Regulation of osteoblast differentiation

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2 Differentiation of murine pre-osteoblastic KS483 cells depends on autocrine BMP signaling during all phases of osteoblast formation

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Bone 2002 31(6): 661-669

Abstract

In this study, we have examined the role of BMP signaling during differentiation of the murine pre-osteoblastic KS483 cell line, which forms ALP positive and mineralized nodules during a three weeks culture period. Semi-quantitative RT-PCR demonstrated the presence of various BMPs (BMP-2, -3, -4, -6, -7, -8a and –8b), BMP type I and II receptors (ALK2, ALK3, ALK4, BMPR-II, ActR-IIA and –IIB), BMP antagonists (DAN, gremlin, chordin, cerberus, noggin and tsg) and Smad 1-8. The mRNA expression of these genes did not change during differentiation, except for BMP-3, BMP-8a and noggin. BMP-3 gradually increased particularly in the matrix formation phase, BMP-8a was induced from the onset of matrix maturation and mineralization, in parallel to the expression of osteocalcin, and noggin tended to decline during the mineralization phase. Treatment of KS483 cells with the BMP antagonists noggin or soluble truncated BMPR-IA either continuously or during distinct periods of osteoblast differentiation, i.e. matrix formation or matrix maturation and mineralization phase decreased ALP positive and mineralized nodule area independent of the phase of osteoblast differentiation. Notably, the antagonists inhibited mineralization of already existing nodules. Similarly, BMP-4 not only stimulated differentiation at the beginning of the culture period, but also stimulated at late stages of differentiation. These data indicate that autocrine BMP signaling is not only involved in KS483 osteoblastic differentiation during the early phase of differentiation, but also during matrix maturation and mineralization. The different expression patterns of components of BMP signaling in the KS483 cells suggest distinct functions of individual BMPs during osteoblast differentiation. In summary, our data suggest that BMP activity is not only required for the initiation of osteoblast differentiation and the further development of early osteoblasts, but is involved in late-stage osteoblast differentiation and matrix mineralization as well.

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) super-family and regulate differentiation of various cells implicated in cartilage and bone formation during skeletal development and fracture repair [1,2]. The role of BMPs in induction of osteoblast differentiation has been well established using various pre-osteoblastic cell lines such as MC3T3, C2C12, C3H10T1/2, ROB-C26, as well as primary cultures of calvarial osteoblasts and human and mouse bone marrow cultures. Using these models, induction of osteoblast differentiation has been described for BMP-2, -4, -5, -6 and –7 [3-6].

Several secreted proteins have been discovered that can antagonize BMP actions, such as noggin, gremlin, cerberus, DAN and chordin, by preventing binding of BMPs to their membrane-bound receptors [7-9].

BMPs exert their effect by binding to an heterodimeric complex consisting of two BMP type I receptors and two BMP type II receptors, which possesses serine/threonine kinase activity. Three mammalian BMP type I receptors have been described: BMPR-IA (Activin receptor Like Kinase 3), BMPR-IB (ALK6) and ALK2 (ActR-I). BMP receptor type II and activin receptor type IIa and -b (ActR-IIa and -b) are type II receptors involved in BMP signaling. Type II receptors are constitutive active, while ligand binding is required for type I receptor kinase activation. Optimal binding is achieved when both receptors are present, although BMPs can bind to each receptor separately and subsequently recruit the other [10]. Triggered by ligand binding, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates intracellular signaling intermediates of the Smad family. The BMP signaling pathway particularly uses Smad 1, 5 and 8, whereas TGF-β leads to phosphorylation of Smad 2 and 3. These receptor-regulated Smads associate with the common mediator Smad 4 and the complex translocates into the nucleus, where they activate BMP target genes such as core binding factor α1 (Cbfα1/RUNX2) by acting as transcription factors (reviewed in [11]).

Overexpression of a truncated BMP type IB receptor (trBMPR-IB) inhibits BMP-2-induced osteoblast differentiation in the osteoblast precursor cell lines 2T3 and C2C12 concomitant with an inhibition of Cbfα1, alkaline phosphatase (ALP) and osteocalcin (OC) mRNA expression [12,13]. Furthermore, pre-osteoblastic MC3T3-E1 cells transfected with trBMPR-IA did not respond to exogenously added BMP-2 and could not differentiate into nodule forming osteoblasts [14]. In contrast, recombinant expression of constitutive active BMPR-IA was shown to be sufficient for osteo-/chondrogenic development of the mesenchymal precursor cell line C3H10T1/2 and a constitutive active form of BMPR-IB induced bone matrix formation in absence of ligand [12,15,16]. Taken together, these observations indicate that BMPs play important roles in initiation and regulation of early osteoblast differentiation.

However, BMPs may also be important during late stages of osteoblast differentiation. In non-skeletal cell types, both pro- and anti-apoptotic effects of BMPs have been reported [17,18]. Recently, a pro-apoptotic role for BMP-2 in osteoblast apoptosis has been described [19]. In contrast, BMP-2 and -4 inhibit TNF-mediated apopotosis in the pluripotent mesenchymal cell line C2C12 [20,] suggesting a role for BMPs in regulation of the life-span of osteoblasts. Furthermore, Wada et al showed BMP-2 and -4 as well as BMP receptor type IA, -IB and II mRNA expression during late phases of differentiation, localized especially in calvarial osteoblasts forming nodules [21]. These data indicate that,

in addition to the well-established effects of BMPs on early osteoblast proliferation and differentiation, BMP activity may also affect mature osteoblasts.
The murine pre-osteoblastic cell line KS483 is a well-established model to study osteoblast differentiation [22,23]. This cell line possesses mesenchymal characteristics, since KS483 cells can differentiate into either adipocytes or mature mineralizing osteoblasts depending on the culture conditions [24]. Differentiation of KS483 cells into osteoblasts occurs in various phases, e.g. proliferation, matrix formation, matrix maturation and mineralization, according to the model proposed by Stein and Lian [25]. In this study, we examined the role of the BMP signaling pathway during the various phases of osteoblast differentiation. Our results demonstrate that osteoblastic differentiation of KS483 cells strictly depends on autocrine BMP signaling. In addition, we demonstrate that BMP activity is not only required for early phases of osteoblast differentiation, but is involved in late stage osteoblast differentiation and matrix mineralization as well.

