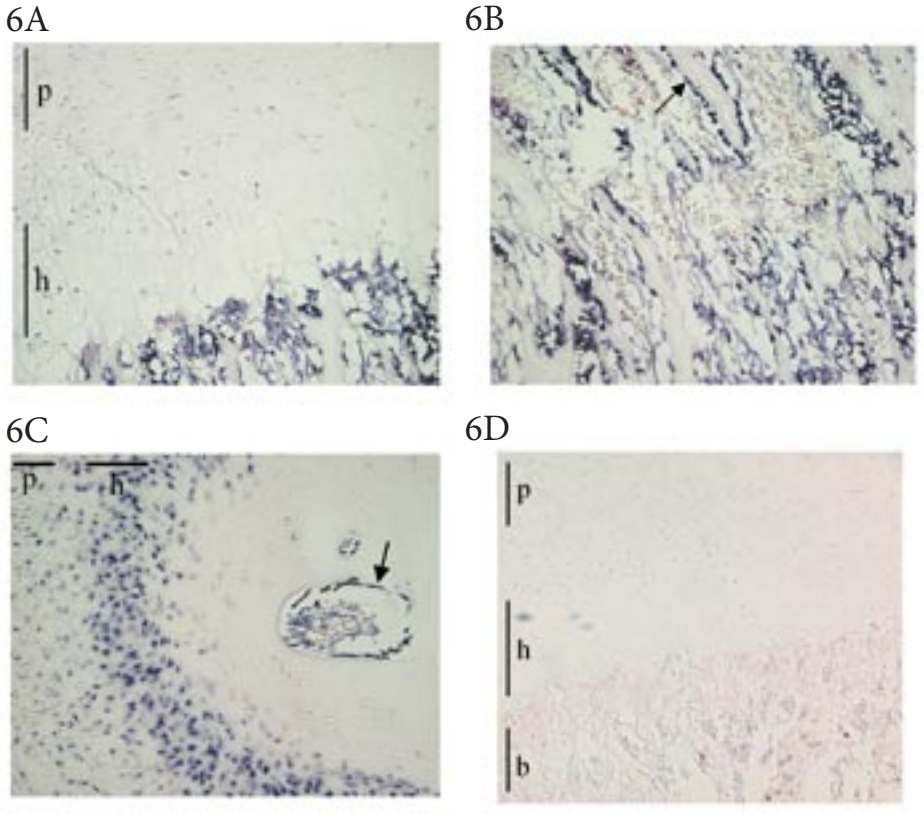


Figure 6 Localization of IHh mRNA in human bone.
In situ hybridization of IHh mRNA in human prenatal and postnatal humerus. Representative sections of trabecular bone are shown (p = proliferative chondrocytes; h = hypertrophic chondrocytes; b = trabecular bone). Growth plate of 22-weeks prenatal humerus showing IHh positive chondrocytes (A). 22 weeks prenatal humerus with IHh mRNA expression in osteoblasts (B; arrow indicates positive osteoblast lining cell) and IHh mRNA expression in hypertrophic chondrocytes and osteoblasts of a 6-months-old postnatal toe (C; arrow indicates positive osteoblast). Control sense experiments (D).

Discussion

In the present study, we analyzed the role of Hh signaling on osteoblastic and adipocytic differentiation of the murine mesenchymal precursor cell line KS483. A systematic survey of mRNA expression during osteoblastic differentiation showed expression of IHh and Hh signaling intermediates. Interestingly, expression of IHh, Gli1 and Ptc1 was regulated and peaked during the maturation phase. Addition of recombinant human sonic hedgehog (rhSHh) potently increased osteoblastic differentiation of KS483 cells dose-dependently as determined by a modest increase in ALP activity and increased mRNA expression of established osteoblast marker genes, as shown previously in other cell lines [26,31,38]. In addition, we show for the first time that Hh strongly increases matrix mineralization. For this effect, addition of Hh during early stages of osteoblastic differentiation was sufficient while addition during later phases had no effect, suggesting that Hh predominantly affects undifferentiated osteoblasts. This is further supported by mRNA expression data demonstrating that undifferentiated KS483 cells showed an induction of Hh target genes in response to Hh, in contrast to differentiated cells, which

did not. These data are in line with previous *in vitro* data showing that only immature pluripotent cells respond to Hh as well as with the effects of Hh on the initiation of osteoblastic differentiation during bone collar formation [7,31]. The effects of rhSHh were specific since they could be completely blocked by the Hh antagonist cyclopamine. Cyclopamine itself slightly induced basal ALP activity only at high concentrations. This high concentration of cyclopamine is known to inhibit cholesterol synthesis [17] and it is likely that this inhibition is not Hh dependent, since low concentrations of cyclopamine are sufficient for Hh inhibition. Addition of cyclopamine itself had no significant effect on basal mineralization, suggesting that autocrine Hh signaling is not essential for osteoblastic differentiation of KS483 cells.

