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Wnt Signaling Acts and Is Regulated in a Human Osteoblast Differentiation Dependent Manner

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Abstract The Wnt signaling pathway is an important regulator of cellular differentiation in a variety of cell types including osteoblasts. In this study, we investigated the impact of Wnt signaling on the function of human osteoblasts in relation to the stage of differentiation. Differentiating osteoblasts were created upon glucocorticoid (GC) treatment, whereas nondifferentiating osteoblasts were created by excluding GCs from the culture medium. GC-induced differentiation suppressed endogenous β-catenin levels and transcriptional activity. During GC-induced osteoblast differentiation, activation of Wnt signaling slightly decreased alkaline phosphatase activity, but strongly suppressed matrix mineralization. In addition, mRNA expression of several Wnt signaling related genes was strongly regulated during GC-induced osteoblast differentiation, including frizzled homolog 8, dickkopf homolog 1, and secreted frizzled-related protein 1. In contrast, in the absence of GC-induced differentiation, Wnt signaling acted positively by stimulating basal alkaline phosphatase activity. Interestingly, pre-stimulation of Wnt signaling in early osteoblasts enhanced their differentiation capacity later on during the GC-induced differentiation process. In conclusion, we showed a differentiation-dependent effect of Wnt signaling on osteoblasts. Wnt signaling stimulated early osteoblasts in their capacity to differentiate, whereas mature osteoblasts were strongly inhibited in their capacity to induce mineralization. Moreover, osteoblast differentiation suppressed endogenous Wnt signaling and changed the expression of multiple Wnt signaling related genes. J. Cell. Biochem. 104: 568–579, 2008.

Key words: osteoblast; differentiation; Wnt signaling; glucocorticoids

The Wnt signaling pathway is an important regulator of cellular differentiation in a variety of cell types [Moon et al., 2004]. It plays a widespread role in skeletogenesis from embryonic skeletal patterning, through fetal skeletal development, and bone remodeling in adults [Hartmann, 2006; Krishnan et al., 2006]. In humans 19 different Wnt proteins have been identified. Wnt proteins activate Wnt/β-catenin signaling upon binding to the frizzled receptor and a co-receptor LRP 5 or 6. During activation of Wnt signaling the degradation of β-catenin is prevented, which results in β-catenin accumulation, its nuclear transport and regulation of several target genes. In the absence of Wnt signal, the level of β-catenin is kept low through degradation of cytoplasmic β-catenin. A multiprotein complex containing glycogen synthase kinase 3β and scaffolding proteins axin and adenomatosis polyposis coli (APC) mediate the degradation of β-catenin by phosphorylation of specific amino acid residues. Wnt signaling is under the control of several extracellular factors that inhibit Wnt signaling. Secreted frizzled related proteins (SFRPs) and Wnt inhibitory factor (WIF) bind directly to Wnt proteins thereby preventing receptor activation. Dickkopf (DKK) proteins inhibit Wnt signaling via binding to LRP5 or 6 and the associated protein Kremen, which dissociates LRP5 or 6 from frizzled preventing formation of an active Wnt receptor complex [Mao et al., 2002; Moon et al., 2004].

Multiple skeletal defects have been described in mice deficient for distinct Wnt signaling genes [Kato et al., 2002; Bodine et al., 2004; Holmen et al., 2004, 2005; Kokubu et al., 2004;

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Glass et al., 2005; Li et al., 2005]. In humans, loss-of-function mutations in LRP5 are associated with osteoporosis-pseudoglioma syndrome, which is characterized by low bone mass and skeletal fragility [Gong et al., 2001]. In contrast, gain of function mutations in LRP5 are associated with a high bone mass phenotype [Boyden et al., 2002; Little et al., 2002]. Moreover, various genetic variants of LRP5 are associated with BMD and risk of fractures in humans [Ferrari et al., 2004, 2005; Koay et al., 2004; van Meurs et al., 2006]. Despite all these data, the precise mechanism of Wnt signaling in bone biology remains unclear. The skeletal defects are at least partially accomplished by regulating osteoblast differentiation. Several reports show that Wnt signaling contributes to osteoblast differentiation [Bain et al., 2003; Rawadi et al., 2003; Hu et al., 2005], whereas other reports show the ability of Wnt signaling to inhibit osteoblast differentiation [Boland et al., 2004; de Boer et al., 2004; van der Horst et al., 2005].

The conflicting data on whether Wnt signaling is stimulating or inhibiting osteoblast differentiation is likely to reflect the diverse function of Wnt signaling at different stages of osteoblast development. To address this we exploited a human pre-osteoblast cell line, SV-HFO, that dependent on the presence or absence of glucocorticoids (GCs) either differentiates in a full active bone forming osteoblast or does not differentiate, respectively. We studied the endogenous Wnt signal and expression of Wnt signaling related genes in differentiating and nondifferentiating osteoblasts. In addition, the impact of Wnt signaling was studied on osteoblast function in both differentiation conditions.

MATERIALS AND METHODS

Cell Culture

SV-HFO cells were cultured in αMEM (Gibco BRL, Paisley, UK) supplemented with 20 mM HEPES, pH 7.5 (Sigma, St. Louis, MO); streptomycin/penicillin; 1.8 mM $CaCl_2$ (Sigma); and heat-inactivated FCS (GIBCO) at 37°C and 5% CO_2 in a humidified atmosphere. Thawed cells were pre-cultured for 1 week in the presence of 10% FCS. In this pre-culture, cells were seeded in a density of 5×10^3 vital cells per cm^2 and were subcultured every week. During this pre-culture, SV-HFO cells remained in an undifferentiated stage. After pre-culturing, cells were seeded in a density of 10×10^3 vital cells per cm^2 in 6- or 12-wells plates (Corning, NY). After seeding, cells were incubated for two days before they were put on differentiating medium (indicated as day 0). Medium was supplemented freshly with 10 mM β-glycerophosphate (Sigma), 100 nM dexamethasone (DEX) (Sigma) or other additives and replaced every 2–3 days. Nondifferentiating cultures were similarly cultured as differentiating cultures, however DEX was excluded. LiCl and Wnt3a were purchased from Sigma and R&D systems (Minneapolis, MN), respectively. For analysis, medium was collected and stored at −20°C and cells were harvested from the culture dish in PBS containing 0.1% Triton X-100 and stored at −80°C. Cell lysates were sonicated on ice in a sonifier cell disrupter for 2×15 s before analysis.

