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Regulation of osteoblast differentiation

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5 Downregulation of Wnt signaling by increased expression of Dkk-1 and –2 is a pre-requisite for late stage osteoblast differentiation of KS483 cells

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Abstract

We have examined the role of Wnt/β-catenin signaling in successive stages of osteoblast differentiation. It is shown that Wnt signaling in mature osteoblasts needs to be downregulated to enable the formation of a mineralized matrix. Using RNA interference, we demonstrate that this is, at least in part, accomplished by upregulation of the Wnt antagonists Dickkopf-1 and –2.

Introduction
The role of Wnt signaling in the initiation of osteoblast differentiation has been well studied. However, the role during late-stage differentiation is less clear. We have examined the role of Wnt/β-catenin signaling in successive stages of osteoblast differentiation.

Materials and Methods
We treated murine bone marrow and MSC-like KS483 cells with either LiCl or Wnt3A during several stages of osteoblast differentiation. In addition, we generated stable KS483 cell lines silencing either the Wnt antagonist Dkk-1 or -2

Results
Activation of Wnt signaling by LiCl inhibits the formation of a mineralized bone matrix in both cell types. While undifferentiated KS483 cells respond to Wnt3A by inducing nuclear β-catenin translocation, differentiated cells do not. This is at least in part accomplished by upregulated expression of the Dkk-1 and -2 during osteoblast differentiation. Using RNA interference, we show that Dkk-1 plays a crucial role in blunting the BMP-induced ALP response and in the transition of an ALP-positive osteoblast in a mineralizing cell. In contrast, Dkk-2 plays a role in osteoblast proliferation and the initiation of osteoblast differentiation.

Conclusion
Our data suggest that Wnt-signaling in maturing osteoblasts needs to be downregulated to enable the formation of a mineralized bone matrix. Furthermore, they suggest that Dkk-1 and Dkk-2 may have distinct functions in osteoblast differentiation.

Key words
Wnt signaling, osteoblast, mineralization, Dickkopf, RNAi

Introduction

Recent studies have provided an increasing amount of evidence that the Wnt/β-catenin signaling pathway is of crucial importance for bone formation and for the establishment of osteoblasts from mesenchymal stem cells (MSC)[12,16]. In the canonical Wnt/β-catenin signaling pathway, Wnt signals are transduced by seven transmembrane receptors of the Frizzled family and single-pass membrane co-receptors, the low-density lipoprotein receptor-related proteins (LRP-5 and -6). Binding of Wnt with these receptors causes β-catenin stabilization through inactivation of glycogen synthase kinase 3β (GSK3β). As a consequence, stabilized β-catenin translocates to the nucleus where it forms a complex with transcription factors of the T-cell factor (TCF)/Leukocyte Enhancer Factor (LEF) family to activate target genes [22,34]. Wnt target genes are involved in a wide range of processes, such as embryogenesis, morphogenesis, organogenesis, and axis specification [34]. Wnt proteins can also transduce signals via the planar cell polarity pathway, in which Jun kinase (JNK) is activated, or via the Wnt/Ca^{2+} pathway. In addition, Wnts can act via both small and heterotrimeric G proteins (reviewed in [33]).

A central role for the canonical Wnt/β-catenin signaling pathway in bone formation has been shown by inactivating and activating mutations in the Wnt co-receptor, LRP5. Inactivation of the receptor decreases bone mass and causes the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome (OPPG) in humans and a similar phenotype in mice, predominantly by decreasing osteoblast proliferation [12,16,17]. These effects on bone formation in LRP5 knockouts are independent of the bone specific transcription factor Runx2. Conversely, an activating mutation in LRP5 is linked to an autosomal-dominant high bone mass trait [1,6,18,32]. This mutation prevents inhibition of Wnt signaling by the Wnt antagonist Dickkopf (Dkk) during bone formation [6].

Several *in vitro* experiments have demonstrated that overexpression of stabilized β-catenin induces the early osteoblast differentiation marker alkaline phosphatase (ALP) in mesenchymal cells [2,12,16,25]. Similarly, Wnts that signal through the canonical β-catenin pathway, such as Wnt1, 2 and 3A, as well as the glycogen synthase kinase inhibitor LiCl [12,25] stimulate ALP activity. In contrast, Wnts that signal through the non-canonical pathway do not activate ALP activity [2,3,12,25]. In addition, experiments with recombinant Dkk-1 protein have established that an autocrine loop of Wnt signaling is required for Bone Morphogenic Protein (BMP)-induced ALP expression in mouse osteoblastic MC3T3 cells [25]. Moreover, Dkk-1 secretion by cancer cells of patients with multiple myeloma results in rapid bone loss, partly due to inhibited osteoblastic bone formation [27].

Whereas all these data point to a stimulatory role of Wnt signaling in bone formation, the role of Wnt/β-catenin signaling in osteoblast differentiation is more complex. Stabilized β-catenin, for example, induced the early osteoblast differentiation marker ALP, but not the late marker osteocalcin [15]. In addition, low doses of LiCl or Wnt3A stimulated proliferation of human bone marrow derived MSC, while higher doses of LiCl or Wnt3A inhibited proliferation and initiated osteoblast differentiation [5,11]. Furthermore, continuous presence of LiCl or Wnt3A during osteoblastic differentiation of these cells inhibited and completely blocked matrix mineralization, respectively [10]. Taken together, these data indicate that Wnt/β-catenin signaling may have distinct dosage-dependent effects at subsequent stages of osteoblast differentiation.

To further examine the mechanism by which Wnt/β-catenin signaling inhibits late stages of osteoblast differentiation, we studied the expression and effects of Wnt signaling on the initiation of as well as on terminal osteoblast differentiation of mouse MSCs and the murine KS483 cell line [31]. The KS483 cell line has MSC-like characteristics, since it can differentiate towards mature mineralizing osteoblasts, fat droplets containing adipocytes and form a cartilaginous matrix [30,31](G. van der Horst et al., in preparation).
Our data suggest that Wnt/β-catenin signaling needs to be downregulated in mature osteoblasts to enable the formation of a mineralized bone matrix. Furthermore, we provided evidence that this is accomplished at least in part by specific upregulation of the Wnt antagonists Dkk-1 and Dkk-2 during late phase osteoblast differentiation.

Materials and methods

Cell culture

KS483 cells were cultured routinely as described previously [31]. C3H10T1/2 cells were cultured in DMEM (GIBCO BRL, Breda, The Netherlands) supplemented with penicillin/streptomycin (Life Technologies, Inc.) and 10% heat inactivated FCS (Integro B.V., Zaandam, The Netherlands). Primary bone marrow cells were derived from tibia and femur of 7 week-old male mice (FVB) and cultured in α-MEM (GIBCO BRL) supplemented with penicillin/streptomycin, 50 μg/ml ascorbic acid and 10% heat inactivated FCS. Stable cell lines were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol. In short, first a KS483 Flp-in host cell line was generated by stable introduction of a single copy of an Flp-Recombinase target (FRT) site as an integral part of an antibiotic resistancy gene in the genome of KS483. One clone was selected that retained full capacity to differentiate into osteoblasts. This clone was used for the generation of the isogenic stable cell lines by transient co-transfection of an FRT-targeting vector and an Flp-recombinase expression vector. The FRT-targeting vector was a modified version of pcDNA5.1 FRT (Invitrogen) in which the CMV promoter was replaced by the human RNAseH1 promoter [7] driving the expression of 64 nucleotides long short hairpin oligonucleotides. Due to Flp-mediated recombination at the genomic FRT-site, this targeting vector was incorporated in the genome. Simultaneously, a shift in antibiotic resistancy was introduced allowing positive selection for integrants in the genomic FRT-site only and negative selection for random integrants in one single step. This method allows the generation of an isogenic stable cell lines after a single transfection by eliminating variability of multiple integrated copies and results in consistent long-term stable expression of the transgene.