Previously we examined the role of BMPs in KS483 osteoblastic differentiation. There are remarkable differences between the stimulatory effects of Hh and BMPs. First, BMPs are effective in all phases of differentiation, while Hh is only effective in initial stages of osteoblastic differentiation. In addition, autocrine Hh signaling does not play a role in osteoblastic differentiation of KS483 cells, whereas autocrine BMP signaling is crucial. Furthermore, BMPs potently stimulate both ALP activity and mineralization, while Hh only had modest effects on ALP activity and strongly induced mineralization to a comparable level as BMP. These data suggest that ALP activity and mineralization can be regulated independently from each other, and that Hh and BMPs have a different mode of action.

The mechanism by which Hh exerts these effects is largely unknown. Several lines of evidence exist for crosstalk between the Hh and the BMP pathway. Therefore, we investigated potential interactions between Hh and BMPs during KS483 osteoblastic differentiation. Hh signaling could be completely blocked by the BMP antagonists soluble truncated BMPR-IA and noggin. In contrast, the BMP-induced differentiation could only be partly inhibited by a high dose of cyclopamine. This concentration of cyclopamine is known to have side effects on cholesterol metabolism, suggesting that activation of Hedgehog plays only a minor role in BMP induced differentiation [17]. These data demonstrate that Hh induced osteoblastic differentiation requires the presence of functional BMP signaling. However, since we found no stimulation of mRNA expression of several BMPs, except for the differentiation marker BMP-8A, and the effects of BMP and Hh on induction of alkaline phosphatase and mineralization differ, Hh does not necessarily exert all its effect via BMPs.

In KS483 cells, Hh and BMP synergistically induced ALP activity, only when sub optimal concentrations of BMP were used. This synergy might be explained by a modulation exerted by BMPs on Hh signaling or vice versa. Members of the ci/Gli family are the major transcriptional effectors mediating Hh signaling [28]. Studies in Drosophila have shown that in absence of Hh, full-length, nuclear Ci, is processed into an N-terminal nuclear repressor form. In the presence of Hh, processing of ci is repressed. In vertebrates similar mechanisms occur, although the activator Gli1 has been shown to be primarily regulated at the transcription level, whereas the bipotential Glis, Gli2 and –3, are post-transcriptionally regulated. Full-length Gli2 and –3 are processed either into an N-terminal repressor region or into a C-terminal positive region, context dependently (reviewed in [27]). Indeed addition of rhSHh to KS483 cells induced Gli1 mRNA expression, but did not affect mRNA expression of Gli2 and –3. Recently, examples of crosstalk between Hh and BMP signaling have been reported.

SHh has been shown to promote chondrogenesis by altering the cellular response to BMP signaling [24]. Subsequent, truncated C-terminal Gli3 has been shown to associate with the BMP mediator, Smad1 [21]. Moreover, synergistic effects of SHh and BMPs in the mesenchymal cell line C3H10T1/2 are at least partly mediated by modulation of Smad1 by Hh. This modulation was independent of Gli1 [31]. However, in KS483 cells, synergism between Hh and BMP did not occur at the level of immediate early BMP response, since no effect of Hh was seen on the expression of the BMP reporter constructs Msx2-luc and BRE-luc. In addition, Hh did not increase the transcriptional activity of the BMP reporters induced by BMP-2 or –6. Subsequently, we investigated whether the synergism of Hh and BMPs could occur at the level of immediate early Hh signaling. Interestingly, both Hh and BMP could induce Gli reporter gene expression. In addition, the combination of hedgehog and BMP synergistically induced Gli reporter expression, suggesting that part of the BMP effect goes via the Hh pathway. These data are supported by *in vitro* data showing induction of IHh mRNA expression by treatment of KS483 cells for 18 days with either BMP-2 or BMP-6 (G. van der Horst et al, unpublished observations). The BMP induced ALP activity as well as the immediate early BMP response were partly inhibited by high doses of cyclopamine only, which are known to have Hh independent effects on cholesterol metabolism. This indicates that Hh only plays a marginal role in BMP induced differentiation. In conclusion, it is likely that Hh and BMP induce osteoblastic differentiation of KS483 cells via separate pathways and that crosstalk between Hh and BMPs can occur at the level of transcription regulation by Gli's e.g. by modulation of regulatory steps in Gli processing by Smads.

