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Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells

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Abstract

Human mesenchymal stem cells (hMSCs) from the bone marrow represent a potential source of pluripotent cells for autologous bone tissue engineering. We previously discovered that over activation of the Wnt signal transduction pathway by either lithium or Wnt3A stimulates hMSC proliferation while retaining pluripotency. Release of Wnt3A or lithium from porous calcium phosphate scaffolds, which we use for bone tissue engineering, could provide a mitogenic stimulus to implanted hMSCs. To define the proper release profile, we first assessed the effect of Wnt over activation on osteogenic differentiation of hMSCs. Here, we report that both lithium and Wnt3A strongly inhibit dexamethasone-induced expression of the osteogenic marker alkaline phosphatase (ALP). Moreover, lithium partly inhibited mineralization of hMSCs whereas Wnt3A completely blocked it. Time course analysis during osteogenic differentiation revealed that 4 days of Wnt3A exposure before the onset of mineralization is sufficient to block mineralization completely. Gene expression profiling in Wnt3A and lithium-exposed hMSCs showed that many osteogenic and chondrogenic markers, normally expressed in proliferating hMSCs, are downregulated upon Wnt stimulation. We conclude that Wnt signaling inhibits dexamethasone-induced osteogenesis in hMSCs. In future studies, we will try to limit release of lithium or Wnt3A from calcium phosphate scaffolds to the proliferative phase of osteogenesis.

Keywords: Human mesenchymal stem cells; Wnt signaling; Osteogenesis; Tissue engineering; Micro-array

Introduction

Human mesenchymal stem cells (hMSCs) are pluripotent cells from the bone marrow, which can be expanded in vitro and differentiated into the osteogenic, chondrogenic, and adipogenic lineages [36]. MSCs were initially identified as the fibroblastic adherent fraction of bone marrow aspirates [6,16] and are also called colony forming units-fibroblasts (CFU-F), marrow stromal cells, bone marrow mesenchymal cells, or mesenchymal progenitor cells. In vitro osteogenic differentiation of hMSCs recapitulates many of the developmental steps during normal in vivo osteogenesis. For instance, in the presence of dexamethasone (dex) and β-glycerol phosphate, hMSCs express osteogenic markers such as bone-specific alkaline phosphatase (ALP) and they deposit an extracellular matrix, which becomes mineralized under appropriate culture conditions [5,8,22,32,37]. Because of their ready availability and well-established in vitro culturing protocols, hMSCs have been the source of cells in autologous bone and cartilage tissue engineering [2,7,18,33]. For bone tissue engineering, we and others have demonstrated ectopic bone formation by seeding hMSCs onto porous calcium phosphate scaffolds and subsequent subcutaneous implantation into immune-deficient mice [11,20].

To further improve bone tissue engineering protocols using hMSCs, we are interested in molecular cues that can stimulate hMSC proliferation and differentiation both in vitro and in vivo. One of the signal transduction pathways that has been associated with bone and cartilage formation, but for which relatively little is known with relation to