Alkaline Phosphatase Activity and Mineralization Assays

For DNA measurements, 100 μl SV-HFO cell lysates were treated with 200 μl heparin (8 IU/ml in PBS) and 100 μl ribonuclease A (50 μg/ml in PBS) for 30 min at 37°C. This was followed by adding 100 μl ethidium bromide solution (25 μg/ml in PBS). Samples were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA) using an extinction filter of 340 nm and an emission filter of 590 nm. For standards, calf thymus DNA (Sigma) was used.

ALPL activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamine buffer supplemented with 1 mM $MgCl_2$ at pH 9.8) in the SV-HFO cell lysates for 10 min at 37°C. The reaction was stopped by adding 0.06 M NaOH and measured at 405 nm. Results were adjusted for DNA content of the cell-lysates.

For quantification of the mineral content cell lysates were incubated overnight in 0.24 M HCl at 4°C. Calcium content was colorimetrically determined after addition of 1 M ethanolamine buffer (pH 10.6) 0.35 mM o-cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline, and 0.6 mM hydrochloric acid at 595 nm. Results were adjusted for DNA content of the cell-lysates.

β-Catenin Western Blot

From SV-HFO cultures, nuclear and cytoplasm fractions were isolated using NE-PER isolation kit (Pierce, Rockford, IL) according to

the manufacturer's description and stored at −80°C. Before analysis, total protein concentration was measured using BCA protein assay reagent (Pierce) according to the manufacturer's description. Next, 10 μg protein was separated on a 10% SDS–PAGE gel, followed by blotting on Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). After blotting, membranes were blocked for 2 h in TBST (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6) containing 5% milk powder. Next, membranes were incubated over-night at 4°C with monoclonal anti-β-Catenin antibody (1:2,000; Cell Signaling, Beverly, MA) in TBST containing 5% bovine serum albumin (Sigma). For detection, membranes were incubated for 2 h with goat-α-rabbit-HRP (1:1,000; DAKO, Carpinteria, CA) in TBST containing 5% milk powder. Next, the ECL detection system (Amersham Pharmacia Biotech) was used and immunoreactive bands were quantified using Quantity One software (Bio-Rad Hercules, CA).

Luciferase Reporter Assays

At day 5 of culture cells were transfected with 200 ng Wnt signaling WRE-Luciferase reporter plasmid (TOPFLASH [van de Wetering et al., 1997]) (12 wells plate) using Fugene6 (Roche, Basel, Switzerland) according to the manufacturer's description. After 24 h cells were lysed by incubating for 20 min in 100–200 μl lysis buffer (Promega, Madison, WI). Luciferase activity was measured using 25 μl cell-lysate and the Steady-Glo Luciferase Assay System (Promega). To correct for transfection efficiency between nondifferentiating and differentiating luciferase values were corrected for the luciferase activity of the pGL3-control vector (Promega).

RNA Isolation and cDNA Synthesis

Total RNA was isolated using RNA-Bee solution (Tel-Test, Friendwood, TX) according to the manufacturer's protocol. To remove calcium (derived from the extracellular matrix), RNA was precipitated by overnight incubation with 4 M LiCl and 50 mM EDTA at −20°C. After precipitation and centrifugation for 30 min at 14,000 rpm and 4°C, the RNA pellet was washed four times with 70% EtOH and subsequently dissolved in H_2O. The total amount of RNA was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). One microgram total RNA was reverse transcribed into cDNA using a cDNA synthesis kit and according to the protocol of the manufacturer (MBI Fermentas, St. Leon-Rot, Germany), using 0.5 μg oligo$(dT)_{18}$ and 0.2 μg random hexamer primers. When purified mRNA was used in the cDNA synthesis, 10 μg of total RNA was purified using a oliotex mRNA mini kit (Qiagen, Stanford, CA) according to the manufacturer's protocol. RNA was eluted in 60 μl form which 10 μl was used in the cDNA synthesis.

Quantitative Real Time PCR (qPCR)

qPCR was carried out using an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Reactions were performed in 25 μl volumes using a qPCR core kit (for assays using a probe) and qPCR kit for SYBR green I (for assays using SYBR green) (Eurogentec, Seraing, Belgium). Primer and probe sets were designed, using the Primer Express software (version 2.0; Applied Biosystems), amplicons overlapped at least one exon–exon boundary. Since the FZD1, 2 and 8 genes consist of a single exon these genes were also analyzed on cDNA derived from purified mRNA to avoid genomic DNA contamination. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer and probe concentrations are listed in Table I. Genes consisting of a single exon were quantified using cDNA derived from purified mRNA.