Short-term osteoblast differentiation studies

KS483, C3H101T1/2 or mouse bone marrow cells were seeded at a density of 12,000-cells/ cm^2 and cultured until confluence. Subsequently, the cells were cultured for 4 days with ascorbic acid in the absence or presence of conditioned medium of recombinant L cells producing Wnt3A (hereafter called Wnt3A conditioned medium) or conditioned medium of control L cells (hereafter called control medium). Effects of conditioned Wnt3A medium at a dilution of 1/50 corresponded to 50 ng/ml Wnt3A protein of R&D systems. At day 11, the supernatants were withdrawn and cells were washed twice with PBS. ALP activity and DNA content were extracted from the cell layer and measured as described previously [31].

Long-term osteoblast differentiation studies

KS483 differentiation studies were performed as described previously [30]. Bone marrow cells were seeded at a density of 500,000 cells/cm^2. In the first eleven days, 2/3 and thereafter the whole amount of medium was changed every 3 to 4 days. When nodules appeared (from day 11 of culture onwards) β-glycerolphosphate (5 mM, Sigma Chemical Co., St Louis, MO) was 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 10 minutes. The cultures were analyzed for osteogenesis and DNA content as described previously [30]. Pictures of the images were taken and digital imaging was performed.

Immunofluorescence of β-catenin

Immunofluorescence was used to detect subcellular localization of β-catenin. Cells were seeded on glass slides (Lab-TEK chamber slide system, Nalge Nunc International, Naperville, USA) and either left untreated or treated with Wnt3A, control medium or LiCl for the indicated time period. After washing with PBS, cells were fixed in 3.7% buffered formaldehyde and quenched for 10 minutes in 50 mM NH_4Cl. Subsequently, cells were incubated o/n at 4˚C in NETGEL (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 0.25% gelatin, and 0.02% azide). The following day, cells were incubated with anti-β-catenin (1:500 in NETGEL, BD Transduction Laboratories) for 1hr at room temperature. After washing with PBS, cells were incubated with goat anti-mouse conjugated with CY3 for 1hr at RT (1:500, Jackson Immune Research Laboratories. Inc, USA). The cells were embedded in Vectashield. Images of the cells were taken with the confocal laser-scanning microscope (Zeiss LSM 510).

Transient transfection assay

Cells were transiently co-transfected with either a minimal Wnt responsive promoter driving expression of a Gal4 VP16 fusion product (TBE) or a mutant thereof (mtTBE) co-transfected with a Gal4 responsive luciferase reporter, using Fugene following the manufacturer's protocol [14] (Roche). To control for transfection efficiency, CMV-renilla luciferase was co-transfected as well. 24 hrs after transfection, cells were stimulated with LiCl or Wnt3A conditioned medium. Luciferase and renilla activity were measured with the dual luciferase reporter assay system as described previously [30]. Values represent ratio TBE / mtTBE corrected for renilla of three independent experiments.

RT-PCR and real-time PCR

RNA isolation was performed by the method described by Chomzynski and Sachi [8] and reverse transcribed into cDNA. For RT-PCR, cDNA was standardized by competitive PCR using an internal standard (pMUS) as described by van Bezooijen et al. [4]. Subsequently, the expression of various genes was examined by semi-quantitative PCR as described previously [31]. For real time PCR, the Eurogentec Kit for SYBR Green I has been used according to the manufacturer's protocol. Subsequently, the relative levels of RNA were determined using the comparative method of Livak and Schmittgen [19]. Data were corrected for β2 microglobulin (β2M) expression and expressed as $\log(2^{-\Delta\Delta Ct})$. For used primer sets see table 1.

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

Wnt/β-catenin signaling in undifferentiated mouse bone marrow and KS483 cells.

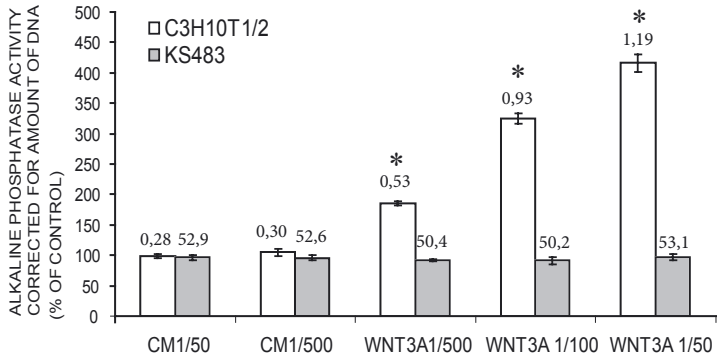
The effect of Wnt/β-catenin signaling on the initiation of osteoblast differentiation was studied in short-term cultures using the KS483 and C3H10T1/2 cell lines and mouse MSCs

Neither LiCl nor Wnt3A affected ALP activity, a marker of early osteoblast differentiation, in short-term experiments in KS483 cells (fig. 1A and 1B). The highest concentration of LiCl had a slightly inhibitory effect on ALP activity, which may have been due to toxicity at this concentration. Similar effects were observed after transfection of Wnt1 and 3 expression vectors, indicating that the absence of an ALP response in KS483 cells was not restricted to Wnt3A (data not shown). Similarly, Wnt3A did not affect ALP activity of primary bone marrow cells (1.03 fold ± 0.16; $p=0.1$ and 0.99 fold ± 0.14; $p=0.1$ stimulation at a concentration of Wnt3A 1/50 and 1/500, respectively). LiCl had a slight, but not significant inhibitory effect (0.86 fold ± 0.11; $p=0.07$ and 0.91 fold ± 0.15 stimulation at a concentration of 10 and 1 mM, respectively). No effects were observed on proliferation in both cell types (data not shown). In contrast, ALP activity was significantly induced in C3H10T1/2 cells by both Wnt3A and LiCl (fig. 1A and 1B), as well as by transient transfection of expression vectors for Wnt1 and –3 (data not shown) [2].

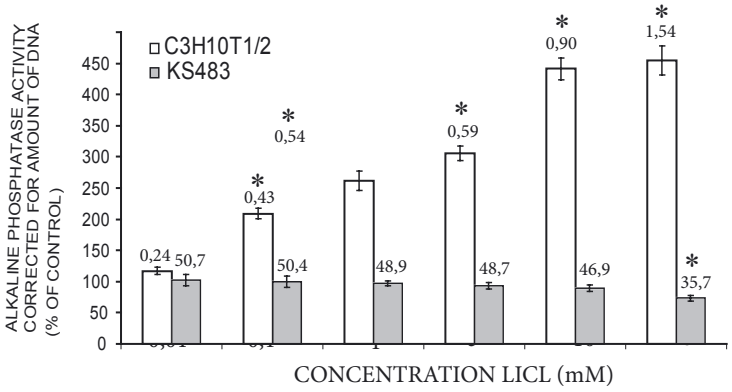
To further explore the absence of ALP induction by Wnt signaling in KS483 cells, we examined the mRNA expression of Wnt signaling components in KS483 cells and found expression of several canonical and non-canonical Wnts (Wnt1, 3, 3A, 4, 5A, 10B) and Wnt receptors (Frizzled 1, 4, 5, 6, 7, 8 and 9; data not shown). In addition, mRNA of the Wnt co-receptors LRP5 and 6 was detected, as well as mRNA of the intracellular signaling molecules Dishevelled and members of the transcription factor family TCF/LEF (Lef-1, TCF3 and TCF7). To study whether Wnt signaling is functional in KS483 and MSC cells, the effect of Wnt3A or LiCl on nuclear translocation of β-catenin was determined in both cell types. Both Wnt3A and LiCl induced β-catenin translocation in KS483, MSC and C3H10T1/2 cells, which served as a control (fig 1C). In addition, Wnt3A and LiCl induced a Wnt-reporter construct in KS483 even more potently than in C3H10T1/2 cells (fig. 1D).

These data demonstrate that undifferentiated KS483 cells are fully capable of responding to Wnts, and suggest that Wnt/β-catenin signaling required for induction of ALP activity may be already sufficient. This is supported by the high basal ALP levels in KS483 cells compared to the low levels in C3H10T1/2 cells (fig. 1A and B) and our observation that reducing autocrine Wnt signaling by addition of the Wnt antagonist Dickkopf-1 inhibited basal as well as BMP-induced ALP levels in KS483 cells after 3 days of treatment (fig. 1E).