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hMSCs, is Wnt signaling. Wnts are a family of secreted glycoproteins that initiate a signal transduction cascade upon binding to the frizzled family of receptors and their low-density lipoprotein-related protein (LRP) co-receptors (see Refs. [4,34] and references therein). The Wnt signaling pathway acts via the bipartite transcription factor β-catenin/T cell factor (TCF), which binds to the promoter of Wnt responsive genes and thus initiates their transcription. In unstimulated cells, cytoplasmic β-catenin is phosphorylated by a complex of proteins containing axin, the adenomatous polyposis coli protein (APC) and glycogen synthase kinase 3 (GSK3), which earmarks β-catenin for degradation by the proteasome. Upon binding of Wnt to frizzled, the axin/APC/GSK3 complex is inactivated, resulting in the accumulation of cytoplasmic β-catenin, which will translocate to the nucleus and activate Wnt responsive genes. The Wnt signal transduction pathway has been implicated in bone formation: patients suffering from osteoporosis–pseudoglioma syndrome have an inactivating mutation in the Wnt co-receptor LRP5 [17], whereas an activating LRP5 mutation is associated with high bone mass syndrome [3,29]. Analysis of LRP5-deficient mice revealed a decreased number of osteoblasts suggesting that Wnt signaling stimulates bone formation at the level of osteoprogenitor proliferation [24]. We previously investigated the effect of Wnt over activation on hMSCs and discovered that Wnt3A and low concentrations (4 mM) of the Wnt mimic lithium stimulated hMSC proliferation [10]. Furthermore, cells that were extensively expanded in the presence of 4 mM lithium chloride could still differentiate into both the osteogenic and adipogenic lineages. We concluded that both lithium and recombinant Wnt3A might be used as mitogenic stimuli during hMSC expansion in vitro or during bone tissue engineering in vivo. In addition to its role in proliferation, some evidence suggests that Wnt signaling can also stimulate osteogenesis. Constitutive activation of Wnt signaling by retroviral transfection of a stabilized form of β-catenin stimulated ALP expression in C3H10T1/2 and ST2 osteogenic cells, whereas wild type β-catenin failed to do so [1,17]. This suggests that high levels of Wnt signaling can stimulate osteoblast differentiation. This is further supported by the fact that ALP is only induced at high concentrations of lithium in both C3H10T1/2 cells [1] and hMSCs [10]. We want to exploit the positive effect of Wnt signaling on both proliferation and differentiation of osteogenic cells by controlled release of lithium from porous calcium phosphate scaffolds and calcium phosphate coatings that we use for bone tissue engineering [11]. Because high lithium concentrations severely inhibit hMSC proliferation [10], we aim at releasing low, mitogenic levels of lithium. To assess the desired duration of lithium release, we set to study how low levels of Wnt signaling affect the osteogenic capacity of hMSCs. In this paper, we report that Wnt signaling inhibits ALP expression during dexamethasone-induced osteogenesis in hMSCs and blocks mineralization of osteogenic hMSCs. Gene expression profiling of Wnt-stimulated hMSCs further supports the conclusion that Wnt signaling inhibits rather than stimulates osteogenic differentiation of hMSCs.

Materials and methods

Cell culturing

Bone marrow aspirates (5–15 ml) were obtained from six donors that had given written informed consent. Donor information is summarized in Table 1. hMSCs were isolated and proliferated as described previously [11]. Briefly, aspirates were resuspended using a 20 G needle, plated at a density of 5×10^5 cells/cm^2, and cultured in hMSC proliferation medium, which contains minimal essential medium (α-MEM, Life Technologies), 10% fetal bovine serum (FBS, Life Technologies), 0.2 mM ascorbic acid (Asap, Life Technologies), L-glutamin (Life Technologies), 100 U/ml penicillin (Life Technologies), 10 μg/ml streptomycin (Life Technologies), and 1 ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C in a humid atmosphere with 5% CO_2. Medium was refreshed twice a week and cells were used for further subculturing or cryopreservation upon reaching near confluence. hMSC basic medium was composed of hMSC proliferative medium without bFGF, and hMSC osteogenic medium was composed of hMSC basic medium supplemented with 10^{-8} M dexamethasone (Sigma) and 0.01 M β-glycerol phosphate (Sigma). A lithium chloride (LiCl, CalBiochem) stock solution of 400 mM in α-MEM and a 400 mM sodium chloride (NaCl, CalBiochem) solution in phosphate-buffered saline solution (PBS) were used throughout the study. L cells and L-Wnt3A cells were obtained from the American Type Culture Collection (CRL-2647 and CRL-2648, respectively). Control- and Wnt3A-conditioned media were prepared essentially as described by the supplier. Briefly, confluent cells were split 1:10, grown in basic hMSC medium, and conditioned medium was collected after 4 and 7 days. Batches were mixed and filter sterilized.