Material and Methods

Differentiation studies

KS483 cells were cultured routinely in α-MEM (GIBCO BRL, Breda, The Netherlands) supplemented with penicillin/streptomycin (Life Technologies, Inc.) and 10 % FCS (Integro B.V., Zaandam, The Netherlands). For differentiation assays, KS483 cells were seeded at a density of 15.000 cells/cm^2. Every 3 to 4 days, the medium was changed. At confluence (from day 4 of culture onwards), ascorbic acid (50 μg/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. Differentiation of KS483 cells can be divided in various phases according to the model of Stein and Lian [25]. From day 0-4 of culture, KS483 cells proliferate, from day 4-11 the cells differentiate and extracellular matrix is formed. From day 11 onwards, the matrix matures and mineralized nodules are formed. At the end of culture (day 18), cells were washed with phosphate buffered saline (PBS) and fixed with 3.7% buffered formaldehyde. Alkaline phosphatase (ALP) activity was determined by staining the cells for 5 minutes with Napthol AS-MX phosphate/Fast blue solution (Sigma). The ALP positive nodule area was determined by measuring the number of pixels per area after binarization of the images using ImageProPlus 3.0 software (Media Cybernetics Maryland, USA). Thereafter, 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). Subsequently, the area of mineralized nodules was measured using ImageProPlus (# of pixels). Alizarin was removed from the cell layer using 10 % cetylpyridinium Cl in 10 mM PO_4 buffer (pH 7.5), and measured against an alizarin standard (0.005-3 μg/ml) at 550 nm as described by Schiller et al [26].

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 ng/ml), BMP-6 (100 ng/ml) or the BMP-antagonists recombinant human BMP receptor IA/Fc chimera (trBMPR-IA), recombinant human BMP receptor IB/Fc chimera (trBMPR-IB)

and recombinant noggin, all purchased at R&D systems (Uithoorn, The Netherlands). At day 7, the supernatants were withdrawn and the cells were washed twice with PBS. The cells were lysed in ALP lysisbuffer (10 mM glycine, 0.1 mM $MgCl_2$, 10 mM $ZnCl_2$, 0.1% Triton X-100) and 10 µl was used to determine ALP activity using 6 mM *p*-nitrophenylphosphate as a substrate (Pierce Chemical Co., Rockford, IL). 400 ml SSC buffer supplemented with proteinase K (100 µg/ml SSC, Gibco) was added and samples were incubated at 56°C to release the DNA from the cells. DNA content was measured using Hoechst 33258 and calibrated against a DNA standard (0.5-10 µg/ml herring sperm DNA). ALP activity was corrected for the amount of DNA in the culture.

gene	sense	antisense
β2M	5'-CCAGCAGAGAATGGAAAGTC-3'	5'-GATGCTGCTTACATGTCTCG-3'
OC	5'-GCAGCTTGGTGCACACCTAG-3'	5'-GGAGCTGCTGTGACATCCAT-3'
ALP	5'-CGGACATCATGAGGGTAAGG-3'	5'-GAGACATTTTCCCGTTCACC-3'
BMP-2	5'-GTTCCCTACAGGGAGAACACC-3'	5'-GCCTGCGGTACAGATCTAGC-3'
BMP-3	5'-CAAAGACCGCAAGAAGAAGG-3'	5'-ATCTTACCGACAGGCACAGG-3'
BMP-4	5'-CCCAGAGAATGAGGTGATCTCC-3'	5'-TGGCAGTAGAAGGCCTGGTAG-3'
BMP-6	5'-CTTGAACCGCAAGAGTCTCC-3'	5'-CAGCCAACCTTCTTCTGAGG-3'
BMP-7	5'-TCCAGGGAAAGCATAATTCG-3'	5'-AGGTCTCGTTGTCAAATCGC-3'
BMP-8a	5'-GTTCGAAGTGGTCCAAGAGC-3'	5'-CCTCCTCTTCAGTGGTCTCG-3'
BMP-8b	5'-TCTCTGTTCTTTTTGCCAAGG-3'	5'-CTTGAACTGCCTCAGCTTCC-3'
Smad1	5'-CATGGAAGAGCTGGAGAACG-3'	5'-ACCAAATGGGAACTCACAGC-3'
Smad2	5'-CCTCCAGTCTTAGTGCCTCG-3'	5'-AACACCACAATGCAGCTTCC-3'
Smad3	5'-CACAGCGACCATGAATTACG-3'	5'-TCACTCAGGTAGCCAGGAGG-3'
Smad4	5'-GTTGCAGATAGCTTCAGGGC-3'	5'-CATCCACAGTCACAACAGGG-3'
Smad5	5'-TGACGAGGAAGAGAAATGGG-3'	5'-GGAGGATAGGGGCTGTTAGG-3'
Smad6	5'-TGAACTGTCGCGAGTAGCAGG-3'	5'-TTGGCATACTGCTCAGCAAG-3'
Smad7	5'-AGGCTCTACTGTGTCCAAGA-3'	5'-CCTCCTCCTTACTCCAGATA-3'
Smad8	5'-GAGCTGAGGGTTGTACTCGC-3'	5'-CAGCAAGTGTGTCACCATCC-3'
DAN	5'-GAACATCACGCAGATTGTGG-3'	5'-CTCTACCAGCTTGTCCACCC-3'
Gremlin	5'-GCTGAGGGGAAAAAGAAAGG-3'	5'-TGGTGGTGAACTTCTTGGC-3'
Chordin	5'-ATCCTGTTTGAACACCCTGC-3'	5'-AAGGGAGCCTGAGCTACTCC-3'
Cerberus	5'-AAGCCACGAAGTACACTGGG-3'	5'-AGTCCAGGGATGAAGGAACC-3'
Tsg	5'-ACCTGGGTCAGGAAGTAGCC-3'	5'-CTGTCCACATCTGTGCATCC-3'
Noggin	5'-TGAGCAAGAAGCTGAGGAGG-3'	5'-GGATCGATCAAGTGTCTGGG-3'
ALK2	5'-AGATGACGTGTAAGACCCCG-3'	5'-ATACTTCTCCATAGCGGCCC-3'
BMPR-IA	5'-TCGTCGTTGTATTACAGGAG-3'	5'-TTACATCCTGGGATTCAACC-3'
BMPR-IB	5'-GCTTTGGACTCATCCTCTGG-3'	5'-CACTGGGCAGTAGGCTAACG-3'
BMPR-II	5'-GGTAGATAGGAGGGAACGGC-3'	5'-CACTGCCATTGTTGTTGACC-3'
ActR-II	5'-GTGCAGAGAAAAGAGGCACC-3'	5'-TATCCTCAGAAATGCGTCCC-3'
ActR-IIB	5'-GAACATCATCACGTGGAACG-3'	5'-ATGTACTCATCGACAGGCCC-3'

Table 1 Primers: Oligonucleotides used in RT-PCR

RT-PCR

RNA isolation was performed by the method described by Chomzynski and Sachi [27] and reverse transcribed into cDNA. cDNA was standardized by competitive PCR using an internal standard (pMUS) as described previously [28]. Subsequently, expression of various genes was examined by semi-quantitative PCR (for primersets, see table 1). cDNA was denatured at 94°C for 3 min followed by repeated cycles of 30 s at 94°C; 45 s at 56°C and 30 s at 72°C. At least three independent experiments were performed for each gene.