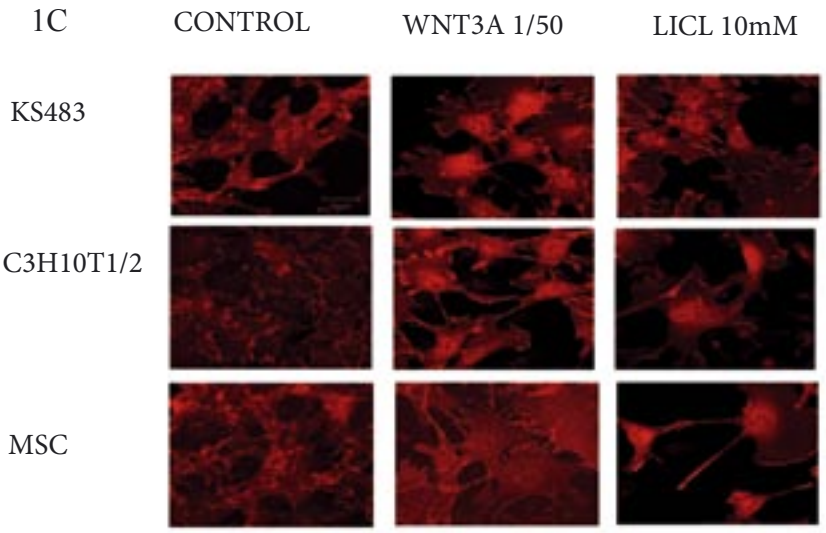
1A


ALKALINE PHOSPHATASE ACTIVITY
CORRECTED FOR AMOUNT OF DNA
(% OF CONTROL)
C3H10T1/2
KS483
0,28 52,9
0,30 52,6
0,53
50,4
0,93
50,2
1,19
53,1
CM1/50
CM1/500
WNT3A1/500
WNT3A 1/100
WNT3A 1/50

1B


ALKALINE PHOSPHATASE ACTIVITY
CORRECTED FOR AMOUNT OF DNA
(% OF CONTROL)
C3H10T1/2
KS483
0,24 50,7
0,43
50,4
0,54
48,9
0,59
48,7
0,90
46,9
1,54
35,7
CONCENTRATION LICL (mM)

1C


CONTROL
WNT3A 1/50
LICL 10mM
KS483
C3H10T1/2
MSC

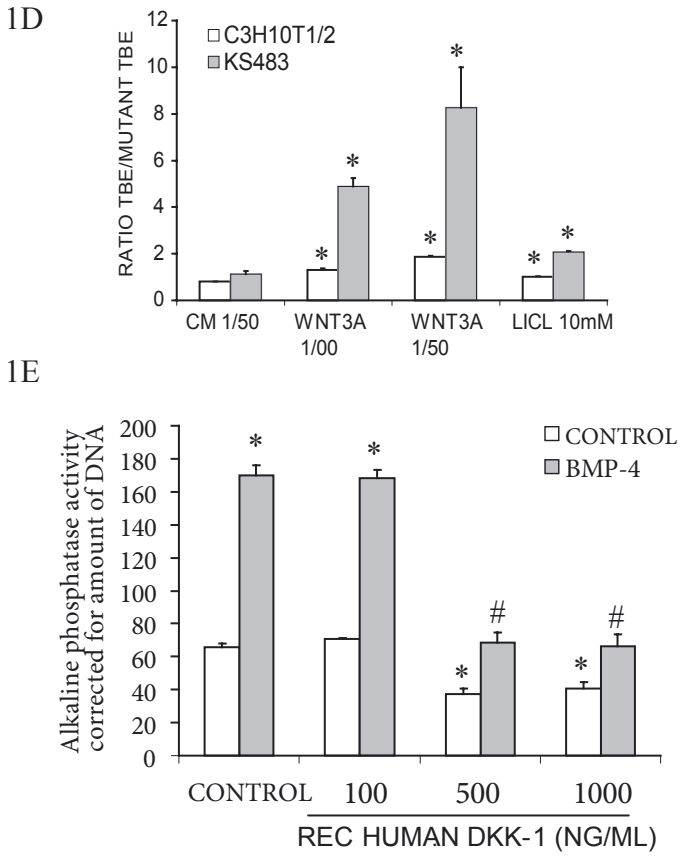


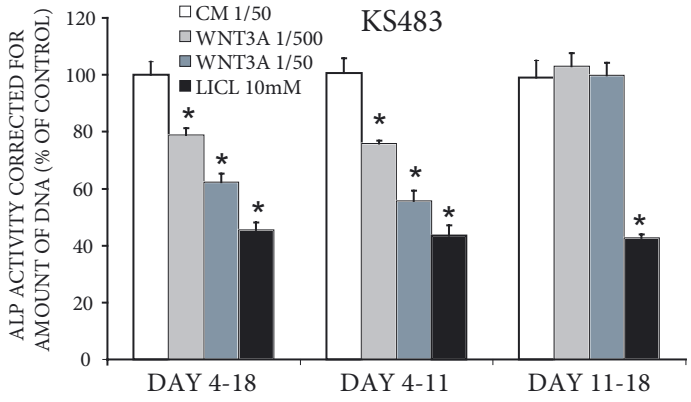
Figure 1 Effects of Wnt3A and LiCl on ALP activity and β-catenin localization in KS483 and C3H10T1/2 cells.
Wnt3A conditioned medium (A) and LiCl (B) induced ALP activity in C3H10T1/2 cells dose-dependently. In contrast, in KS483 cells, Wnt3A conditioned medium (A) or LiCl (B) did not affect ALP activity. Cultures were treated from confluency (day 4) onwards and stopped at day 11. Values represent the average ± SEM of three independent triplicate experiments corrected for DNA content and are expressed as percentage of control. Above the bars, the actual values of ALP activity corrected for DNA content are shown. (C) Wnt3A and LiCl induced nuclear translocation of β-catenin after one day of culture in C3H10T1/2, KS483 and murine bone marrow cells. Representative images are shown. Bar represents 20 μm. Wnt3A and LiCl dose dependently only activated a wild type Wnt reporter (TBE), but not a mutant (mtTBE) in both KS483 and C3H10T1/2 cells (D). Values were corrected for renilla luciferase and expressed as ratio TBE/mt TBE. Addition of recombinant human Dkk-1 dose-dependently inhibited basal and BMP-4 (50 ng/ml) induced ALP activity in KS483 cells (E). Cultures were treated from confluency (day 4) onwards and stopped at day 11. Values represent the average ± SEM of three independent triplicate experiments corrected for DNA content. * significant vs. control; # significant vs BMP-treated control($p<0.05$).

Wnt/β-catenin signaling inhibits bone nodule formation and matrix mineralization in KS483 and murine bone marrow cells.

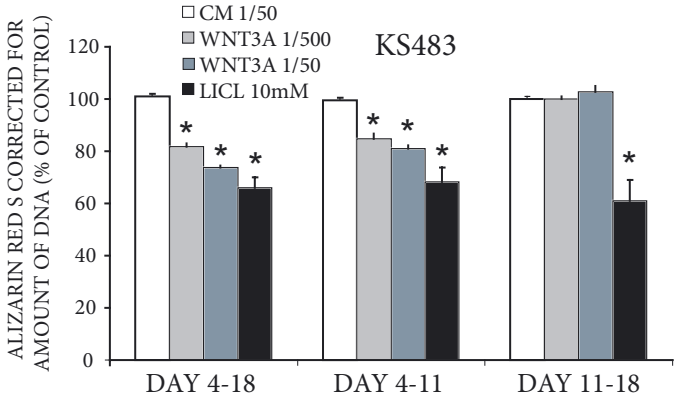
To study the effect of Wnt signaling on more mature stages of osteoblast differentiation, we examined the effects of Wnt/β-catenin signaling on bone nodule formation and matrix mineralization in KS483 and mouse bone marrow cells. Interestingly, continuous addition (from day 4-18) of Wnt3A conditioned medium to KS483 cells significantly reduced ALP activity and LiCl treatment resulted in an even higher reduction (fig. 2A). In addition, both Wnt3A and LiCl significantly reduced the amount of mineral as assayed by extraction of alizarin red from the cultures (fig. 2B). This inhibition was dose dependent (dose range Wnt3A 1/50, 1/100 1/500 and LiCl 1 – 20 mM; data not shown). These data suggest that prolonged exposure to Wnt / β-catenin signaling decreased late stage osteoblast differentiation and the formation of a mineralized matrix.