Flow cytometry

The effect of Wnt signaling on ALP expression was studied by flow cytometry on cells seeded at 1000 cells/

Table 1
hMSC donor information

Donor	Age	Sex	Source	Passage	Frozen/fresh
1	25	m	iliac	2	frozen
2	66	f	iliac	4	frozen
3	100	f	iliac	2	frozen
4	72	f	iliac	3	frozen
5	72	m	iliac	2	frozen
6	77	f	iliac	2	frozen

cm^2 in 6-well plates and grown under various conditions for 4 to 5 days. Each experiment was performed in triplicate and included a negative control (cells grown in basic medium), a positive control (cells grown in osteogenic medium), and one or more experimental conditions (4 mM LiCl, different concentrations of control- and Wnt3A-conditioned medium). The effect of 4 mM LiCl on ALP levels was analyzed in cells of donors 2, 3, and 4, the effect of Wnt3A was studied in cells of donors 3, 4, and 5. After 4 or 5 days of culture, cells were trypsinized and incubated for 30 min in PBS/5% bovine serum albumin (BSA, Sigma) and incubated in PBS/1% BSA plus primary antibody (anti-ALP B4-78 [Developmental Studies Hybridoma Bank, University of Iowa, USA]) for 30 min, washed three times in PBS/1% BSA, and incubated with secondary antibody (goat anti-mouse IgG PE, DAKO) for 30 min. Mouse IgG2a (DAKO) was used as an isotype control. Cells were washed three times and suspended in 250 μl PBS/1% BSA plus 10 μl Viaprobe (PharMingen) for live/dead cell staining. Staining was analyzed on a FACS Calibur (Becton Dickinson Immunocytometry Systems) and ALP levels were analyzed on living cells only, with a minimum of 7500 gated events.

Mineralization

For mineralization, hMSCs were seeded in duplo at 1000 cells/cm^2 in T25 culture flasks and grown under various conditions. In every experiment, osteogenic medium was used as a positive control and basic medium as negative control. Cells were rinsed with PBS and fixed overnight in 4% paraformaldehyde (Merck) when extensive mineralization was observed in the positive control by phase contrast microscopy, which usually occurred between 21 and 28 days of culture. Next, the cells were rinsed with demineralized water and incubated in 5% silver nitrate (Sigma) until a distinct black stain was observed in the positive control. The mineralization experiment was performed with cells of donors 3, 5, and 6. Mineralization was quantified by image analysis of the total T25 area. Images were taken using a Sony Mavica model MUV-FD85 digital camera and mineralized area was expressed as percentage of total area using ImageJ imaging software (http://rsb.info.nih.gov/ij/).

Micro-array analysis

To assess the effect of Wnt pathway activation at the transcriptional level, hMSCs of donor 1 were grown for 4 days in basic medium, basic medium supplemented with 4 mM LiCl, basic medium supplemented with 10% control-conditioned medium, and basic medium supplemented with 10% Wnt3A-conditioned medium. RNA was isolated using a RNeasy midi kit (Qiagen) and 30 μg of total RNA was used for probe labeling according to the manufacturer's protocol (Affymetrix). Probe quality was verified using lab-on-chip technology (Agilent Technologies) and samples were hybridized to Human Genome Focus arrays according to manufacturer's protocol (Affymetrix). Data analysis was performed using Affymetrix GENECHIP 4.0 software.

Quantitative PCR

The effect of LiCl and Wnt3A on S100A4 mRNA levels was determined by seeding hMSCs of donors 3, 4, and 5 at 5000 cells/cm^2 in T25 culture flasks in 5 ml of basic medium, basic medium supplemented with 4 mM LiCl, basic medium supplemented with 10% control-conditioned medium, and basic medium supplemented with 10% Wnt3A-conditioned medium. Total RNA was isolated using an RNeasy mini kit (Qiagen) and on column DNase treated with 10U RNase-free DNase I (Gibco) at 37°C for 30 min. DNase was inactivated at 72°C for 15 min. The quality and quantity of RNA was analyzed by gel electrophoresis and spectrophotometry. Two micrograms of each DNase-treated RNA sample was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturers' protocol. One microliter of 100× diluted cDNA was used for 18s rRNA control amplification, and 1 μl of undiluted cDNA was used for S100A4 amplification. PCR reactions were performed and monitored on a Light Cycler real time PCR machine (Roche) using the SYBR Green I master mix (Eurogentec) with primers for 18s rRNA (18srRNA-F 5′cggctaccacatccaaggaa3′ and 18srRNA-R 5′gctggaattaccgcgggt3′) and for S100A4 (S100A4-F 5′agcttcttggggaaaaggac3′and S100A4-R 5′ccccaaccacatcaa-gagg3′). Data was analyzed using Light Cycler software version 3.5.3, using the fit point method by setting the noise band to one. Expression of S100A4 was calculated relative to 18s rRNA levels by comparative ΔC_T method [30]. Each sample was analyzed at least in duplicate and averages were used for further calculations.