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 BMPs, BMP antagonists, BMP receptors and Smads during differentiation of KS483 cells.

To examine the expression of various components of the BMP signaling pathway, semi-quantitative RT-PCR was performed using RNA isolated at representative stages of osteoblast differentiation. cDNA was first standardized using competition PCR as described previously [28]. As shown in figure 1A, expression of alkaline phosphatase mRNA increased from day 4 to day 18 and decreased thereafter. At least 6 members of the BMP family were expressed during osteoblast differentiation. BMP-4, -6, -7 and –8b transcripts were expressed at comparable levels at all stages of differentiation. Expression of BMP-2 was very low and did not change during differentiation (data not shown). In contrast, no BMP-5 mRNA was detected (data not shown). Furthermore, expression of BMP-3 mRNA gradually increased particularly in the matrix formation phase (days 4 and 7). Interestingly, BMP-8a expression was induced during late differentiation of the KS483 cells. Expression of BMP-8a started at the onset of mineralization in parallel to the expression of the differentiation marker osteocalcin (fig 1A) [22].

KS483 cells also expressed various BMP-antagonists (fig 1B). The expression of DAN, gremlin, chordin, cerberus and twisted gastrulation did not change with differentiation. In contrast, the levels of noggin mRNA tended to decline during the mineralization phase (day 18 and 21).

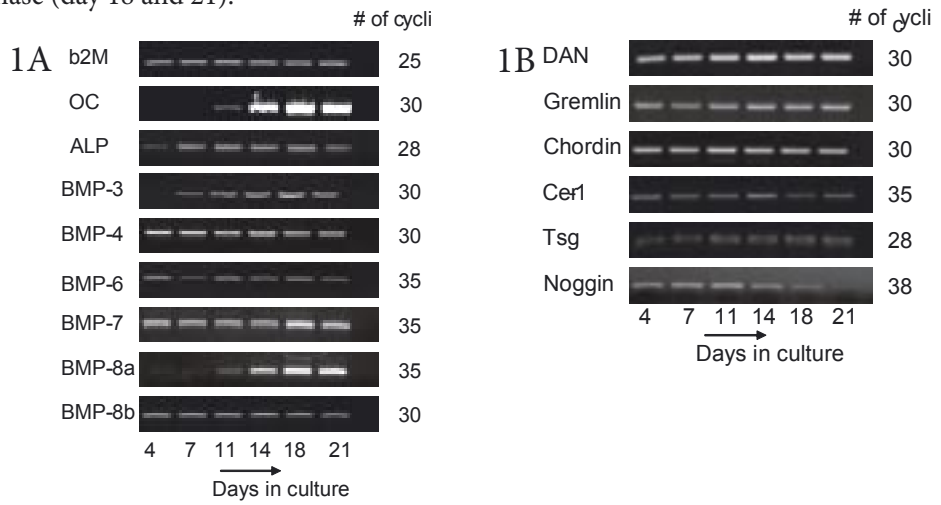


Figure 1 Expression of BMP and BMP antagonists during KS483 differentiation

Expression of various BMPs (A) and BMP antagonists (B) during differentiation of osteoblastic KS483 cells. At day 4, 7, 11, 14, 18 and 21 of culture, total cellular RNA was isolated and cDNA was prepared and standardized for housekeeping gene expression (β2 microglobulin) by competition PCR. These data are confirmed by semi-quantitative PCR for β2M (1A). 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

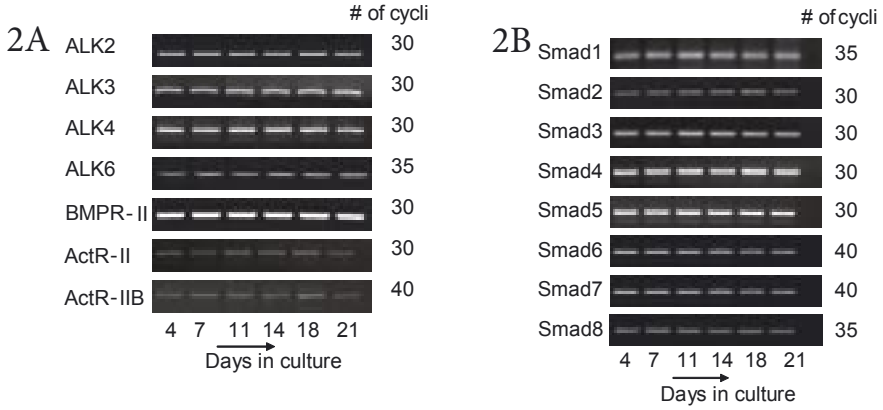


Figure 2 Expression of BMP receptors and Smads during KS483 differentiation
Expression of BMP receptors (A) and intracellular signaling molecules (B). Semi-quantitative PCR was performed for the indicated genes as described in the legend of figure 1.
As a negative control H_2O was used. No bands were 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

As shown in figure 2A, mRNA of the BMP type I receptors, ALK2, BMPR-IA and BMPR-IB and the BMP type II receptors, BMPR-II and ActR-IIa and -b), was detected continuously during differentiation of KS483 cells. Likewise, mRNAs of the intracellular signaling proteins of the BMP pathway, Smad 1, 5 and 8, and of the TGF-β signaling pathway, Smad 2 and 3, the common mediator Smad 4, as well as the inhibitory Smads 6 and 7 were expressed continuously during osteoblastic differentiation (fig.2B).

Noggin and trBMPR-IA inhibit BMP-induced osteoblast differentiation.