Long-term treatment with LiCl of primary mouse bone marrow cultures also resulted in reduced ALP activity and mineral content (respectively fig. 2C and 2D). In addition, the morphology of the cells changed dramatically into thin elongated fibroblast-like cells (data not shown). In contrast, continuous addition of Wnt3A (1/50) to primary mouse bone marrow cultures had no significant effect on ALP activity and matrix mineralization (respectively fig. 2C and 2D).

2A



2B



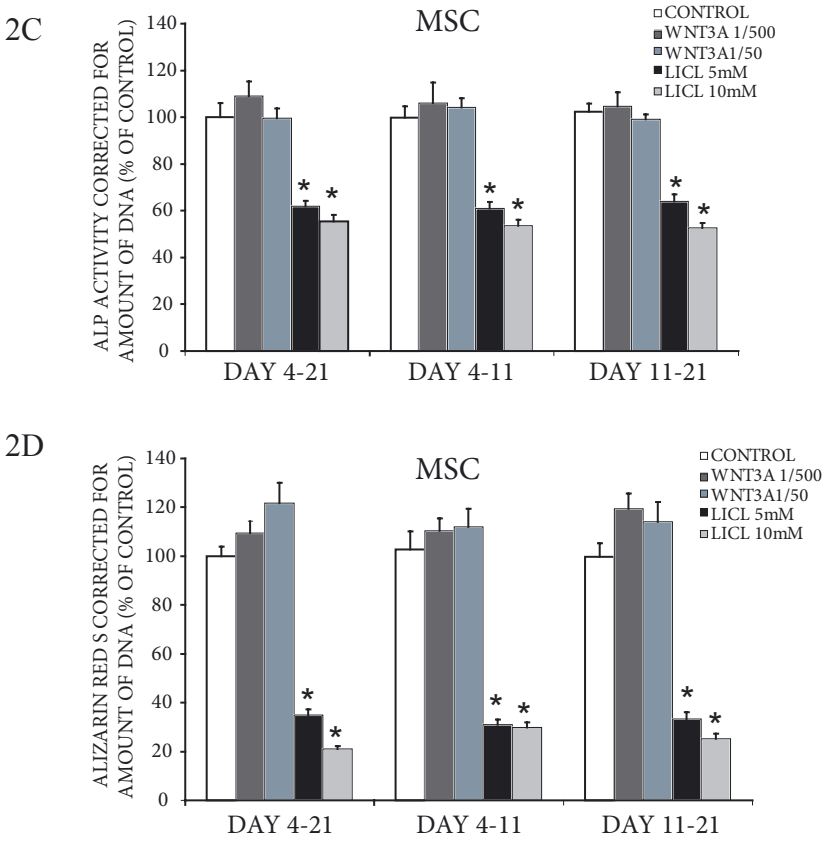


Figure 2 LiCl inhibits matrix mineralization in KS483 and murine bone marrow cells.
KS483 cells were cultured for 18 days in osteogenic culture conditions and treated for the indicated time periods with either Wnt3A conditioned medium (1/50 and 1/500), control medium (1/50) or LiCl (10mM). Alkaline phosphatase activity was measured kinetically at day 18 and corrected for amount of DNA (A). Alizarin Red S was extracted from stained cultures at day 18 and amount of alizarin/well was quantitatively measured and corrected for amount of DNA (B). Wnt3A inhibited ALP activity and the formation of mineralized nodules only when added to undifferentiated cells, whereas LiCl inhibited irrespectively of differentiation stage. Murine bone marrow cells were cultured in osteogenic culture conditions for 21 days, and treated from confluency (day 4) onwards with either Wnt3A, LiCl or control medium for the indicated time periods. Alkaline phosphatase activity was measured kinetically at day 21 and corrected for amount of DNA (C). Alizarin Red S was extracted from stained cultures at day 21 and amount of alizarin /well was quantitatively measured (D). LiCl, but not Wnt3A inhibited ALP activity and matrix mineralization in murine bone marrow cells. Values represent the average ± SEM of 3 independent triplicate experiments corrected for DNA content and are expressed as percentage of control. * significant vs. control ($p<0.05$).

To further define the period in which Wnt3A affects KS483 osteoblast differentiation, a time window experiment was performed. Therefore, KS483 were cultured until confluence and thereafter treated with Wnt3A or LiCl during the first week of culture (predominantly matrix formation) or during the last week of culture, in which matrix maturation and mineralization occurs. Mouse bone marrow cells were only treated with LiCl, since Wnt3A had no effect on these cells. The inhibitory effect of Wnt3A on ALP activity and mineralization was only observed when it was added to undifferentiated KS483 cells, early in the differentiation process, while addition to mature osteoblasts was ineffective. This was in marked contrast to LiCl, which inhibited ALP activity and

formation mineralized nodules irrespective of the differentiation stage in both KS483 and mouse bone marrow cells (fig. 2).

β-catenin localization during osteoblast differentiation of KS483 cells

To further investigate the lack of an effect of Wnt3A in mature osteoblasts, KS483 cells were cultured in osteogenic conditions on glass slides for 4, 11 or 18 days. At each of these time points, cultures were treated for 2 hrs with either control medium (1/50), Wnt3A (1/50) or LiCl (10 mM) and β-catenin localization was examined. In KS483 cells treated with control medium, localization of β-catenin was predominantly membrane-bound and cytoplasmic (fig. 3A, D and G). Wnt3A and LiCl treatment of undifferentiated and KS483 cells at day 11 of differentiation induced nuclear translocation of β-catenin in almost all cells (respectively 3B, C, E and F). Remarkably, Wnt3A was not able to induce β-catenin translocation in differentiated cells at day 18 of culture (compare fig. 3B, E and H), while LiCl also induced β-catenin translocation in these differentiated cells (compare fig. 3C, F and I).

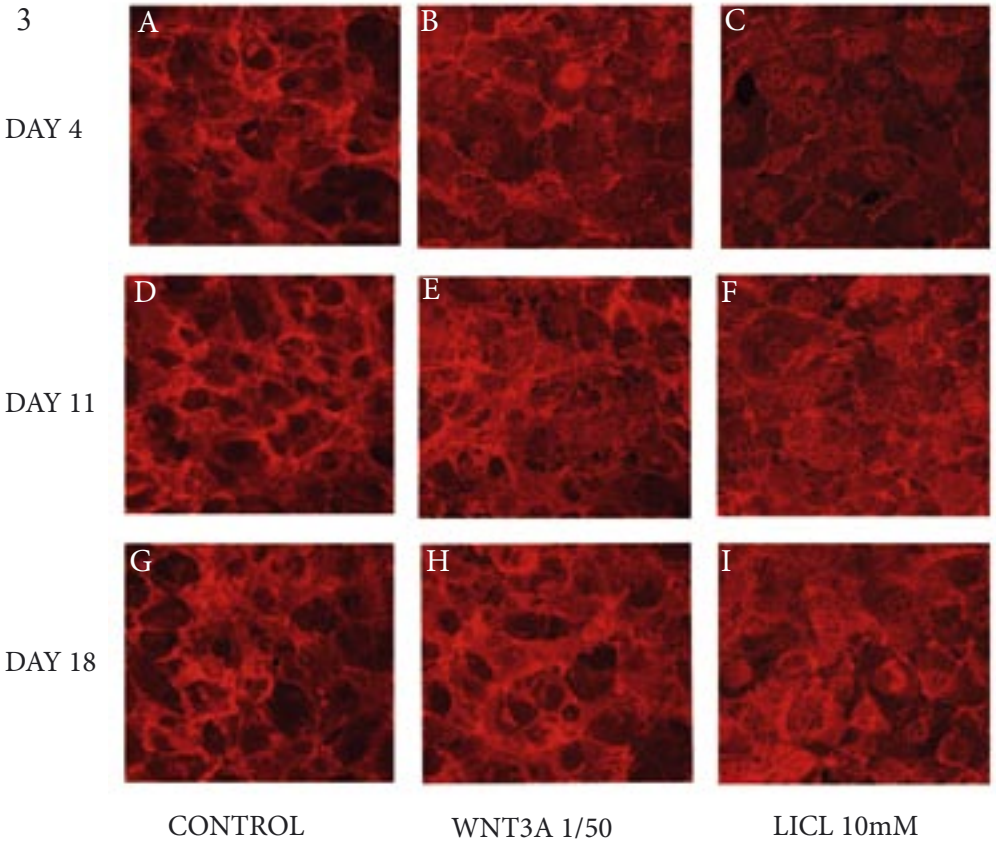


Figure 3 Wnt3A only induces β–catenin nuclear translocation in undifferentiated KS483 cells, whereas LiCl induces translocation regardless of differentiation stage.
KS483 cells were cultured until the indicated time period, and treated for 2 hrs with either LiCl, Wnt3A conditioned medium or control medium. In control medium, predominantly membrane bound staining of β-catenin was found (A, D and G).