IL-6 ELISA

The effect of LiCl and Wnt3A on IL-6 secretion by hMSCs was determined by seeding hMSCs of donors 3 and 4 in triplicate at 5000 cells/cm^2 in T25 culture flasks in 5 ml of basic medium, basic medium supplemented with 4 mM LiCl, basic medium supplemented with 10% control-conditioned medium, and basic medium supplemented with 10% Wnt3A-conditioned medium. Conditioned media were collected from the hMSC cultures after 4 days and IL-6 levels were determined using a human IL-6 ELISA kit (Pierce) according to the manufacturers' protocol.

Results

Wnt signaling inhibits ALP expression in differentiating hMSCs

In previous studies, we noticed that conditioned medium from mouse L cells, which we used as control-conditioned

medium (see Materials and Methods), induces the expression of the osteogenic marker alkaline phosphatase (ALP) in hMSCs (see Ref. [10] and compare ALP levels among the negative control, 10 c, and 50 c in Fig. 1). Interestingly, conditioned medium from Wnt3A transgenic L cells (Wnt3A-conditioned medium) did not show an increase in ALP expression. Therefore, we hypothesized that Wnt signaling could inhibit ALP expression in hMSCs. To test this hypothesis, we grew hMSCs in osteogenic medium, which contains dexamethasone, and analyzed ALP expression after 5 days. As expected, dexamethasone stimulated ALP expression in hMSCs (see Fig. 1). To induce Wnt signaling during osteogenic differentiation, we added lithium chloride, which inhibits the negative Wnt signaling regulator GSK3 and thus stimulates Wnt signaling [26]. As depicted in Fig. 1, 4 mM LiCl reduced ALP induction in osteogenic medium from 328 ± 30% to 206 ± 17% of ALP expression in control medium. Next, we stimulated Wnt signaling by supplementing osteogenic medium with 10% and 50% Wnt3A- or control-conditioned medium, respectively. The strongest effect was seen with 50% Wnt3A-conditioned medium, which inhibited ALP expression to levels comparable to those observed in unstimulated hMSCs (Fig. 1).

Wnt signaling inhibits mineralization of hMSCs

Inhibition of ALP by lithium and Wnt3A in hMSCs undergoing osteogenesis suggests that Wnt signaling might block dexamethasone-induced osteogenesis. We therefore investigated the effect of Wnt signaling on the endpoint of osteogenesis, that is, mineralization. In Fig. 2A, we show that hMSCs grown in the presence of dexamethasone and β-glycerol phosphate display extensive mineralization whereas cells grown in the absence of dexamethasone do not. We then studied how mineralization is affected upon stimulation of Wnt signaling. As shown in Fig. 2A and Table 2, 10% Wnt3A-conditioned medium, but not control-conditioned medium, completely blocked dexamethasone-induced mineralization of hMSCs in cells from all three donors studied. Similarly, mineralization in lithium-treated cultures of donors 3 and 5 was strongly reduced compared to cells grown in osteogenic medium although some mineralization could still be observed in cells from donor 5. Surprisingly, lithium had a stimulatory effect on mineralization in cells of donor 6 (data not shown). As a control, mineralization in hMSC grown in osteogenic medium supplemented with 4 mM NaCl was not noticeably affected in cells from any donor studied.