BMPs are potent inducers of ALP activity in various osteoblastic cells [3-5]. BMP-4 and -6 treatment of KS483 cells for 3 days strongly induced ALP activity dose-dependently (dose-range: 10-300 ng/ml for both BMPs; data not shown). Doses of 50 and 100 ng/ml for BMP-4 and –6 respectively, were used for further experiments.
ALP measurements were corrected for DNA content, since BMP-4 and BMP-6 treatment of KS483 cells for 3 days had a slight effect on proliferation as shown by DNA measurements (control: 7.14 ± 0.4 μg DNA/well; BMP-4: 9.09 ± 0.38 μg DNA/well; BMP-6: 9.97 ± 0.29 μg DNA/well).
Induction of ALP activity by BMP-4 and -6 was blocked by addition of trBMPR-IA dose-dependently (fig.3A). Another BMP antagonist, trBMPR-IB, also blocked BMP-4 and -6 induced alkaline phosphatase activity but less efficiently (data not shown). The antagonist noggin blocked BMP-4 induced ALP activity, while BMP-6 activity was hardly affected (fig. 3B).
Interestingly, addition of trBMPR-IA or noggin also inhibited ALP activity of untreated KS483 cells dose-dependently (fig.3A and B, respectively). This suggested that autocrine BMP signaling was involved in induction of ALP activity during osteoblastic differentiation of KS483 cells.

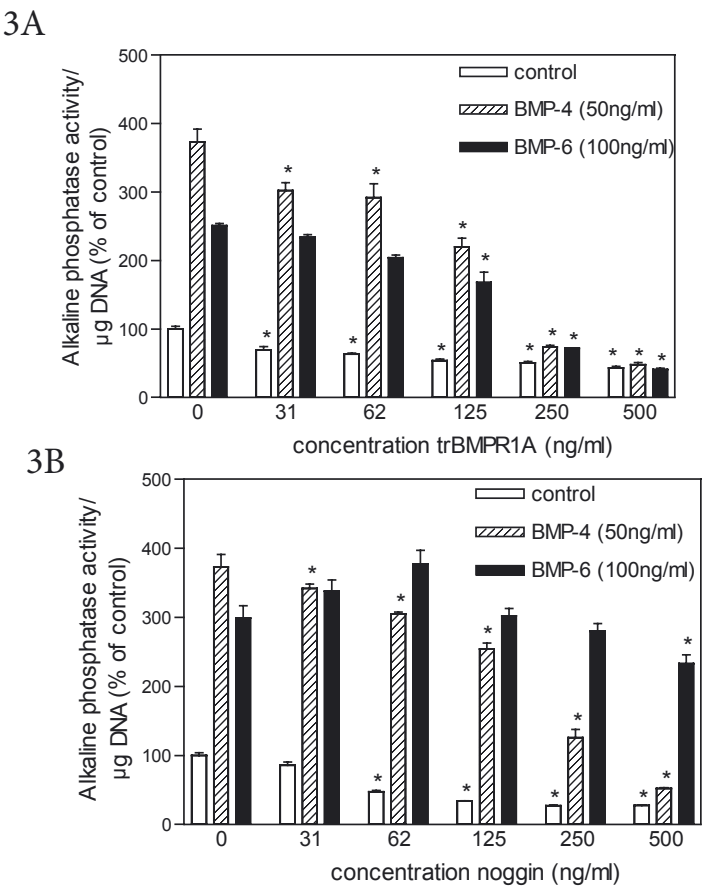
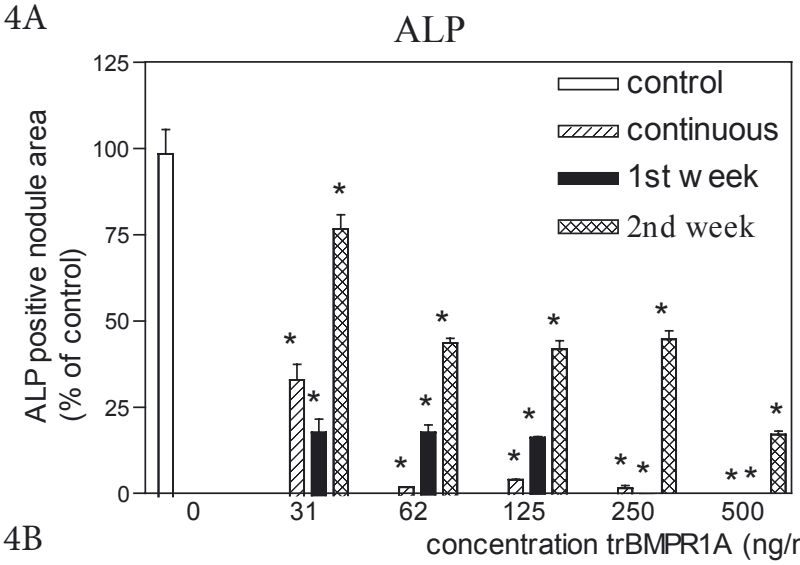


Figure 3 Inhibition of BMP-4 and -6 induced osteoblast differentiation by noggin or trBMPR-IA.
Inhibition of BMP-4 and -6 induced osteoblast differentiation by the BMP antagonists noggin and trBMPR-IA. KS483 cells were cultured until confluence and thereafter cultured for 72 hrs in the absence or presence of either 50 ng/ml BMP-4 or 100 ng/ml BMP-6 in combination with a concentration range of BMP antagonist trBMPR-IA (A) or noggin (B). Cells were lysed and alkaline phosphatase activity was measured as described in material and methods. Values represent the average ± SEM of 2 independent triplicate experiments corrected for DNA content and are expressed as percentage of control. * Significant vs. control ($p<0.05$).

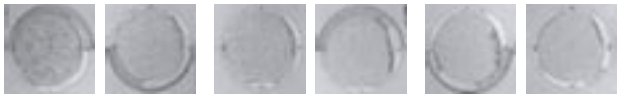
Effects of the BMP antagonists noggin and trBMPR-IA on KS483 differentiation.

To investigate the role of autocrine BMP signaling during the whole osteoblastic differentiation process, KS483 cells were cultured for 18 days in the presence or absence of noggin or trBMPR-IA. The level of differentiation was scored by measuring the ALP positive area, as well as by the mineralized nodule area of the cultures at day 18. As shown in figure 4, continuous treatment (day 4-18) with trBMPR-IA (fig. 4A) or noggin (fig. 4E) blocked formation of ALP positive nodules. Furthermore, trBMPR-IA and noggin concomitantly blocked formation of mineralized nodules (fig.4C and G, respectively).