Treatment with rhSHh not only induced KS483 osteoblastic differentiation, but simultaneously inhibited adipogenesis. Furthermore, indomethacin induced adipogenesis could be reversed by rShh, suggesting that Hh is involved in directing differentiation of mesenchymal precursor cells towards osteoblasts on the expense of adipocyte differentiation. This is supported by recent data showing that SHh differentially regulates osteoblast and adipocyte commitment in the pluripotent cell line C3H10T1/2 31. Overall, these data imply that Hh can regulate mesenchymal cell fate decisions.

Our *in vitro* data clearly indicate a role for Hh in osteoblastic differentiation. However, until now, no data have been described showing IHh expression in bone *in vivo*. In this study, we have demonstrated for the first time, IHh mRNA expression in osteoblasts and lining cells in the humerus of the developing human skeleton. Based on our *in vitro* data and the mRNA expression of IHh in osteoblasts *in vivo*, we propose the following model for Hh action. IHh, produced by mature osteoblasts, recruits early progenitors towards the osteoblast lineage and additionally inhibits adipogenesis. Furthermore, Hh induces early osteoblasts to differentiate into mature osteoblasts, thereby providing a positive feedback loop. Interestingly, IHh induced not only the expression of the known target genes Ptc1 and Gli1, but also of IHh itself, providing additional evidence for the existence of a positive feedback loop.

Thus far, we only examined the expression of IHh in the developing skeleton. Whether IHh also plays a role in the remodeling skeleton is not clear yet. In addition, under conditions leading to bone loss, e.g. osteoporosis or osteopenia, more adipocytes are generated in the bone marrow at the expense of osteoblasts [2]. Whether Hh is also involved in this process is presently unclear and will be subject of future studies.

Acknowledgements

The authors thank Prof. Dr. S. E. Papapoulos, LUMC, The Netherlands, for critically reading the manuscript. This work was supported by a grant from the Netherlands Organization of Scientific Research (NWO).

gene	Sense	Antisense
β2M	5'-CCAGCAGAGAATGGAAAGTC-3'	5'-GATGCTGCTTACATGTCTCG-3'
OC	5'-GCAGCTTGGTGCACACCTAG-3'	5'-GGAGCTGCTGTGACATCCAT-3'
ALP	5'-CGGACATCATGAGGGTAAGG-3'	5'-GAGACATTTTCCCGTTCACC-3'
PTH1R	5'-TGCTTGCCACTAAGCTTCG-3'	5'-TCCTAATCTCTGCCTGCACC-3'
Osx	5'-CTTAACCCAGCTCCCTACCC-3'	5'-AGAGCGAGTGAACCTCTTGC-3'
Cbfa1(MASN)	5'-CTAAGCTTCCACCATGCTTCATTCGCCTCAC-3'	5'-CAGGAAGTTGGGACTGTCGG-3'
Cbfa1(MRIP)	5'-CCCTCGATTTCCTCCTCCT-3'	5'-CAGGAAGTTGGGACTGTCGG-3'
BMP-3	5'-CAAAGACCGCAAGAAGAAGG-3'	5'-ATCTTACCGACAGGCACAGG-3'
BMP-4	5'-CCCAGAGAATGAGGTGATCTCC-3'	5'-TGGCAGTAGAAGGCCTGGTAG-3'
BMP-8a	5'-GTTCGAAGTGGTCCAAGAGC-3'	5'-CCTCCTCTTCAGTGGTCTCG-3'
Msx-2	5'-ACCACATCCCAGCTTCTAGC-3'	5'-TCTGGTCCATCTGGTCTTC-3'
IHh	5'-TGGATATCACCACCTCAGAC-3'	5'-GATTGTCCGCAATGAAGAGC-3'
Ptc1	5'-GCATCGGAGTGGAGTTCACC-3'	5'-CTCACTGATGCCAGACACCG-3'
Smo	5'-AACTATCGGTACCGTGCTGG-3'	5'-CATCATGGGAGACAGTGTGC-3'
Gli1	5'-GGTCCAACCAACTATGG-3'	5'-GCTGGGGATGATGTGAAAAC-3'
Gli2	5'-GCGTAATGATGTGCATGTCC-3'	5'-TGTCAGGTCTCCCAGACTCC-3'
Gli3	5'-GCTCTTCAGCAAGTGGTTCC-3'	5'-GTGCTGCTCACTGCAGACTC-3'