Affymetrix GeneChip-Based Gene Expression

Purity and quality of isolated RNA was assessed by RNA 6000 Nano assay on a 2100 Bioanalyzer (Agilent Technologies). Per analyzed condition total RNA of three different biological samples was pooled. Synthesis of first and second strand cDNA from total RNA was performed according to the One-Cycle Target Labeling protocol (Affymetrix; 701024 Rev. 3). In total, 4.0 μg of total RNA was reverse transcribed using Superscript ds-cDNA Synthesis Kit according to the manufacturer's description (Invitrogen). Subsequently, double-stranded cDNA was purified using GeneChip Sample Cleanup Module (Affymetrix) and served as a template in the in vitro transcription reaction using BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). Next, the RNA (cRNA) was purified using GeneChip Sample Cleanup Module. In total, 20 μg of biotin-labeled cRNA was fragmentized by metal-induced

TABLE I. Primer and Probe Sequences and Concentration Used for qPCR Analysis

	nM forward primer 5′–3′	nM reverse primer 5′–3′	nM Probe 5′-FAM–3′ TAMRA
APC	200 TTTTGGAACAGATACGCGCTTAC	200 AGAACACACACAGCAGGACAGATC	
DKK1	500 AATGTATCACACCAAAGGACAAGAAG	500 GATCTTGGACCAGAAGTGTCTAGCA	100 TCTGTTTGTCTCCGGTCATCAGACTGTGC
FZD1	100 CACTCCCTTCTCTCCCACCTTAGTT	100 CACCCCTCAAAAAATTAGACTTCAAAA	
FZD2	200 TTTCTGGGCGAGCGTGAT	200 AAACGCGTCTCCTCCTGTGA	
FZD6	600 GCGGTGGAAATGGAGCATT	600 ATGCTTTGCAGAGGAAAGTTTCA	100 CTCTCGCAAATCTGGAATGTTCACCAAACA
FZD8	200 AAGACAGGCCAGATCGCTAACT	200 CGCTCGTCCTGGCTGAA	
GAPDH	150 ATGGGGAAGGTGAAGGTCG	150 TAAAAGCAGCCCTGGTGACC	150 CGCCCAATACGACCAAATCCGTTGAC
JUP	100 TCAGCAGCAAGGGCATCA	100 CCCTGGCTGTTTGTGGACATC	
LRP6	200 GTGAGAGAAGAGAACGCGAGAAG	200 GTCCCGTCTGTTTTGCATAAAGC	
SFRP1	200 CGTCTGCATCGCCATGAC	200 ATGTTCAATGATGGCCTCAGATTT	

hydrolysis at a final concentration of 0.5 μg/μl for 35 min at 94°C. Fragmentation was checked on an Agilent 2100 Bioanalyzer confirming an average size of approximately 100 nt. In total, 15 μg fragmented biotinylated cRNA was hybridized to GeneChip Human Genome U133 Plus 2.0 oligonucleotide GeneChips (Affymetrix) according to the manufacturer's protocol (Affymetrix, 701025 Rev 5). Staining, washing and scanning procedures were carried out as described in the GeneChip Expression Analysis technical manual (Affymetrix). Data acquisition was performed using the GeneChip Scanner 3000.

In order to examine the quality of the different arrays, measured intensity values were analyzed using the GeneChip Operating Software (GCOS, Affymetrix). The percentage of present calls (about 40%), noise, background, and ratio of GAPDH 3′ to 5′ (<1.4) all indicated high quality of the samples and an overall comparability. Probe sets that were never present (according to Affymetrix MAS5.0 software) in any of the GeneChips were omitted from further analysis. Raw intensities of the remaining probe sets (30336) of each chip were log 2 transformed and normalized using quantile normalization. After normalization the data was back transformed to normal intensity values.

Gene Nomenclature

Gene names and gene symbols were used as provided by HUGO Gene Nomenclature Committee [Wain et al., 2002].

Statistics

Data were only presented if multiple independent experiments showed similar results. Experiments were performed at least in triplicate. Values are indicated as means ± SEM. Significance was calculated using the student's *t*-test.

RESULTS

Glucocorticoid Induced Human Osteoblast Differentiation

Wnt signaling was studied in human osteoblasts representing two functionally different stages of differentiation. To create these two different staged osteoblasts the human preosteoblast cell line SV-HFO was treated with or without 100 nM dexamethasone (DEX). DEX

treatment induced osteoblast differentiation and created cultures having high alkaline phosphates activity (ALPL) that initiated matrix mineralization after 2 weeks of culture. Cultures that were not treated with DEX showed low ALPL activity and showed no matrix mineralization [Eijken et al., 2006]. Throughout the remainder of the study non-DEX-treated and DEX-treated cultures will be referred as nondifferentiating and differentiating cultures, respectively.

Endogenous Wnt Signaling Is Reduced in Differentiating Osteoblasts

To explore the relationship between differentiation and Wnt signaling, endogenous Wnt signaling was measured in nondifferentiating and differentiating osteoblasts. β-catenin levels were compared between nondifferentiating and differentiating osteoblast cultures at days 5, 12, and 19 of culture. This showed that differentiated osteoblasts had clearly lower β-catenin levels compared to their nondifferentiating counterparts in both cellular fractions (Fig. 1A). Quantification of multiple independent Western blot assays showed that β-catenin was significant reduced in differentiating cultures in both the cytosolic and the nuclear fraction at day 12 and 19 (Fig. 1B). In addition, short-term DEX treatment (10^{-7} M, 48 h) did not change β-catenin levels (data not shown), demonstrating that the difference found in β-catenin levels is a consequence of the GC-induced differentiation program.

Reduced β-catenin levels suggest reduced endogenous Wnt signaling. A Wnt signaling luciferase reporter construct was used to measure endogenous Wnt signal in osteoblasts. Indeed, this showed that differentiating osteoblasts had reduced Wnt signaling compared to nondifferentiating cultures (Fig. 1C).