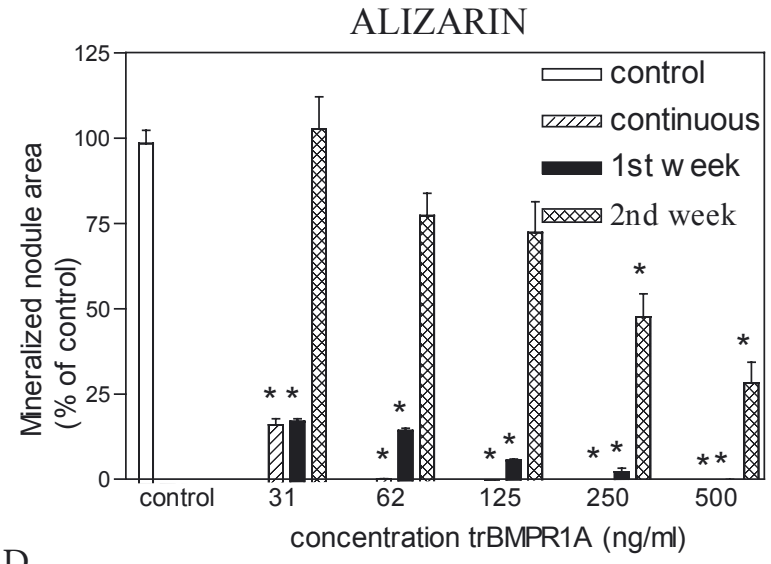
4A


ALP
ALP positive nodule area (% of control)
125
100
75
50
25
0
control
continuous
1st w eek
2nd week
0
31
62
125
250
500
concentration trBMPR1A (ng/ml)

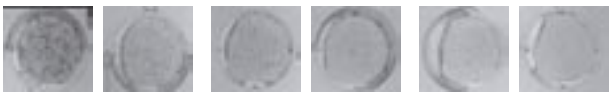
4B


1st week trBMPR1A (ng/ml)
control
31
62
125
250
500

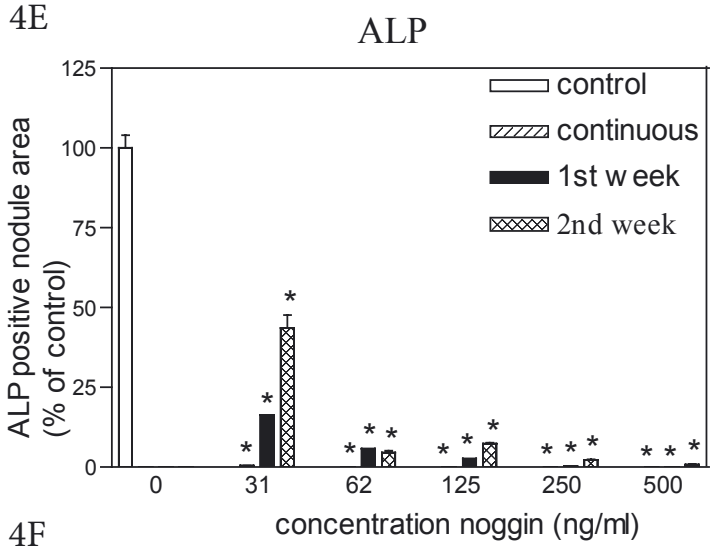
4C


ALIZARIN
Mineralized nodule area (% of control)
125
100
75
50
25
0
control
continuous
1st w eek
2nd week
control
31
62
125
250
500
concentration trBMPR1A (ng/ml)

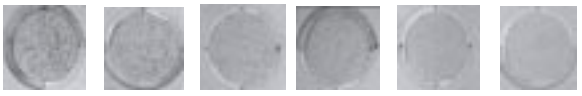
4D


1st week trBMPR1A (ng/ml)
control
31
62
125
250
500

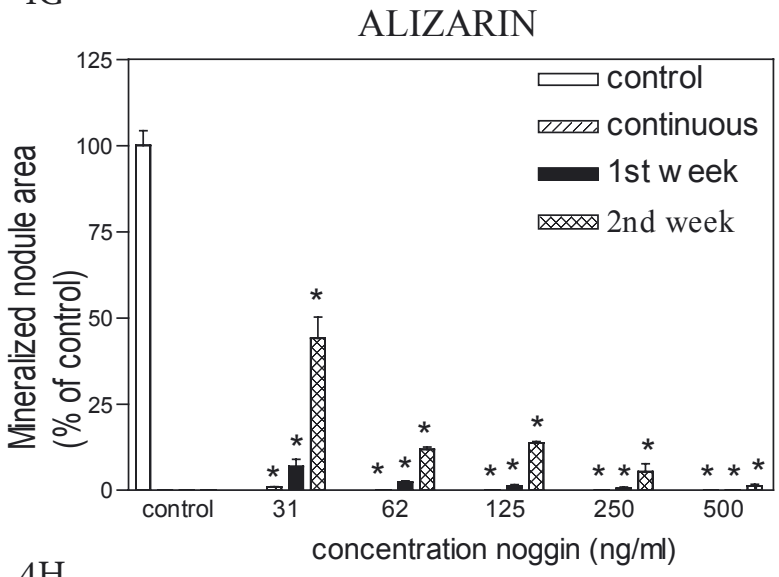
4E


ALP
ALP positive nodule area (% of control)
125
100
75
50
25
0
control
continuous
1st w eek
2nd week
0
31
62
125
250
500
concentration noggin (ng/ml)

4F


2nd week noggin (ng/ml)
control
31
62
125
250
500

4G


ALIZARIN
Mineralized nodule area (% of control)
125
100
75
50
25
0
control
continuous
1st w eek
2nd week
control
31
62
125
250
500
concentration noggin (ng/ml)

4H

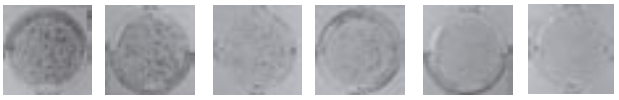
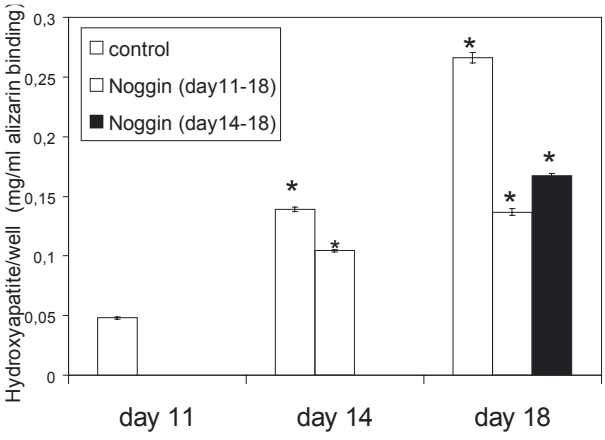