Table 1 Primers: Oligonucleotides used in RT-PCR

References

1. Aza-Blanc,P. and Kornberg, T. B., Ci: a complex transducer of the hedgehog signal. Trends Genet 15: 458-462: 1999.

2. Benayahu,D., The Hematopoietic Microenvironment: The Osteogenic Compartment of Bone Marrow: Cell Biology and Clinical Application. Hematol 2000;4 (5):427 -435 4: 427-435:

3. Bezooijen van,R.L., Farih-Sips, H., Papapoulos, S. E., and Lowik, C. W. G. M., IL-1a. IL-1b, IL-6 and TNF-a steady state mRNA levels analyzed by reverse transcriptase competitive PCR in bone marrow of gonadectomized mice. J Bone Min Res 13: 185-194: 1998.

4. Chiang,C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A., Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383: 407-413: 1996.

5. Chomczynski,P. and Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. Anal Biochem 162: 156-159: 1987.

6. Chung,U., Schipani, E., McMahon, A. P., and Kronenberg, H. M., Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. Journal of Clinical Investigation 107: 295-304: 2001.

7. Chung,U.I., Schipani, E., McMahon, A. P., and Kronenberg, H. M., Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. J Clin Invest 2001 Feb ;107 (3):295 -304 107: 295-304:

8. Cooper,M.K., Porter, J. A., Young, K. E., and Beachy, P. A., Teratogen-mediated inhibition of target tissue response to Shh signaling. science 280: 1603-1607: 1998.
9. Dang,Z.C., Van Bezooijen, R. L., Karperien, M., Papapoulos, S. E., and Lowik, C. W., Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. J Bone Miner Res 2002 Mar ;17 (3):394 -405 17: 394-405:
10. Deckers,M.M.L., Karperien, M., Bent van der, C., Yamashita, T., Papapoulos, S. E., and Lowik, C. W. G. M., Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology 141: 1667-1674: 2000.
11. Eerden van der,B.C.J., Karperien, M., Gevers, E. F., Lowik, C. W. G. M., and Wit, J. M., Expression of indian hedgehog, parathyroid hormone related protein and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. J Bone Min Res 15: 1045-1055: 2000.
12. Fietz,M.J., Concordet, J. P., Barbosa, R., Johnson, R., Krauss, S., McMahon, A. P., Tabin, C., and Ingham, P. W., The hedgehog gene family in Drosophila and vertebrate development. Dev Suppl :43-51.: 43-51: 1994.
13. Fietz,M.J., Concordet, J. P., Barbosa, R., Johnson, R., Krauss, S., McMahon, A. P., Tabin, C., and Ingham, P. W., The hedgehog gene family in Drosophila and vertebrate development. Dev Suppl :43-51.: 43-51: 1994.
14. Grimsrud,C.D., Romano, P. R., D'Souza, M., Puzas, J. E., Schwarz, E. M., Reynolds, P. R., Roiser, R. N., and O'Keefe, R. J., BMP signaling stimulates chondrocyte maturation and the expression of Indian hedgehog. J Orthop Res 2001 Jan ;19 (1):18 -25 19: 18-25:
15. Hammerschmidt,M., Brook, A., and McMahon, A. P., The world according to hedgehog. Trends Genet 13: 14-21: 1997.
16. Incardona,J.P., Gaffield, W., Kapur, R. P., and Roelink, H., The teratogenic Veratrum alkaloid cyclopamine inhibits Sonic hedgehog signal transduction. Development 125: 3553-3562: 1998.
17. Incardona,J.P., Gaffield, W., Lange, Y., Cooney, A., Pentchev, P. G., Liu, S., Watson, J. A., Kapur, R. P., and Roelink, H., Cyclopamine inhibition of Sonic hedgehog signal transduction is not mediated through effects on cholesterol transport. Dev Biol 2000 Aug 15 ;224 (2):440 -52 224: 440-452:
18. Iwamoto,M., Enomoto-Iwamoto, M., and Kurisu, K., Actions of hedgehog proteins on skeletal cells. Crit Rev Oral Biol Med 10: 477-486: 1999.
19. Katsuura,M., Hosono-Sakuma, Y., Wagatsuma, M., Yanagisawa, S., Okazaki, M., and Kimura, M., The NH2-terminal region of the active domain of sonic hedgehog is necessary for its signal transduction. FEBS Lett 447: 325-328: 1999.
20. Kinto,N., Iwamoto, M., Enomoto-Iwamoto, M., Noji, S., Ohuchi, H., Yoshioka, H., Kataoka, H., Wada, Y., Yuhao, G., Takahashi, H. E., Yoshiki, S., and Yamaguchi, A., Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. FEBS Lett 404: 319-323: 1997.
21. Liu,F., Massague, J., and Ruiz., Carboxy-terminally truncated Gli3 proteins associate with Smads. Nat Genet 20: 325-326: 1998.
22. Minina,E., Kreschel, C., Naski, M. C., Ornitz, D. M., and Vortkamp, A., Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. Dev Cell 2002 Sep ;3(3):439 -49 3: 439-449:
23. Minina,E., Wenzel, H. M., Kreschel, C., Karp, S., Gaffield, W., McMahon, A. P., and Vortkamp, A., BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development 128: 4523-4534: 2001.
24. Murtaugh,L.C., Chyung, J. H., and Lassar, A. B., Sonic Hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. Genes & Development 13: 225-237: 1998.
25. Murtaugh,L.C., Chyung, J. H., and Lassar, A. B., Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. Genes Dev 13: 225-237: 1999.
26. Nakamura,T., Aikawa, T., Iwamoto-Enomoto, M., Iwamoto, M., Higuchi, Y., Pacifici, M., Kinto, N., Yamaguchi, A., Noji, S., Kurisu, K., Matsuya, T., and Maurizio, P., Induction of osteogenic differentiation