Regulation of Wnt Related Genes During Osteoblast Differentiation

Affymetrix GeneChips were used to identify the expression of a broad range of Wnt signaling related genes in human osteoblasts. A gene was selected as a Wnt signaling related gene on basis of data on http://www.stanford.edu/~rnusse/wntwindow.html. First, genes were identified as expressed in human osteoblast if they were detected by multiple independent GeneChip experiments. An overview of the presence of various Wnt signaling related genes in human

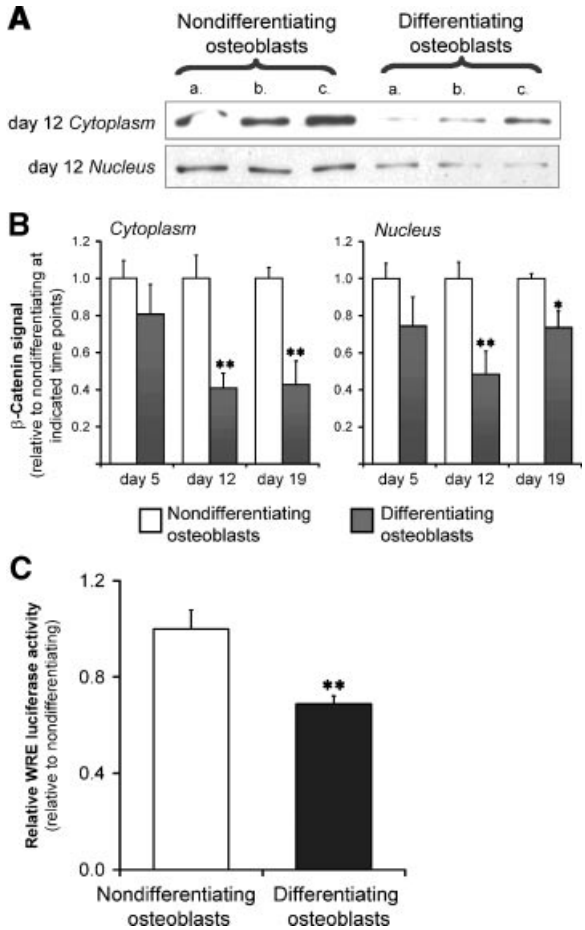

Fig. 1. Endogenous Wnt signaling in differentiating and nondifferentiating osteoblast cultures. (**A**) β-catenin levels of the cytosolic and nuclear fractions of both nondifferentiating and differentiating osteoblast cultures (day 12). Of both conditions 10 μg of protein was loaded in triplicate. (**B**) Quantification of β-catenin in the cytosolic and nuclear fraction in nondifferentiating and differentiating osteoblast cultures at day 5, 12, and 19 of culture. (**C**) Wnt signaling measured by a Wnt responsive luciferase reporter construct in nondifferentiating and differentiating cultures at day 7. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

osteoblasts is shown in Table II. Second, Wnt signaling genes were selected that showed differential expression between nondifferentiating and differentiating osteoblasts. Genes were selected if the expression was 1.6-fold-different between nondifferentiating and differentiating cultures at at least one of the time points analyzed. Since a 1.6-fold difference is a relative mild cut-off, these genes were only identified as potentially regulated. Quantitative real time PCR (qPCR) was used to confirm the differential mRNA expression of these genes.

TABLE II. Wnt Signaling Related Genes Expressed in Human Osteoblasts Identified by Affymetrix GeneChips

Gene symbol	Detected by GeneChip	Regulated[a]	qPCR
Wnt proteins			
WNT1	N	—	
WNT2	N	—	
WNT2B	Y	N	
WNT3	Y	N	
WNT3A[b]	—[b]	—[b]	
WNT4	N	—	
WNT5A	Y	N	
WNT5B	Y	—	
WNT6	Y	Y	
WNT7A	N	—	
WNT7B	N	—	
WNT8A	Y	N	
WNT8B	N	—	
WNT9A	Y	N	
WNT9B	N	—	
WNT10A	N	—	
WNT10B	N	—	
WNT11	N	—	
WNT16	Y	N	
Receptors			
FZD1	Y	Y	Fig. 2A
FZD2	Y	Y	Fig. 2B
FZD3	Y	N	
FZD4	Y	N	
FZD5	N	—	
FZD6	Y	Y	Fig. 2C
FZD7	Y	N	
FZD8	Y	Y	Fig. 2D
FZD9	N	—	
FZD10	N	—	
KREMEN1	Y	N	
KREMEN2	N	—	
LRP5	Y	N	
LRP6	Y	Y	Fig. 2E
Inhibitors			
DKK1	Y	Y	Fig. 2F
DKK2	Y	N	
DKK3	Y	N	
DKK4	N	—	
SFRP1	Y	Y	Fig. 2G
SFRP2	N	—	
FRZB	Y	N	
SFRP4	Y	N	
SFRP5	N	—	
WIF	N	—	
Nuclear regulators			
CTNNB1	Y	N	
JUP	Y	Y	Fig. 2I
LEF1	Y	N	
TCF7	Y	N	
TCF7L1	Y	N	
TCF7L2	Y	N	
Cytoplasmic regulators			
GSK3A	Y	N	
GSK3B	Y	N	
APC	Y	Y	Fig. 2H
APC2	Y	N	
DVL1	Y	N	
DVL2	Y	N	
DVL3	Y	N	
DVL1L1[b]	—[b]	—[b]	
AXIN1	N	—	
AXIN2	Y	N	
FRAT1	Y	N	
FRAT2	Y	N	
NKD1	N	—	
NKD2	N	—	
PORCN	N	—	

[a]According to GeneChip data using a cut-off value of 1.6-fold difference.
[b]No probeset was present on the GeneChip to detect these genes.

The following 10 Wnt signaling related genes were identified as potentially regulated and analyzed using qPCR (Fig. 2); wingless-type MMTV integration site family member 6 (WNT6), frizzled homolog 1/2/6 and 8 (FZD1/2/6/8), low density lipoprotein receptor-related protein 6 (LRP6), dickkopf homolog 1 (DKK1), secreted frizzled-related protein 1 (SFRP1), APC, and junction plakoglobin (JUP).

In total, five Wnt receptors were differentially expressed between nondifferentiating and differentiating cultures. FZD1, 2 and 8 were all suppressed in differentiating cultures, whereas FZD6 together with LRP6 were upregulated in differentiating cultures. The Wnt inhibitor DKK1 was also increased in differentiating cultures at all days of culture, whereas another inhibitor of Wnt signaling SFRP1 was strongly suppressed in differentiating cultures. APC, which also acts negative on Wnt signal increased during the final stages of GC-induced differentiation. The β-catenin homolog JUP [Kolligs et al., 2000] was decreased in differentiating cultures. Differential expression of WNT6 could not be confirmed using qPCR (data not shown).