2nd week noggin (ng/ml)
control
31
62
125
250
500

Figure 4 Effects of noggin and trBMPR-IA on differentiation of KS483 cells.
Effects of the BMP antagonists noggin and trBMPR-IA on differentiation of KS483 cells. KS483 cells were treated with the indicated concentrations of BMP antagonist continuously (day 4-18; open bars), during the first week of culture (day 4-11; dashed bars) or during the last week of culture (day 11-18; black bars). Cultures were fixed at day 18 with 3.7% PBS-buffered formaldehyde and stained for alkaline phosphatase (ALP). The ALP positive area was measured using Image ProPlus software. ALP staining was washed out and cultures were stained with alizarin, after which the mineralized nodule area was determined. (A) ALP positive area of cultures treated with the indicated concentrations of trBMPR-IA. (B) ALP stained cultures treated with the indicated concentrations of trBMPR-IA during the first week of culture. (C) Mineralized nodule area of cultures treated with the indicated concentrations of trBMPR-IA. (D) Alizarin stained cultures treated with trBMPR-IA during the first week of culture. (E) ALP positive area of cultures treated with the indicated concentrations of noggin. (F) ALP stained cultures treated with the indicated concentrations of noggin during the last week of culture. (G) Mineralized nodule area of cultures treated with the indicated concentrations of noggin (H). Alizarin stained cultures treated with noggin during the last week of culture. Data are expressed as percentage of the control and are representative for two independent triplicate experiments (mean ± SEM) * significant vs. control ($p<0.05$). Representative images of the cultures are shown in 4B,D, F and H.

To investigate further the effect of BMPs at different phases of differentiation, noggin and trBMPR-IA were added early during differentiation (from day 4-11 of culture) or during the matrix maturation and mineralization phase (day 11-18 of culture). TrBMPR-IA (fig. 4A-D) and noggin (fig. 4E-H) inhibited the formation of ALP positive and mineralized nodules dose-dependently during the early phase as well as during the late phase of differentiation, although the effects during the last week of culture were less pronounced. Similar findings were found using trBMPR-IB (data not shown).
Nodules appeared at day 11 in non-treated cells and developed further into mature mineralized nodules at day 18. Treatment of the cells with noggin from day 11 onwards, resulted in less mature nodules that were smaller in size and contained less mineral (fig.5B-C). The effects of noggin on mineralized nodule area were confirmed by extraction of alizarin staining from the cell layer as a measure of hydroxyapatite incorporation. This quantitative determination of alizarin staining also showed a dose-dependent decrease in mineral content (fig. 5A). To exclude whether the effects of the BMP antagonists were caused by an effect on proliferation, the DNA content of each well was measured.
Noggin only decreased DNA content slightly at the highest concentration (500 ng/ml, 1.2 fold reduction) and no significant differences were seen with trBMPR-IA (data not shown).

5A



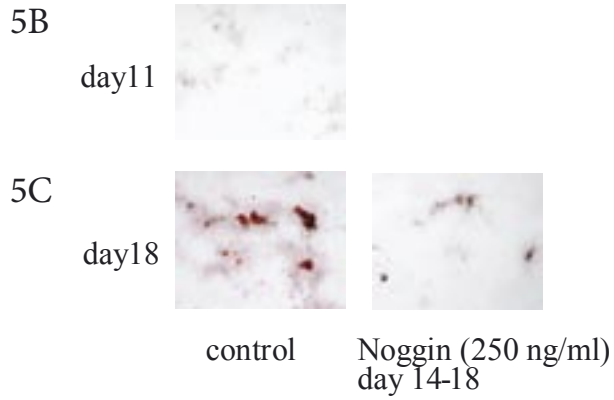


Figure 5 Noggin and trBMPR-IA inhibit nodule mineralization.
(A) KS483 cells were treated for the indicated time periods with noggin (250 ng/ml) and fixed with 3.7 % PBS-buffered formaldehyde at day 11,14 and 18 of culture. Cultures were stained for hydroxyapatite, and the amount of alizarin was measured. Data are representative for two independent triplicate experiments (mean ± SEM) * significant vs. control ($p<0.05$). (B) Representative image of an alizarin stained culture at day 11. (C) Representative images of alizarin stained cultures at day 18.

Effects of exogenous BMP on differentiation of KS483 cells.

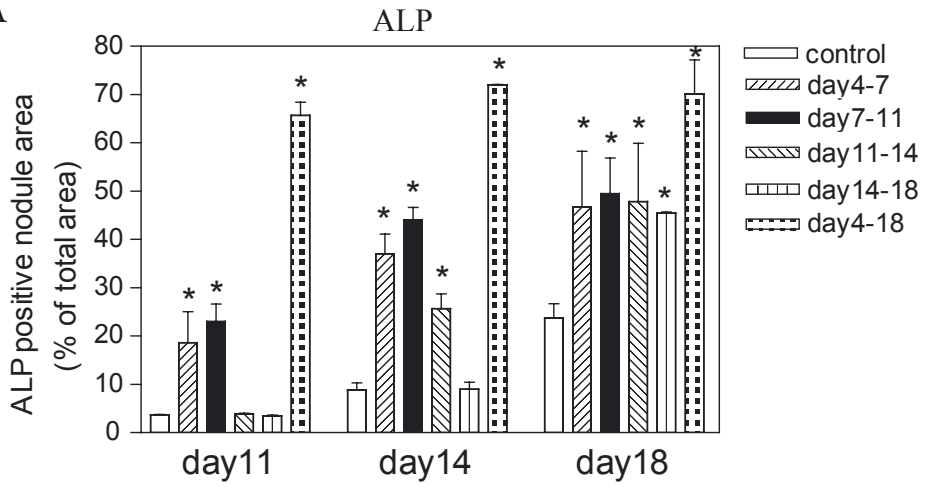
The specific induction of BMP-8a as well as the effects of the BMP antagonists during late stages of differentiation suggested that BMP activity was required for the early phases of osteoblast differentiation as well as for matrix maturation and mineralization. To test this hypothesis, the effects of exogenously added BMP-4 during different phases of KS483 osteoblastic differentiation were investigated. KS483 cells were cultured in presence of BMP-4 (50 ng/ml) during different periods of differentiation. Cultures were stopped at representative stages of differentiation (day 11, 14, and 18 of culture), and the ALP positive and mineralized nodule area were measured.