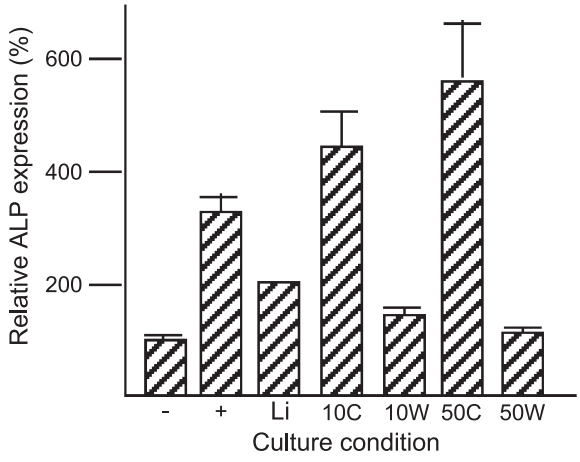

Fig. 1. Lithium chloride and Wnt3A inhibit dex-induced ALP expression in hMSCs. ALP levels determined by flow cytometry in hMSCs of donor 3, grown in different culture conditions: −, basic medium; +, osteogenic medium; Li, osteogenic medium supplemented with 4 mM LiCl; 10 c, osteogenic medium supplemented with 10% control-conditioned medium; 10 w, osteogenic medium supplemented with 10% Wnt3A-conditioned medium; 50 c, osteogenic medium supplemented with 50% control-conditioned medium; 50 w, osteogenic medium supplemented with 50% Wnt3A-conditioned medium. ALP is expressed relative to the level in the negative control. Error bars represent standard deviation of triplicate experiments. Data was analyzed by Student's *t* test and ALP levels differed significantly between + and Li, 10 c and 10 w, and 50 c and 50 w, respectively ($P < 0.01$).

To delineate the developmental window in which Wnt signaling can block hMSC mineralization, we allowed the cells to grow in osteogenic medium. Examination of the cultures showed that the first signs of mineralization appeared after 12 days of culture, with small areas of mineral deposition (data not shown). After 17 days, the culture was fully mineralized with large areas of mineralization covering the cell sheet (see Fig. 2B). In parallel, we allowed the cells to grow in osteogenic medium for 0, 4, 8, and 12 days before adding 10% Wnt3A- or control-conditioned medium and determined the effect on mineralization after 17 days. As expected, Wnt3A exposure from day 0 onwards completely blocked mineralization. Similarly, mineralization was inhibited when Wnt3A was added at days 4 and 8. In contrast, when Wnt3A was added at day 12, we observed a fully mineralized cell sheet at day 17. Control-conditioned medium did not have an effect on mineralization (data not shown).

To examine whether Wnt3A affects extracellular matrix deposition, hMSCs were grown on titanium discs for 21 days and analyzed for matrix formation by scanning electron microscopy. As expected, both hMSCs grown in basic medium and hMSCs grown in osteogenic medium deposited an extensive extracellular matrix (Figs. 2C, D). Although we did not observe gross abnormalities in morphology of the collagen fibers deposited by cells grown in osteogenic medium supplemented with Wnt3A-conditioned medium, the matrix did appear slightly disorganized (Fig. 2E).

Micro-array analysis of hMSCs treated with lithium and Wnt3A

We previously discovered that Wnt signaling stimulates proliferation of hMSCs but does not noticeably affect hMSC pluripotency [10]. To acquire a global view of the cellular