Wnt Signaling Controls Osteoblast Differentiation and Function

Together these data show that during GC osteoblast differentiation, osteoblasts lower their endogenous Wnt signal and change the expression of several genes involved in Wnt signaling. To reveal the impact of enhanced Wnt signaling in both differentiating and nondifferentiating osteoblasts Wnt signaling was activated in both conditions. Wnt signaling was induced by culturing SV-HFO cells in the presence of recombinant Wnt3a or lithium chloride (LiCl), which stimulates Wnt signaling by inhibiting β-catenin degradation [Klein and Melton, 1996]. First, Wnt signaling was activated in nondifferentiating pre-osteoblast cultures. These cultures were continuously treated with LiCl (4 and 8 mM LiCl) or Wnt3a (2 and 10 ng/ml), and subsequently ALPL activity was measured at days 5, 12, and 19 of culture. This showed that activation of Wnt signaling significantly induced basal ALPL activity with the most prominent effect after 19 days of culture (Fig. 3A). Although ALPL activation was increased, this stimulation of ALPL in the absence of DEX was not sufficient to induced mineralization (data not shown).

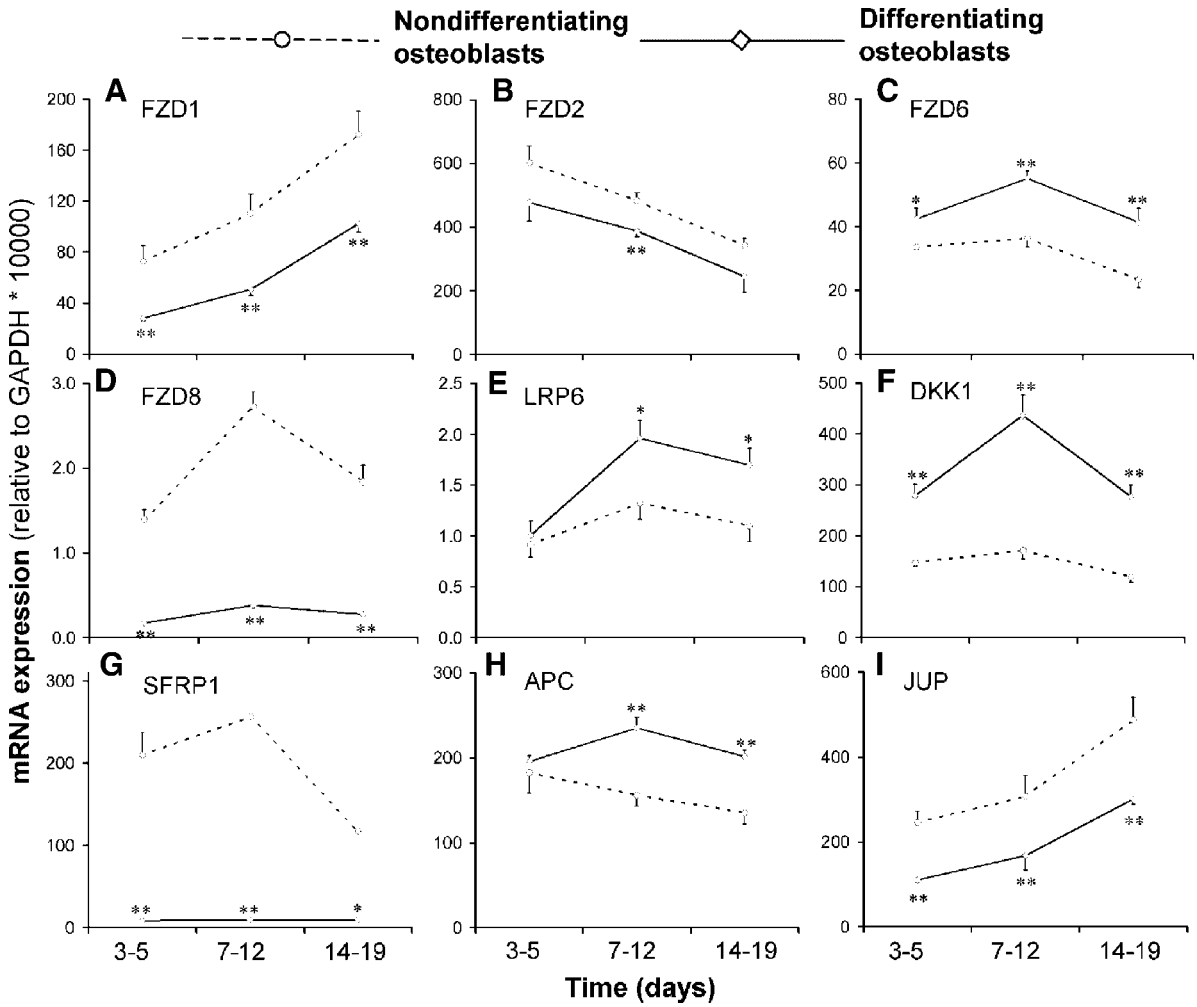

Fig. 2. Gene expression of various Wnt signaling genes during osteoblast differentiation. (**A**–**I**) qPCR data of nine Wnt signaling related genes that were differentially expressed between nondifferentiating and differentiating osteoblast cultures. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to non-differentiating osteoblasts.

Second, Wnt signaling was activated in differentiating osteoblast cultures. Remarkably, in these differentiating cultures an opposite effect was observed. LiCl and Wnt3a treatment slightly decreased ALPL activity (Fig. 3B). In order to confirm this discrepancy in ALPL regulation by Wnt signaling we measured mRNA levels in differentiating and nondifferentiating cultures after LiCl treatment. This also showed that LiCl stimulated ALPL mRNA in nondifferentiating osteoblasts, whereas LiCl decreased ALPL mRNA in differentiating osteoblasts after 12 days of culture (Fig. 3C).