Wnt3A induced significant nuclear translocation of β-catenin at day 4. Translocation appeared a little bit less abundant at day11, whereas no positive nuclei were found at day 18 (B, E and H). LiCl induced β-catenin translocation in almost all cells at all time points (C, F and I). Representative images are shown. Bar represents 20 μm.

Expression of Dickkopf –1 and –2 increased during osteoblast differentiation of KS483 cells.

Our data show that mature differentiated osteoblasts have lost their response to Wnt3A, but not to LiCl. This could be either due to loss of expression of Wnt receptors or by the induction of secreted Wnt antagonists, blocking the activity of exogenously added Wnt3A. We did not find any evidence for decreased Wnt receptor expression, since mRNA expression of various Frizzled receptors (Fzd 1, 4, 5, 6, 7, 8 and 9) and Wnt co-receptors (LRP5 and-6) did not change during osteoblast differentiation (data not shown).

In contrast, expression of the Wnt antagonists Dkk-1 and Dkk-2 was tightly regulated during differentiation, each with its own specific expression profile. The expression patterns were compared with the induction of osteocalcin mRNA, a marker for mature osteoblasts, which was induced from day 11 onwards (fig. 4A), using real time PCR. Expression of Dkk-1 was induced at day 7 of culture and increased until day 14. Its expression decreased thereafter (fig. 4A). Dkk-2 mRNA expression showed a similar biphasic pattern, which peaked at day 11 of culture. Compared to Dkk-1, the expression of Dkk-2 was much higher in undifferentiated cells.

In addition, we examined the regulation of Wnt antagonists mRNA expression in terminally differentiated KS483 cultures continuously treated with known modulators of osteoblast differentiation, i.e. either BMP-2 (50 ng/ml), 1,25$(OH)_2$ vitamin D3 (10-7 M), parathyroid hormone related peptide (PTHrP 1-34; 10^{-7} M), transforming growth factor- β (TGF-β; 10 ng/ml), recombinant human Sonic Hedgehog (rSHh; 100 ng/ml) or BMP-6 (100 ng/ml) (fig. 4B). Osteocalcin expression was upregulated by the osteoblast differentiation stimulating factors, BMP-2, -6 and rSHh and downregulated by osteoblast differentiation inhibiting factors, such as PTHrP and TGF-β and by 1,25 $(OH)_2$ vitamin D3.

Dkk-1 mRNA expression was not affected by BMP-2, BMP-6 nor 1,25$(OH)_2$ vitamin D3, downregulated by PTHrP (1-34) and TGF-β, and upregulated by rSHh (fig. 4B). Dkk-2 mRNA expression was not affected by BMP-2, BMP-6 nor rSHh and downregulated by PTHrP, TGF-β and 1,25 $(OH)_2$ vitamin D3.

Knockdown of Dkk-1 and -2 by RNAi results in decreased matrix mineralization.

Induction of Dkk-1 and –2 during osteoblast differentiation may be responsible for the absence of a Wnt3A response in mature osteoblasts. This downregulation of Wnt/β-catenin signaling may be required to induce final maturation and mineralization of the bone matrix. To test this hypothesis, we generated stable cell lines that have decreased Dkk-1 or Dkk-2 expression by targeting Dkk-1 or Dkk-2 RNAi vectors to an unique FRT recombination site introduced in the genome of the KS483 cell line using the Flp-In method (Invitrogen). The RNAi vector contained an hRNAseH1 promoter driving the expression of 64-nucleotides long short hairpin oligonucleotides. The sequences used for silencing Dkk-1 (Dkk-1_{si}), Dkk-2 (Dkk-2_{si}) and their respective mutant constructs

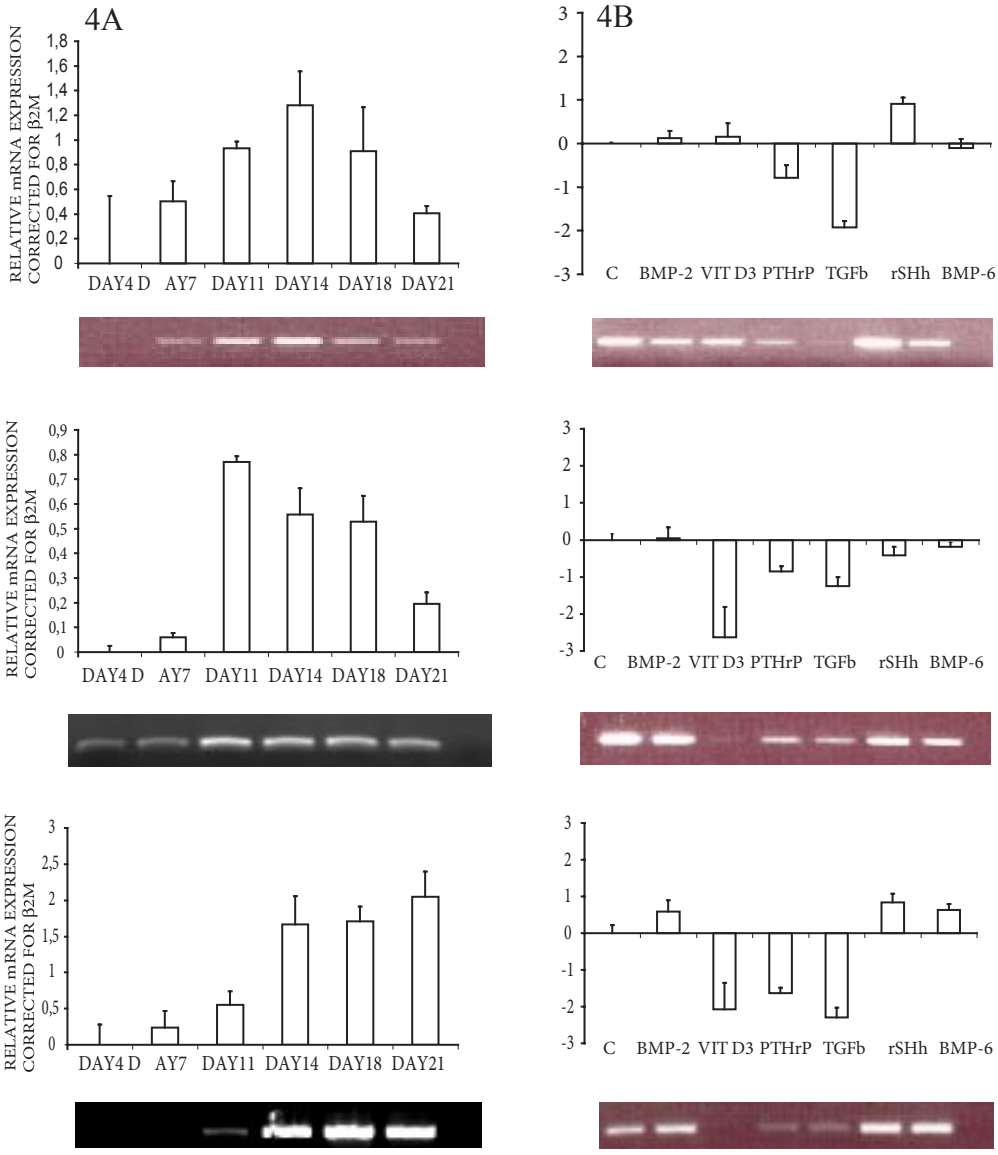


Figure 4 Expression of Dkk-1 and Dkk-2 during KS483 osteoblastic differentiation
Real time RT-PCR analysis of Dkk-1, Dkk-2 mRNA expression during KS483 osteoblastic differentiation in relation to the induction of osteocalcin mRNA expression and corrected for the housekeeping gene β2 microglobulin (β2M). Values are relative to day 4 and expressed as log ($2^{-\Delta\Delta Ct}$). Data are representative for three separate experiments (A). Real time RT-PCR analysis of Dkk-1, Dkk-2 and OC corrected for β2M mRNA expression at day 18 of KS483 cells continuously stimulated with either BMP-2 (100 ng/ml), 1,25$(OH)_2$ vitamin D3 (10^{-7}M), PTHrP 1-34 (10^{-7}M), TGFβ (50 ng /ml), rhSHh (100 ng/ml) or BMP-6 (100 ng/ml). Values are relative to control and expressed as log ($2^{-\Delta\Delta Ct}$). Data are representative for three separate experiments (B). Insets show PCR bands visualized by ethidium bromide staining of a separately performed semi-quantitative PCR reaction.