As shown in figure 6A and B, continuous treatment with BMP-4 (day 4-18) significantly increased the ALP positive nodule area, which reached the maximal level already at day 11 of culture. Moreover, the mineralized nodule area was significantly increased at day 14 and 18 of culture in continuously treated KS483 cells compared to control (fig.6C and D).

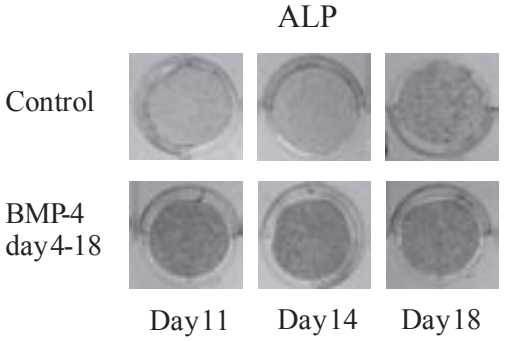
Treatment of cells during early osteoblast differentiation and matrix formation phase (BMP-4 present from day 4 - 7 or day 7-11 of culture) also resulted in an increased ALP positive and mineralized nodule area, although less effective than continuous treatment with BMP-4. In addition, osteoblastic differentiation was increased when the cells were cultured with BMP-4 during the matrix maturation phase or mineralization phase (day 11-14 or 14-18 of culture respectively).

To exclude whether the effects of BMP-4 were caused by inducing osteoblast proliferation, the DNA content was measured. At day 18, differences up to maximal 1.3 fold were seen. Even after correction for DNA content, BMP addition significantly stimulated matrix mineralization, suggesting a direct effect on mature osteoblasts.

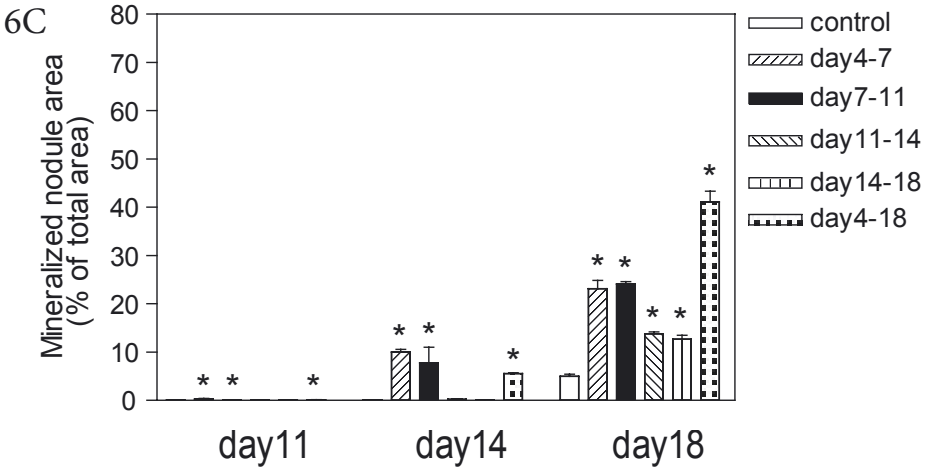
6A


ALP
ALP positive nodule area
(% of total area)
0
10
20
30
40
50
60
70
80
day11
day14
day18
control
day4-7
day7-11
day11-14
day14-18
day4-18

6B


ALP
Control
BMP-4
day4-18
Day11
Day14
Day18

6C


Mineralized nodule area
(% of total area)
0
10
20
30
40
50
60
70
80
day11
day14
day18
control
day4-7
day7-11
day11-14
day14-18
day4-18

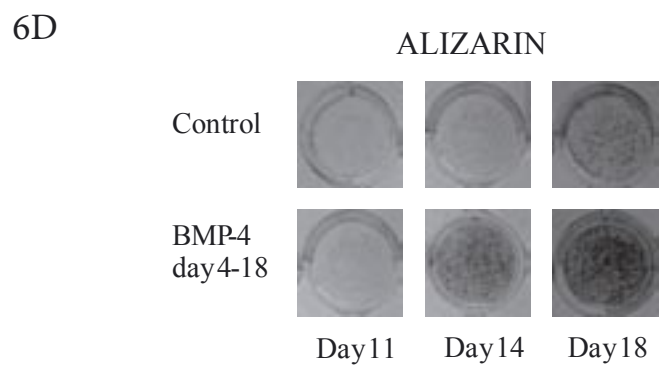


Figure 6 The effect of BMP-4 on differentiation of KS483 cells.
KS483 cells were treated for the indicated time periods with 50 ng/ml BMP-4. Cultures were fixed at day 11, 14 and 18 of culture with 3.7 % PBS-buffered formaldehyde and stained for alkaline phosphatase. (A) ALP positive nodule area expressed as percentage of the total area. (B) ALP stained cultures in the presence or absence of BMP-4 fixed at day 11, 14 or 18. The cells were also stained for hydroxyapatite with alizarin and the % of mineralized nodule area was measured (C). Hydroxyapatite stained cultures in the absence or presence of BMP-4 fixed at day 11, 14 or 18 (D). Data are expressed as percentage of the total area and are representative for three independent duplicate experiments (mean ± SEM). * Significant vs. control isolated at the same day ($p<0.05$). Representative images of the cultures are shown in 6B and D

Discussion

The murine KS483 cell line is a mesenchymal precursor cell line, which differentiates into mature mineralizing osteoblasts during a three-week culture period, when cultured under osteogenesis inducing conditions. This differentiation process can be divided in a proliferation, a matrix formation, a matrix maturation and finally a mineralization phase, according to the model of Stein and Lian [23,25]. In this study, we examined the role of BMPs during the different stages of this differentiation process. While the role of BMPs as inducers of osteoblastic differentiation of various uncommitted mesenchymal precursor cells has been well established [1,3], it is less clear whether BMPs are also involved in regulation of later stages of osteoblastic differentiation such as matrix maturation and mineralization [4].

First, we characterized the expression of various components of the BMP signaling pathway during all differentiation stages of KS483 cells. Expression of BMP type I and II receptors in other pre-osteoblastic cells such as C2C12, 2T3 and MC3T3-E1 cells has been shown earlier, although the temporal expression of these genes has not been studied extensively [13,21,29]. In addition, Smad 1, 2, 4 and 5 expression in C2C12 cells has been shown earlier by Yamamoto et al. [30]. Similarly, we showed constitutive expression of ALK2, ALK3 and ALK6, and BMPR-II, ActR-IIa and ActR-IIb as well as Smad 1-8 during all stages of KS483 osteoblastic differentiation.