In addition, we measured the effects of Wnt signaling on matrix mineralization. This showed that Wnt signaling in differentiating osteoblasts strongly inhibited matrix mineralization (day 19, Fig. 4).

In order to exclude any discrepancy in β-catenin activation between nondifferentiating and differentiating osteoblasts, β-catenin activation by LiCl treatment was measured in both differentiating conditions. This showed that 8 mM of LiCl clearly induced β-catenin levels in both differentiation conditions (data not shown). Moreover, in all experiments NaCl (4 and 8 mM) was used as a negative control for LiCl, which showed no effect on β-catenin levels, ALPL activity, and mineralization irrespective of the differentiation condition.

Wnt Signaling Controls Osteoblast Function in an Differentiation Dependent Manner Independent of Glucocorticoids

Differentiating osteoblasts were created upon continuous treatment with DEX. Using this

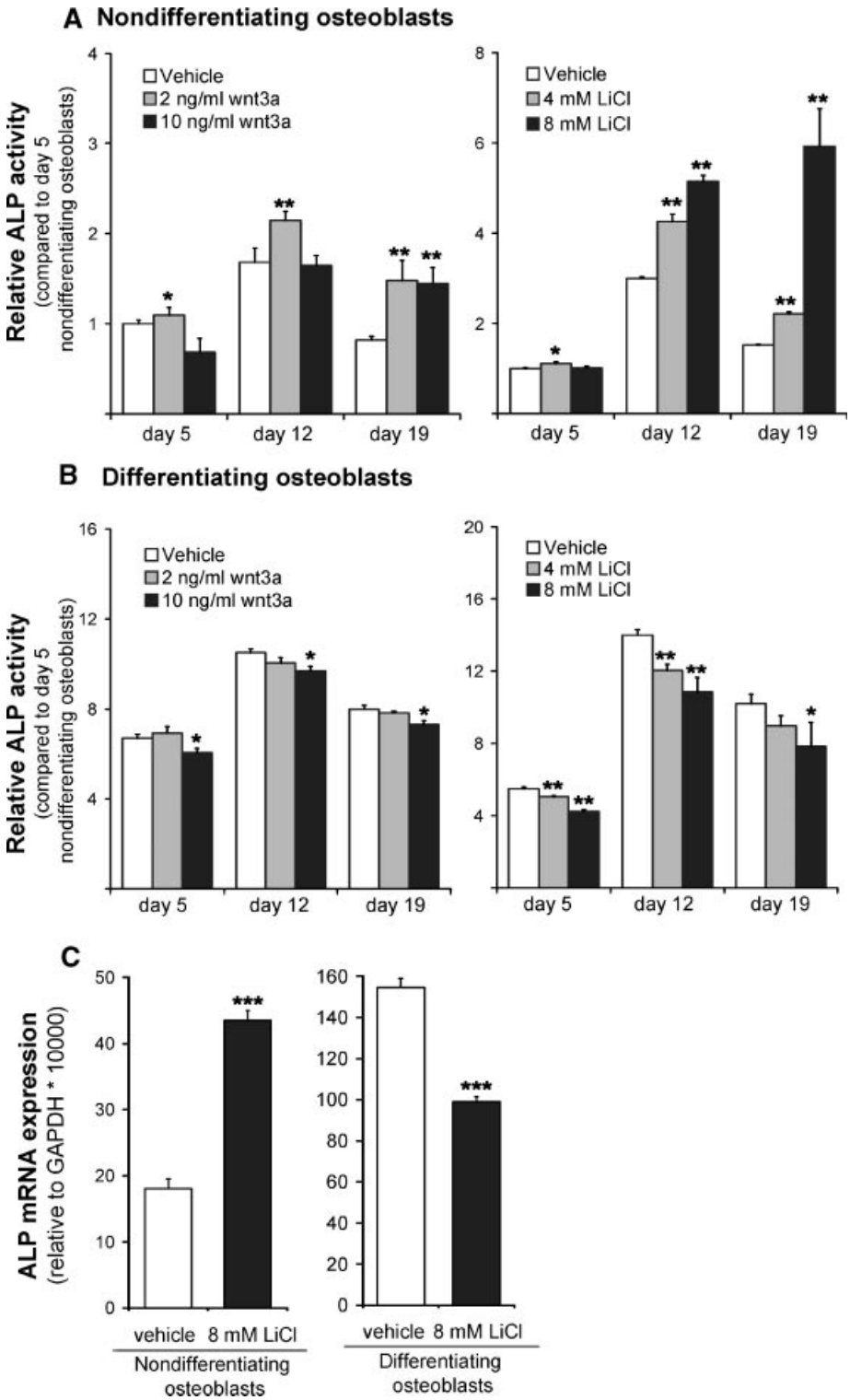

Fig. 3. Effect of Wnt signaling on osteoblast differentiation. (**A**) Nondifferentiating osteoblasts were continuously treated with LiCl or Wnt3a to induce Wnt signaling, subsequently ALPL activity was measured at day 5, 12, and 19. (**B**) Differentiating osteoblasts (DEX treated) were continuously treated with LiCl or Wnt3a to induce Wnt signaling, subsequently ALPL activity was measured at day 5, 12, and 19. (**C**) Quantification of ALPL mRNA expression at day 12 upon continuously LiCl treatment in nondifferentiating and differentiating cultures. Expression was quantified using qPCR. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle.

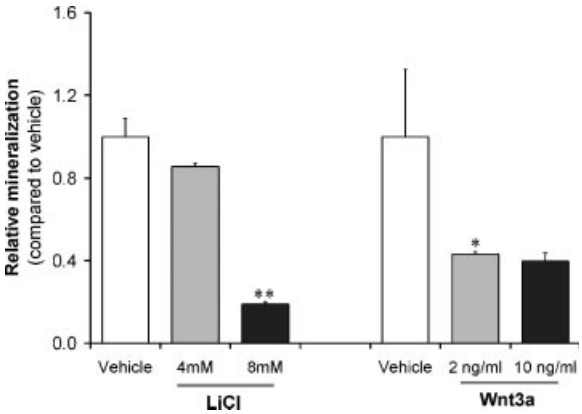

Fig. 4. Effect of Wnt signaling on matrix mineralization. Differentiating osteoblasts (DEX treated) were continuously treated with LiCl or Wnt3a to induce Wnt signaling, subsequently matrix mineralization was measured at day 19. Values are means ± SEM. $*P < 0.05$, $**P < 0.01$ compared to vehicle.