are shown in figure 5A. Specific silencing of Dkk-1 and -2 was confirmed by real time RT-PCR analysis in both undifferentiated (day 4) and more mature osteoblasts (day11) (fig. 5B). No effects on Dkk-1 and –2 expression were found in control cells, expressing mtRNAi constructs or an empty vector.

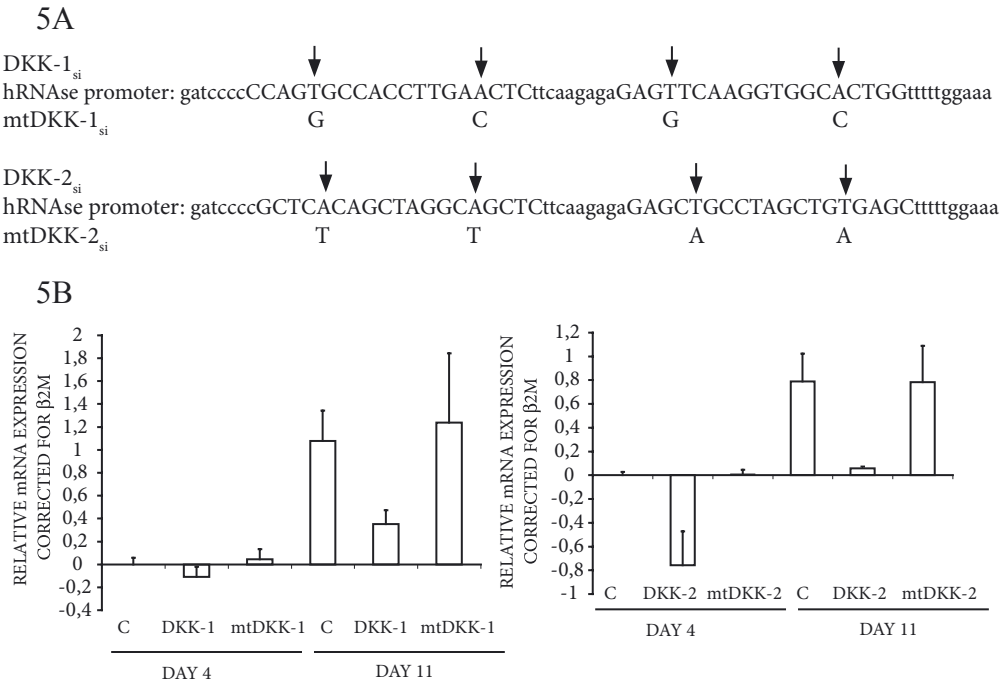


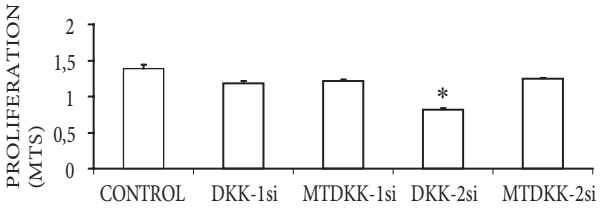
Figure 5 Generation of Frt clones silencing Dkk-1 or Dkk-2.
Isogenic stable cell lines were generated by targeting short hairpin vectors silencing Dkk-1 or –2 in a unique FRT site introduced in the genome of KS483 cells by stable transfection. In addition, mutant clones were generated with two nucleotide changes in the hairpin construct (A). From these clones and the control empty vector clone, RNA was isolated at day 4 and 11, and a real time PCR was performed. Values are relative to control day 4 and expressed as log ($2^{-\Delta\Delta Ct}$). Dkk-1 mRNA expression was decreased in the Dkk-1_{si} clones at day 11 and not in the control and mtDkk-1_{si} clone. Dkk-2 mRNA expression was decreased at day 4 and 11, while mRNA expression of Dkk-2 remained unchanged in the mtDkk-2_{si} and the control clone (B).

We subsequently determined the effect of Dkk-1_{si} or Dkk-2_{si} on cell proliferation, basal and BMP-induced ALP activity, nodule formation and matrix mineralization. No effects were found in control experiments using empty vector or mutant RNAi clones. Dkk-2_{si}, but not Dkk-1_{si}, had a modest inhibiting effect on cell proliferation (fig. 6A). Dkk-1_{si} had no effect on the induction of ALP activity, but further increased the BMP-4-induced ALP response at day 11 (fig. 6B). This is in line with the observed inhibitory role of rhDkk-1 protein in this process [25].

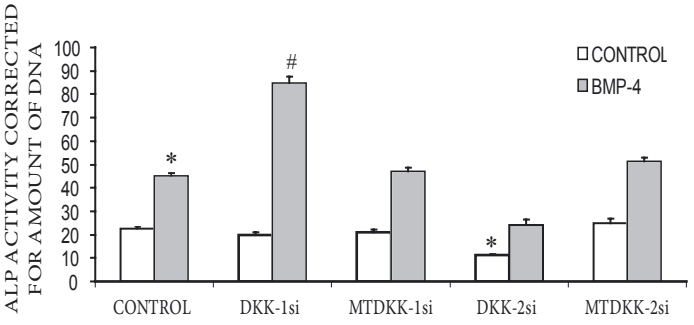
Remarkably, Dkk-2_{si} significantly decreased basal ALP activity, whereas the response to BMP-4 remained relatively unchanged (fold induction control cells 2.0; Dkk-2_{si} cells 2.2). In long-term cultures, silencing of either Dkk-1 or Dkk-2 completely inhibited the formation of mineralized bone nodules (fig. 6C). Furthermore, we noted a decrease in ALP staining in these clones. The effect of Dkk-1, but not Dkk-2 silencing, could be rescued by addition of recombinant Dkk-1. These data were further confirmed by semi-quantitative RT-PCR analysis of osteoblast differentiation-dependent genes at day 11 of

differentiation.
The lack of an effect of Dkk-1_{si} on ALP activity was confirmed at the transcriptional level, showing no effect on ALP mRNA expression (fig. 6D). Dkk-2_{si} that inhibited ALP activity also lowered ALP mRNA expression levels. Similarly, PTH/PTHrP receptor (PTH1R), also a marker of early osteoblast differentiation, was not affected by Dkk-1_{si}, while Dkk-2_{si} decreased its expression. The expression of osteocalcin and BMP-8A, two late markers of osteoblast differentiation, was decreased by both Dkk-1_{si} and Dkk-2_{si}, which confirmed the inhibiting effect of both on late stage differentiation and mineralization.