by hedgehog proteins. Biochem Biophys Res Commun 237: 465-469: 1997.
27. Ruiz., Gli proteins and Hedgehog signaling: development and cancer. Trends Genet 15: 418-425: 1999.
28. Ruiz i Altaba,A., Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development 126: 3205-3216: 1999.
29. Sasaki,H., Hui, C., Nakafuku, M., and Kondoh, H., A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development 124: 1313-1322: 1997.
30. Sasaki,H., Nishizaki, Y., Hui, C., Nakafuku, M., and Kondoh, H., Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126: 3915-3924: 1999.
31. Spinella-Jaegle,S., Rawadi, G., Kawai, S., Gallea, S., Faucheu, C., Mollat, P., Courtois, B., Bergaud, B., Ramez, V., Blanchet, A. M., Adelmant, G., Baron, R., and Roman-Roman, S., Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. J Cell Sci 114: 2085-2094: 2001.
32. St-Jacques,B., Hammerschmidt, M., and McMahon, A. P., Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes & Development 13: 2072-2086: 1999.
33. Stein,G.S. and lian, J. B., Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14: 424-442: 1993.
34. Taipale,J., Chen, J. K., Cooper, M. K., Wang, B., Mann, R. K., Milenkovic, L., Scott, M. P., and Beachy, P. A., Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 2000 Aug 31 ;406 (6799):1005 -9 406: 1005-1009:
35. Van der Eerden,B.C., van Til, N. P., Brinkmann, A. O., Lowik, C. W., Wit, J. M., and Karperien, M., Gender differences in expression of androgen receptor in tibial growth plate and metaphyseal bone of the rat. Bone 2002 Jun ;30 (6):891 -6 30: 891-896:
36. van der Horst,G., Van Bezooijen, R. L., Deckers, M. M., Hoogendam, J., Visser, A., Lwik, C. W., and Karperien, M., Differentiation of murine preosteoblastic KS483 cells depends on autocrine bone morphogenetic protein signaling during all phases of osteoblast formation. Bone 2002 Dec ;31 (6):661 -9 31: 661-669:
37. Yamashita,T., Ishii, H., Shimoda, K., Sampath, T. K., Katagiri, T., Wada, M., Osawa, T., and Suda, T., Subcloning of three osteoblastic cell lines with distinct differentiation phenotypes from the mouse osteoblastic cell line KS-4. Bone 19: 429-436: 1996.
38. Yuasa,T., Kataoka, H., Kinto, N., Iwamoto, M., Enomoto-Iwamoto, M., Iemura, S., Ueno, N., Shibata, Y., Kurosawa, H., and Yamaguchi, A., Sonic hedgehog is involved in osteoblast differentiation by cooperating with BMP-2. J Cell Physiol 2002 Nov ;193 (2):225 -32 193: 225-232:
39. Zehenter,B.K., Leser, U., and Burtscher, H., BMP-2 and sonic hedgehog have contrary effects on adipocyte-like differentiation of C3H10T1/2 cells. DNA and Cell biology 19: 275-281: 2000.