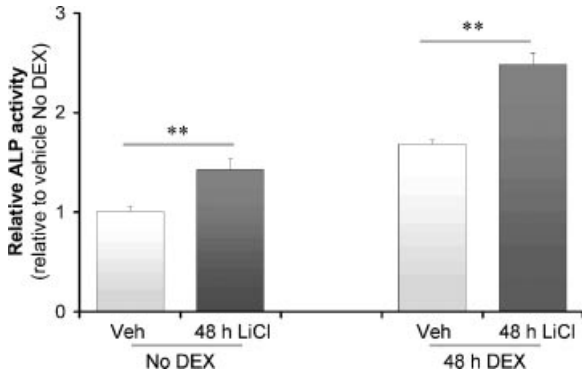

Fig. 5. Short-term LiCl and DEX treatment in nondifferentiating osteoblast cultures. Nondifferentiating osteoblasts were cultured until day 10. At day 10 the cultures were treated for 48 h with 8 mM LiCl and 100 nM DEX. In addition, LiCl treated cultures were co-treated with DEX. At day 12 of culture ALPL activity was measured. Values are means ± SEM. $**P < 0.01$.

method the observed differentiation dependent effects of Wnt signaling on ALPL activity might be caused by two different processes; (1) direct interaction with GC signaling or (2) as a consequence of the osteoblast differentiation program.

In order to study the role of DEX-treatment in more detail, experiments were performed in which LiCl treated cultures were co-incubated with DEX for 48 h (Fig. 5). This should reveal whether the presence of DEX results in a direct inhibitory effect of Wnt signaling as observed during DEX-induced differentiation. SV-HFO cultures were cultured for 10 days in non-differentiating conditions. Subsequently, these cultures were treated for 48 h with LiCl (8 mM), DEX (100 nM) or a combination of both. DEX and LiCl treatment independently stimulated ALPL activity. Importantly, Wnt signaling-stimulated ALPL activity was independent of the presence or absence of DEX (Fig. 5). This proves that the different effects of Wnt signaling as shown in Figures 3A,B are due to differences in osteoblast differentiation between the two conditions. In other words, Wnt signaling enhances ALPL activity when pre-osteoblasts are still undifferentiated, whereas Wnt signaling inhibits ALPL activity in differentiated osteoblasts.

These data led to the hypothesis that activation of Wnt signaling in the early stages of osteoblast differentiation will enhance differentiation later on. In order, to test this hypothesis we activated Wnt signaling in non-differentiating osteoblasts before they were induced to differentiate. This was achieved by pre-treating the cultures with LiCl (8 mM) for 5 days. After these 5 days the cultures were induced to differentiate by DEX treatment (Fig. 6 condition II). As a control, pre-osteoblast cultures were treated with LiCl (8 mM) only during DEX-induced differentiation from day 5 onwards (Fig. 6 condition III). At day 19, we quantified the level of differentiation by measuring ALPL activity. This revealed that pre-treatment with LiCl until day 5 enhanced ALPL activity at day 19 compared to the nonpre-treated controls (Fig. 6 condition I). In contrast, cultures that were treated with LiCl only during DEX-induced differentiation had slightly reduced ALPL activity (Fig. 6 condition III).

DISCUSSION

Conflicting data has been published whether Wnt signaling acts stimulatory or inhibitory on osteoblast differentiation. Using human pre-osteoblasts we showed that Wnt signaling acts either negative or positive on osteoblast function. Most importantly, these effects were dependent on the differentiation stage of the osteoblast. In human osteoblasts, differentiation can be initiated after exposure to GCs. During this GC-induced osteoblast differentiation process Wnt signaling strongly inhibited matrix mineralization. This negative effect of Wnt signaling on osteoblast differentiation and matrix mineralization was also demonstrated by other studies using osteoblasts from various origin [Boland et al., 2004; de Boer et al., 2004;

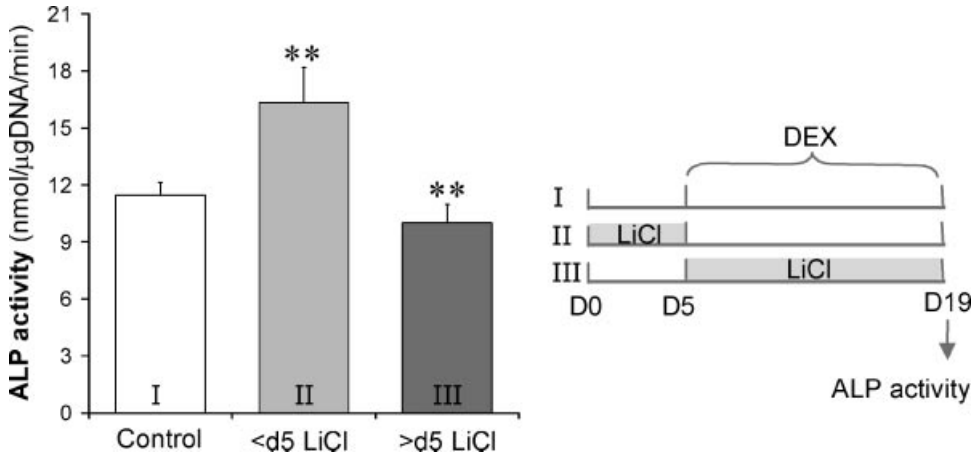

Fig. 6. Activation of Wnt signaling in early osteoblasts. Nondifferentiating cultures were pre-treated with 8 mM LiCl for 5 days (II). After these 5 days differentiation was initiated by DEX treatment. In contrast, osteoblast cultures were treated with 8 mM LiCl only during DEX-induced differentiation from day 5 onwards (III). ALPL activity was measured at day 19 of culture. Values are means ± SEM. $^{**}P < 0.01$ compared to control (I).

van der Horst et al., 2005]. In contrast, Wnt signaling acted positive on osteoblasts when, in the absence of GCs, they were not induced to differentiate. Smith and Frenkel (2005) demonstrated that GCs can directly influence Wnt signaling by inhibiting the transcriptional activity of LEF/TCF in differentiating murine osteoblasts. However, we showed that the effects of Wnt signaling in differentiating osteoblasts are not due to a direct effect of GCs on Wnt signaling but the result of an altered cellular response to Wnt signaling as a consequence of the GC-induced differentiation program.