During differentiation, KS483 cells expressed mRNA of BMP-2, -3, -4, -6, -7 (OP-1), -8a (OP-2) and -8b, and their expression did not change during differentiation. Whereas, in differentiating fetal rat calvarial cells, BMP-2 and –7 mRNA expression did not change, BMP-4 expression increased and reached a peak prior to matrix formation, BMP-6 mRNA increased throughout culture and expression of BMP-5 gradually increased, reaching a maximum at the mineralization phase [31,32].

The expression patterns of BMP-3 and –8a were of special interest. BMP-3 has been

shown to be expressed in primary cultures of fetal rat calvarial osteoblasts and to antagonize osteogenic BMPs [33,34]. Interestingly, expression of BMP-8a in KS483 cells was induced from the onset of matrix maturation and mineralization, in parallel to osteocalcin expression. Two closely linked BMP-8 genes have been found on the mouse chromosome 4. BMP-8a has been shown to be involved in maintenance of spermatogenesis and the integrity of the epididymis, whereas BMP-8b is required for both initiation and maintenance of spermatogenesis [35,36]. At present no role for BMP-8a and –b has been described in bone formation. This is supported by absence of a bone phenotype in the BMP-8a or -8b knock-out mice. Nevertheless, it has been shown by Dileone et al, that BMP-8a is expressed primarily in developing skeletal tissue, especially in ossifying regions, suggesting a role for BMP-8a in late stages of osteoblast differentiation *in vivo* as well [37].

KS483 cells also expressed various BMP antagonists, such as DAN, gremlin, chordin, tsg, noggin and cerberus. This suggested that the BMP antagonist feedback system is functional during all stages of differentiation. The expression of the antagonists did not change during differentiation except for noggin, which tended to decline during mineralization. This was in contrast to the increased expression of noggin in differentiating bone marrow UAMS-33 cells [7]. The expression of cerberus is of particular interest, since it can antagonize both BMPs and Wnts. A role for Wnt in osteoblast differentiation has recently been suggested by identification of a mutation in LRP5, a coreceptor of Wnt, which causes osteoporosis [38].

The different expression patterns of components of BMP signaling in KS483 cells suggests distinct functions of individual BMPs during osteoblast differentiation.

In addition, the presence of the components of the BMP signaling pathway during the whole differentiation period and the specific induction of BMP-8a suggested functional BMP signaling not only during early phases of differentiation, but in late-stage osteoblast differentiation as well.

To further elucidate the role of BMP signaling during late stages of osteoblastic differentiation, we treated KS483 cells with BMP-4 or BMP antagonists during different stages of differentiation. Consistent with previous data in other cell types, BMP-4 and -6 stimulated initiation of KS483 differentiation [3,39]. In addition, BMP-4 and –6-induced ALP activity could be efficiently blocked by trBMPR-IA, while noggin, another BMP antagonist, blocked BMP-4 but not BMP-6 induced ALP activity [12,13]. Noggin only inhibited BMP-6 induced ALP activity at high concentrations. It has been shown earlier that noggin can bind to BMP-6/-7, but with lesser affinity than binding to BMP-2/-4 [9]. Interestingly, trBMPR-IA and noggin also blocked ALP activity of untreated KS483 cells, whereas proliferation was not affected. In 2T3 cells trBMPR-IA blocked adipocyte differentiation, whereas trBMPR-IB inhibited osteoblast differentiation and mineralized bone nodule formation [12]. However, in the osteogenic culture conditions we have used for the KS483 cells, we did not observe a difference in action between trBMPR-IA and –IB. The inhibitory effects of the BMP antagonists on ALP activity in the absence of exogenous BMPs suggested that endogenously produced BMPs are involved in initiation of osteoblastic differentiation. Also in bone marrow cultures it was shown that addition of noggin inhibited osteoblastic and osteoclastic differentiation by blocking endogeneous BMP activity [7]. In addition, endogenously produced BMPs are also required for normal membranous ossification [40]. These data demonstrate that endogenous BMP signaling is

required for osteoblast differentiation.
To investigate the role of autocrine BMP signaling during all phases of osteoblast differentiation, KS483 cells were treated with antagonists continuously, during early differentiation or during the matrix maturation and mineralization phase. Both noggin and trBMPR-IA inhibited differentiation dose-dependently during all phases, although the effects during the last period of differentiation were less pronounced. This indicated that autocrine BMP signaling is not only required for initiation of differentiation, but is also involved in matrix maturation and mineralization. Furthermore, noggin blocked the effects of BMP-4, but not of BMP-6 and also blocked osteoblastic differentiation of untreated KS483 cells. It is, therefore likely that BMPs belonging to the BMP-4 subgroup are more important for regulation of differentiation of these cells. It has been shown earlier by Shukunami et al. that autocrine BMP-4 signaling is required for chondrogenic differentiation of ATDC5 cells [41].
To determine the role of BMPs on distinct phases of osteoblast differentiation, KS483 cells were treated with BMP-4 during various periods. Continuous BMP-4 treatment and BMP-4 treatment during distinct phases of osteoblast differentiation, i.e. matrix formation or matrix maturation and mineralization, both stimulated ALP positive and mineralized nodule area. These data, together with the inhibiting effect of BMP antagonists in the late stage KS483 cells cultures, suggest that BMP activity in mature osteoblasts is involved in matrix mineralization. Alternatively, BMP activity may be involved in the regulation of the fate of mature osteoblasts. Mature osteoblasts differentiate either into lining cells or osteocytes or undergo apoptosis. Recent studies have suggested a role for BMPs in the regulation of osteoblast apoptosis, and both pro- and anti-apoptotic effects have been reported, providing additional support for a role of BMPs in late stages of osteoblast differentiation [19,42]. In summary, our data indicate that osteoblastic differentiation of KS483 cells depends on autocrine BMP signaling. The presence of BMP signaling pathway components during all phases of differentiation, the specific induction of BMP-8a mRNA during the mineralization phase, and the stimulatory and inhibitory effects of BMP-4 and the BMP antagonists, respectively, during late differentiation suggest that BMP signaling is involved in all stages of osteoblast differentiation, including the matrix maturation and mineralization phase.

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