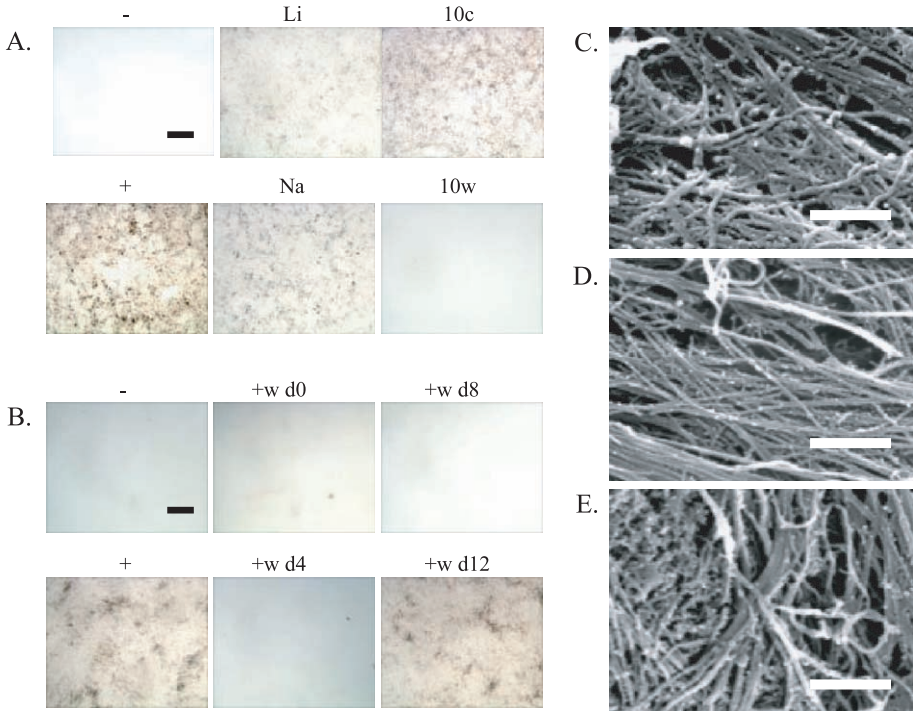

Fig. 2. Lithium chloride and Wnt3A inhibit dex-induced mineralization of hMSCs. (A) Mineralization in hMSCs of donor 5 grown under various conditions for 27 days: −, basic medium; +, osteogenic medium; Li, osteogenic medium supplemented with 4 mM LiCl; Na, osteogenic medium supplemented with 4 mM NaCl; 10 c, osteogenic medium supplemented with 10% control-conditioned medium; 10 w, osteogenic medium supplemented with 10% Wnt3A-conditioned medium. Mineralization was visualized by von Kossa staining. The scale bar is 2 mm. (B) Mineralization in hMSCs of donor 5 grown for 17 days in basic medium (−), osteogenic medium (+), or in osteogenic medium supplemented with 10% Wnt3A-conditioned medium at different time points after the start of the experiment (+w d0, Wnt3A added at day 0; etc.). Mineralization was visualized by von Kossa staining. The scale bar is 2 mm. (C, D, E) Scanning electron microscopical images of hMSCs of donor 5 grown for 21 days in basic medium (C), osteogenic medium (D), or osteogenic medium supplemented with 10% Wnt3A-conditioned medium (E). Note the fibers that indicate extensive matrix deposition. The scale bar is 1 μm.

processes affected by Wnt activation, we decided to study gene expression profiles of hMSCs grown for 4 days in basic medium, basic medium supplemented with 4 mM lithium chloride, with 10% control-conditioned medium, and with 10% Wnt3A-conditioned medium. RNA was isolated and gene expression was analyzed on Affymetrix Human Genome Focus arrays. Gene expression was compared between cells grown in control- to Wnt3A-conditioned medium, and in basic versus lithium chloride-supplemented medium. We then focused on the genes similarly regulated for at least 1.3-fold by both lithium and Wnt3A (summarized in Table 3). Out of the approximately 9000 transcripts on the array, only 37 genes matched these criteria and only 4 genes were regulated more than twofold in both conditions analyzed (see Table 3). Of the 37 differentially regulated genes, 32 were downregulated and 5 genes were upregulated when compared to nonsupplemented medium. IL-6 was one of the most strongly regulated genes on the array, and to validate our micro-array data, we analyzed IL-6 secretion by hMSCs of two different donors, grown in basic medium, basic medium supplemented with lithium, or basic medium supplemented with either control- or Wnt3A-conditioned medium for 4 days. As shown in Fig. 3A, both Wnt3A and lithium significantly inhibit IL-6 secretion by hMSCs. To further confirm our micro-array data, we isolated RNA from the cells described in the previous experiment and analyzed S100A4 expression using quantitative PCR. As expected, S100A4 RNA levels were upregulated by both Wnt3A- and lithium-exposed cells (Fig. 3B).