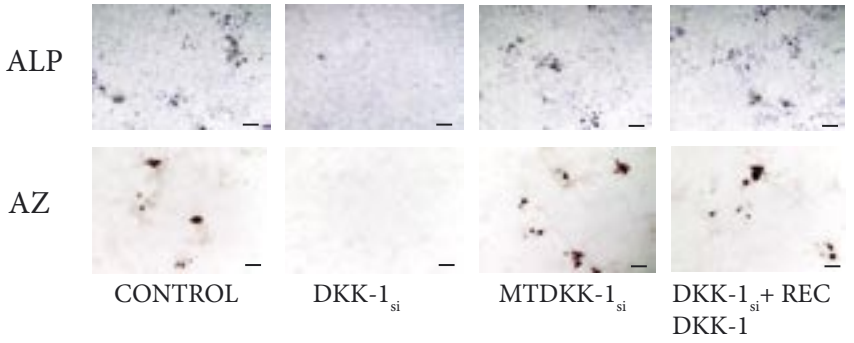
6A



6B



6C



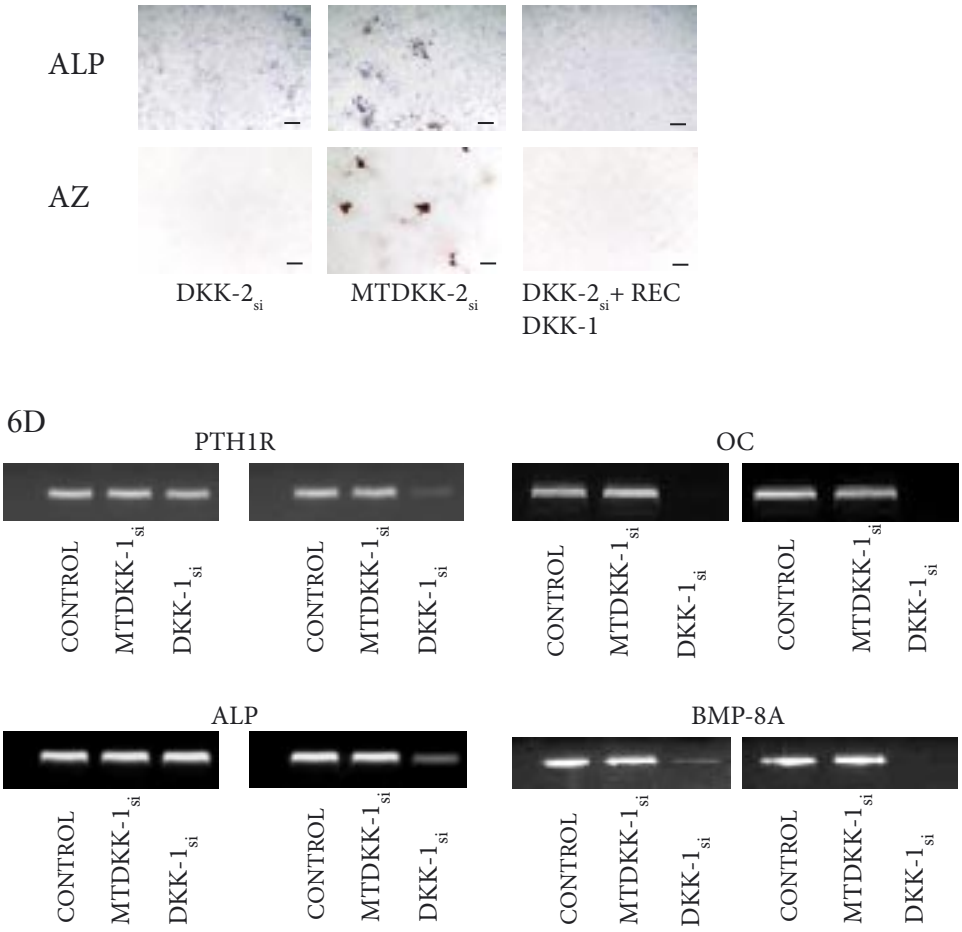


Figure 6 Dkk-1_{si} and Dkk-2_{si} have differential effects on proliferation and the initiation of osteoblastic differentiation, but completely block ALP positive and mineralized nodule formation

Dkk-1_{si} did not affect proliferation, whereas Dkk-2_{si} had a modest effect on proliferation, as measured with a MTS proliferation assay. The mtDkk-1_{si} and mtDkk-2_{si} were indistinguishable from control, empty vector clones (A). Basal ALP activity of Dkk-1_{si} was not changed, whereas the BMP-4 induced ALP activity was augmented. In contrast, Dkk-2_{si} inhibited both basal and BMP-4 induced ALP activity. No effect was observed in the mutant clones. Cultures were stopped at day 11 and values represent averages ± SEM of three independent duplicate experiments corrected for DNA content. * significant vs control; # significant vs BMP-treated control (B). KSFrt clones were cultured in osteogenic culture conditions for 18 days. Cultures were fixed with 3.7% buffered formaldehyde and stained for ALP and amount of mineral. Dkk-1_{si} and Dkk-2_{si} completely blocked the formation of ALP positive and mineralized nodules. The mtDkk-1_{si} and mtDkk-2_{si} were indistinguishable from control cells. Addition of rec. human Dkk-1 (1 mg/ml) reversed the phenotype in the Dkk-1_{si}, but not the Dkk-2_{si} clones. Representative images of both stainings are shown. Bar represents 100 μM (C). mRNA expression of PTH1R, OC, BMP-8a and ALP in control, mtDkk$_{si}$ and Dkk$_{si}$ clones measured with semi-quantitative RT-PCR at day 11 of culture (D).

Discussion

In the present study, we analyzed the effect of Wnt/β-catenin signaling on matrix mineralization and compared this with its effect on the induction of ALP in murine bone marrow cells and the MSC-like KS483 cell line. Previously, it has been shown that

the Wnt/β-catenin pathway increased ALP activity in the mesenchymal C3H10T1/2, ST2 and C2C12 cell lines [12,25]. As expected, we found an induction of ALP activity in C3H10T1/2 cells by Wnt3A and LiCl; however, Wnt3A or LiCl did not induce ALP activity in bone marrow and KS483 cells. The absence of an ALP response in KS483 and bone marrow cells was not due to aberrations in the Wnt/ β-catenin pathway, since Wnt3A and LiCl efficiently induced nuclear β-catenin translocation in both cell types and activated a Wnt reporter construct in the KS483 cells. Furthermore, all relevant signaling pathway components were present in these cells at least at the mRNA level. These data suggest that autocrine Wnt/β-catenin signaling, required for ALP expression is already maximal in KS483 cells. This is furthermore supported by the reduction in ALP activity when Wnt signaling was reduced in undifferentiated KS483 cells by treatment with Dkk-1. Similarly, overexpression of Wnt1, Wnt2 or Wnt3A in the mesenchymal MC3T3 cell line had no effect on ALP expression [25]. These data suggest that the level of Wnt signaling required for ALP activity is bound to a maximum, above which higher signaling will not induce more ALP activity, at least in KS483 and MC3T3-E1 cells. Alternatively, co-factors or additional signaling cascades, required for Wnt-induced osteoblast differentiation, may be limiting factors blunting the ALP response.

Remarkably, Dkk-1_{si} potentiated the BMP-induced ALP response in KS483, suggesting involvement of Dkk-1 in an autocrine signaling cascade antagonizing BMP activity [25]. In contrast, Dkk-2_{si} did not effectively change the BMP response. These data are another example of the complex interactions between the osteogenic BMP pathway and the Wnt signaling pathway.

Several explanations can be given for the inhibitory effects of Dkk-2_{si} on osteoblast proliferation and the initiation of differentiation. First, Dkk-2_{si} may result in overactivation of the Wnt signaling pathway. It has been shown that high β-catenin signaling is able to induce cell cycle arrest or even apoptosis in various cell types [23]. However, addition of LiCl to KS483 cells did not result in decreased proliferation and high levels of LiCl only inhibited the initiation of differentiation. This is in disagreement with the induction of cell cycle arrest by high levels of β-catenin. Alternatively, rather than acting as an antagonist, Dkk-2 may act as an agonist of Wnt signaling in KS483 cells. This depends on the expression of the membrane-bound Dickkopf receptor Kremen2 [20,21]. Whether Kremen2 is indeed absent in undifferentiated KS483 cells and Dkk-2 can thus act as an agonist is subject of further study.