We hypothesized that Wnt signaling stimulates early osteoblasts in their capacity to differentiate and subsequently to progress to mature matrix synthesizing osteoblasts. This is proven by our pre-treatment studies in which Wnt signaling was induced for 5 days in the early stage of culture and next induced to differentiate by GCs. The differentiation capacity of the Wnt signaling pre-treated cells was higher than the nonpre-treated cells. However, other additional signals, that is, GCs were still necessary to induce full differentiation. This positive property of Wnt signaling on osteoblast differentiation capacity is supported by LRP5 deficient mice that showed a reduced number of osteoblasts and by reports showing stimulation of osteoblast differentiation by Wnt signaling in vitro [Kato et al., 2002; Bain et al., 2003; Rawadi et al., 2003; Hu et al., 2005]. These early effects together with the observations of a controlling or limiting effect on the final differentiation and mineralization of osteoblasts shows a dual control mechanism of osteoblast differentiation by Wnt signaling. Such a dual control mechanism might act as an elegant feedback mechanism to control proper bone formation by controlling the number of osteoblast precursors that will become mature osteoblasts and at the same time controlling the activity of the mature osteoblasts. In case this control mechanism is strongly out of balance this results in impaired bone formation, as demonstrated by various mice models that are deficient for distinct Wnt signaling genes [Kato et al., 2002; Bodine et al., 2004; Kokubu et al., 2004; Glass et al., 2005; Holmen et al., 2005; Li et al., 2005].

The diverse function of Wnt signaling at different stages of osteoblast development makes the control of Wnt signaling during these different stages of great importance to ensure proper osteoblast function. Suppressed β-catenin levels in differentiating osteoblasts points to such a control mechanism. In mouse ES cells β-catenin levels are correlated with the differentiation capacity of these cells [Kielman et al., 2002]. Suppressed β-catenin levels in differentiating osteoblasts might help to facilitate osteoblast differentiation and matrix mineralization. One candidate to suppress Wnt signaling during human osteoblast differentiation is the Wnt signaling inhibitor DKK1. DKK1 was significantly upregulated during GC-induced differentiation and is a well-known inhibitor of

Wnt signaling. A functional role of DKK proteins in osteoblasts has been described for DKK2 in murine osteoblasts. DKK2 expression is strongly increased during murine osteoblast differentiation and silencing of DKK2 clearly reduced matrix mineralization [van der Horst et al., 2005]. Moreover, DKK2 deficient mice show reduced matrix mineralization, supporting the negative effects of Wnt signaling on mature osteoblasts in vivo [Li et al., 2005]. However, it is likely that several other regulators are involved as well in suppressing Wnt signaling in mature osteoblasts.

The fact that there are multiple Wnt proteins, Wnt inhibitors and Wnt receptors makes it yet difficult to unravel the role, regulation and function of Wnt signaling in osteoblasts. More information about which Wnt signaling genes are expressed and functional in osteoblasts is important for better understanding the role and complexity of Wnt signaling in bone. One challenge is to reveal the impact of endogenous expression of Wnt proteins, for example, which Wnt proteins activate early differentiation and which Wnt proteins inhibit later stages of differentiation and via which Frizzled or LRP receptors these actions take place.

Our data show that several Wnt signaling genes were differentially expressed between nondifferentiating and differentiating osteoblasts. A similar kind of study was performed by Boland et al. [2004] in human mesenchymal stem cells (MSCs) focusing on FZD on WNT genes. They found differential mRNA expression of FZD6, FZD7, WNT11, and WNT9A during osteoblast differentiation. We found differential expression of four Frizzled receptors, LRP6, and the Wnt signaling inhibitors DKK1 and SFRP1. SFRP1 was the most strongly regulated Wnt signaling gene found in our study. Interestingly, in mice this Wnt antagonist has been shown to be a negative regulator of trabecular bone formation [Bodine et al., 2004]. Although we cannot we explain the precise consequence of all the differentially expressed genes for osteoblast function we can correlate them with decreased β-catenin levels and decreased Wnt signal. Moreover, it might well be that due to the differentially expressed Wnt receptors and Wnt antagonists osteoblasts have a change sensitivity and specificity for the various Wnt proteins during differentiation.

It is still unclear, however, how the differentiation dependent effects of Wnt signaling are mediated. In this article we excluded a direct interaction of GCs. Moreover, LiCl experiments showed that the differentiation dependent effects were independent of any Wnt signaling receptor, Wnt protein, or Wnt antagonist. Therefore, it is tempting to speculate that differentiation-dependent epigenetic activation and silencing of Wnt signaling-regulated genes takes place. However, to answer this question detailed target gene promoter analyses should be performed in Wnt activated early and mature osteoblast cultures.

In conclusion, we showed that Wnt signaling stimulates the early steps of osteoblast differentiation and inhibits more mature osteoblasts. Importantly, we demonstrated these contradicting findings within the same osteoblast differentiation model, precluding possible cell dependent or species dependent effects. Furthermore, we found that differentiating osteoblasts have suppressed Wnt signal and changed expression of various Wnt signaling genes, which might help facilitate the differentiation and mineralization process in osteoblasts.

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