In long-term culture experiments, continuous presence of Wnt3A blocked nodule formation and matrix mineralization in KS483, but not in bone marrow cells. More pronounced and cell type independent effects were found with LiCl, which was a potent inhibitor of nodule formation with strongest effects on matrix mineralization. Similar observations have been made previously in human bone marrow cultures [10]. In contrast to our observations, Wnt3A potently inhibited matrix mineralization in the human bone marrow cells [5,10]. Cell type dependent differences in susceptibility for Wnt3A may also explain the differences in response between KS483 and bone marrow cells. These differences may be caused by cell type dependent expression of specific co-factors required for Wnt3A actions or of specific antagonists blocking Wnt3A. In addition, the experiments in the human and mouse bone marrow cells as well as in KS483 and MC3T3 cells indicate that activation of the Wnt-pathway not automatically results in increased osteoblast differentiation. The outcome apparently depends on the cellular context and

possibly on the commitment of the cells towards the osteoblast lineage at the time of exposure to the Wnt-signals. We have found evidence for the spatio-temporal regulation of various Wnt-antagonists during osteoblast differentiation in KS483 cells which may contribute to these differences. The Wnt signaling pathway during differentiation is very complex and is determined by various factors and complex interactions with other pathways. Further studies are required to determine the relative contributions of each factor. To explain the inhibitory effect on mineralization, it is possible that Wnt/β-catenin signaling in osteoblastic cells interferes with Runx2 signaling, although in LRP5 knock out mice no effects were found on Runx2 expression [16]. However, in another study it was shown that the downstream Wnt effector Lef-1 repressed Runx2 dependent activation of the osteocalcin promoter. Moreover, they showed that constitutive active β-catenin enhanced Lef-1 mediated repression of the bone specific transcription factor Runx2 [15]. These data suggested that the Wnt/β-catenin signaling pathway might decrease osteoblastic differentiation by inhibiting Runx2 activity. Posttranslational repression of Runx2 after prolonged exposure to Wnt/β-catenin signaling may explain the inhibitory effect on terminal osteoblast differentiation in KS483 and murine bone marrow cells.

A remarkable finding was that differentiated KS483 osteoblasts had lost their response to Wnt3A. Comparison with LiCl suggested that the loss of response to Wnt3A could either be caused by decreased expression of receptors involved in Wnt signaling or by increased expression of Wnt antagonists. In primary osteoblasts the expression of Fzd2 and Fzd6 decreased during mineralization[16], while the expression of the Wnt antagonist Wif-1 was increased after BMP-2 treatment in C2C12 and MC3T3 cells [28]. We did not find decreased expression of Frizzled receptors during osteoblast differentiation, at least at the mRNA level, but interestingly, we noted a sharp increase in the expression of Dkk-1, Dkk-2, and Wif-1 (data not shown). The induction of Wnt antagonists can explain the lack of effect of Wnt3A on differentiated cells, since exogenously added Dkk-1 completely blocked Wnt3A-induced nuclear β-catenin translocation in undifferentiated KS483 cells (data not shown) and Dkk-1 inhibited Wnt3A-induced increase in ALP activity in C3H10T1/2, ST2, and C2C12 cells [25]. The differential expression of Dkk-1 and Dkk-2 during osteoblast differentiation is noteworthy with respect to the role of LRP5 in regulating bone mass [18]. In KS483 cells, Dkk-1 and Dkk-2 expression peaked respectively at the onset and during matrix mineralization, suggesting that differentiated mature osteoblasts might be the prime source of Dkk in bone regulating LRP5 activity. Interestingly, the expression of Dkk-1 and Dkk-2 is differentially regulated by known modulators of osteoblast differentiation. This suggests that these signaling pathways may affect osteoblast differentiation at least in part by modulating Wnt signaling by regulating the expression of Wnt antagonists. Furthermore, it indicates that differentiating osteoblasts may actively change sensitivity for Wnt/ β-catenin signaling by expressing the Wnt antagonists Dkk-1 and –2, as well as Wif-1 [28].

Downregulation of Wnt signaling in mature osteoblasts might be required to enable nodule formation and matrix mineralization. In long-term culture experiments, silencing of the Wnt antagonists Dkk-1 or Dkk-2 completely blocked nodule formation and matrix mineralization in KS483 cells.

The silencing of Dkk-1 in the KSFrt clones was specific, since no effects were found in empty vector control and mutant Dkk_{si} clones. Moreover, recombinant human Dkk-1 could reverse the effect of $Dkk\text{-}1_{si}$. In contrast, recombinant Dkk-1 could not reverse

Dkk-2_{si}, suggesting that Dkk-1 and Dkk-2 exert their effects by antagonizing specific subsets of Wnts.
Besides a complete block in nodule formation, silencing of Dkk-1 or –2 differentially affected cell proliferation and the initiation of differentiation, which were not affected by Dkk-1_{si}, but decreased by Dkk-2_{si}. These differences may be explained by the absence of Dkk-1 mRNA expression at the beginning of KS438 osteoblastic differentiation, whereas low levels of Dkk-2 mRNA expression are already found at day 4 of culture.
Limited data are available on the role of Wnt antagonists in late-stage osteoblast differentiation. It has been shown that Wif-1 is upregulated during osteoblast differentiation [28]. Furthermore, addition of secreted frizzled related protein sFRP3, another Wnt antagonist, increased ALP activity as well as the amount of mineralized nodules in MC3T3-E1 cells [9]. The authors proposed that the increase in mineralization is due to activation of a β-catenin independent pathway. Our data, however, suggest that this effect might also be explained by a specific blockade of Wnt(s) inhibiting the transition of an ALP-positive osteoblast in a mineralizing osteoblast.
Transient overexpression of Dkk-1 in MC3T3-E1 cells significantly reduced the mineralizing capacity of these cells, and blocked BMP-induced ALP activity [25]. In KS483 cells, exogenously added Dkk-1 tended to decrease the number of mineralized nodules, but this was not significant (data not shown). These effects could be caused by reduced initiation of osteoblast differentiation by Dkk-1.
Current data suggest that the level of Wnt/β-catenin signaling is tightly regulated during different phases of osteoblast differentiation. First, in osteoprogenitor cells, low levels of Wnt signaling increase cell proliferation and thereby increase the amount of osteoprogenitors [12,16]. Then, higher levels of Wnt/β-catenin signaling are required for the initiation of osteoblast differentiation to a certain cut-off point [10]. Finally, in this paper and in a previous study in human MSCs, evidence is provided that downregulation of Wnt/β-catenin signaling is required for the induction of terminal osteoblast differentiation and matrix mineralization [10]. These data are in line with data in the central nervous system, where Wnt is required for the increase of the progenitor cell population in the central nervous system by promoting proliferation and inhibiting differentiation of these cells [35]. Furthermore, blocking Wnt-1 induced inhibition of neuronal differentiation by adding the Wnt antagonist sFRP2 is sufficient for neurite outgrowth from ES cells [13]. In addition, these data are in line with studies in transgenic mice and colorectal cancer cell lines demonstrating that canonical Wnt signaling is essential for the continuation of intestinal stem cells by stimulating proliferation and blocking differentiation. Furthermore, Wnt signaling needs to be downregulated to enable terminal differentiation of enterocytes in the intestine [24,26,29]. Our data suggest that similar mechanisms may play a role in osteoblast differentiation, where mature mineralizing osteoblasts downregulate Wnt/β-catenin signaling at least in part by increasing the expression of the extracellular Wnt antagonists, Dkk-1 and Dkk-2. Moreover, our data suggest that Dkk-1 and Dkk-2 may have distinct functions in osteoblast differentiation: Dkk-1 may preferentially antagonize Wnt(s) which stimulate the induction of ALP and simultaneously block the transition of an ALP-positive in a mineralizing osteoblast, while Dkk-2 specifically antagonizes Wnt(s) involved in cell proliferation and the initiation of osteoblastic differentiation.

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gene	Sense	Antisense
β2M	CACTGACCGGCCTGTATGC	GAATTCAGTGTGAGCCAGGATATAGA
OC	ACAGACAAGTCCCACACAGCAGC	TGAAGGCTTTGTCAGACTCAGGGC
Dkk-1	ACTACCAGCCCTACCCTTGC	CTTGGACCAGAAGTGTCTTGC
Dkk-2	CTGGGATGGCAGAATCTAGG	AATCCAGGTTTCCATCATGC
PTH1R	TGCTTGCCACTAAGCTTCG	TCCTAATCTCTGCCTGCACC
ALP	CGGACATCATGAGGGTAAGG	GAGACATTTTCCCGTTCACC
BMP-8a	GTTCGAAGTGGTCCAAGAGC	CCTCCTCTTCAGTGGTCTCG

Table 1 PCR primers (5' - 3')

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