Lithium- and Wnt3A-treated hMSCs display a distinct increase in cell proliferation, and accordingly, four genes

Table 2
Mineralization in osteogenic hMSCs[a]

	Donor 3[b]	Donor 5	Donor 3	Donor 5	Donor 6	
−dex 1	0	0	0	0	2	−dex 1
−dex 2	0	0	0	0	1	−dex 2
dex 1	45	13	21	12	16	dex + control 1
dex 2	41	20	16	18	17	dex + control 2
dex + li1	0	4	3	0	2	dex + wnt3A 1
dex + li2	0	3	1	0	6	dex + wnt3A 2

[a] Mineralisation in osteogenic cultures, indicated as percentage mineralized area of total area. Duplicate experiments are shown. −dex, basic medium; dex, osteogenic medium; dex + li, osteogenic medium plus 4 mM lithium; dex + control, osteogenic medium plus 10% control-conditioned medium; dex + wnt3A, osteogenic medium plus 10% wnt3A-conditioned medium.

[b] Every column represents an independent experiment, for donor numbers, refer to Table 1.

Table 3
Genes regulated by both Wnt3A and lithium chloride in hMSCs

Osteogenesis
ENPP1 (−1.5/−1.3)[a]
ID2 (−1.7/−1.4)
Transglutaminase (−1.9/−1.4)
VitD3 upregulated (−1.5/−1.4)
Leptin receptor (−1.6/−2.1)
S100A4 (1.9/1.3)
Proenkephalin (−1.3/−3.2)

Hematopoiesis
SDF-1 (−1.9/−1.7)
SLIT-2 (1.3/1.4)
EPAS-1 (−1.3/−1.3)
IL-6 (−2.3/−2.5)

Chondrogenesis
GDF-5 (−1.6/−1.7)
Collagen X (−1.6/−1.9)
Sox-4 (−1.7/−1.3)

Proliferation
meox-2 (−2.3/−1.6)
cpr8 (−1.4/−1.5)
btg-1 (−1.4/−1.3)
CREG (−1.4/−1.3)

Other mesenchymal genes
EFEMP-1 (−2.1/−2.5)
Dystrophin (−1.9/−2.5)
Laminin α4 (−1.4/−1.3)
SM22 (1.4/1.4)
dsc54 (−1.5/−1.5)

Miscellaneous
PTX3 (−2.3/−3.0)
Cytochrome *P*450 (−1.5/−1.5)
Phosphatidic acid phosphatase 2A (−1.9/−1.4)
HLA-DMA (−1.4/−1.5)
MHC gamma chain (−1.4/−1.5)
Keratin 14 (1.6/1.4)
TEM7 (2.3/2.1)
Cited-2/MRG1 (−1.3/−1.6)
Dihydropyrimidine dehydrogenase (−1.5/−1.3)
Myomegalin (−1.9/−1.3)
PKC μ (−1.3/−1.3)
Desmoplakin (−1.5/−1.4)
Histamin *N*-methyltransferase (−1.4/−1.3)
lmcd-1 (−1.5/−1.6)

[a] Fold regulation in control- versus Wnt3A-treated cells and non- versus lithium-treated cells is indicated between parentheses (left and right, respectively).

involved in proliferation were differentially regulated. Mesenchymal-specific homeobox gene 2 (MEOX-2), btg-1 [38] and CREG [44] are negative regulators of proliferation [19,41] and were downregulated. However, a presumed positive regulator of the cell cycle [13], cell cycle progression 8 protein, was also downregulated. Notably, undifferentiated hMSCs express many osteogenesis- or chondrogenesis-specific genes (e.g., ID-2 [21,27,31], transglutaminase [23], collagen type X and Sox-4 [39], most of which are downregulated by both Wnt3A and lithium. This suggests that Wnt signaling inhibits the differentiated phenotype of hMSCs, which is further supported by the fact that the negative

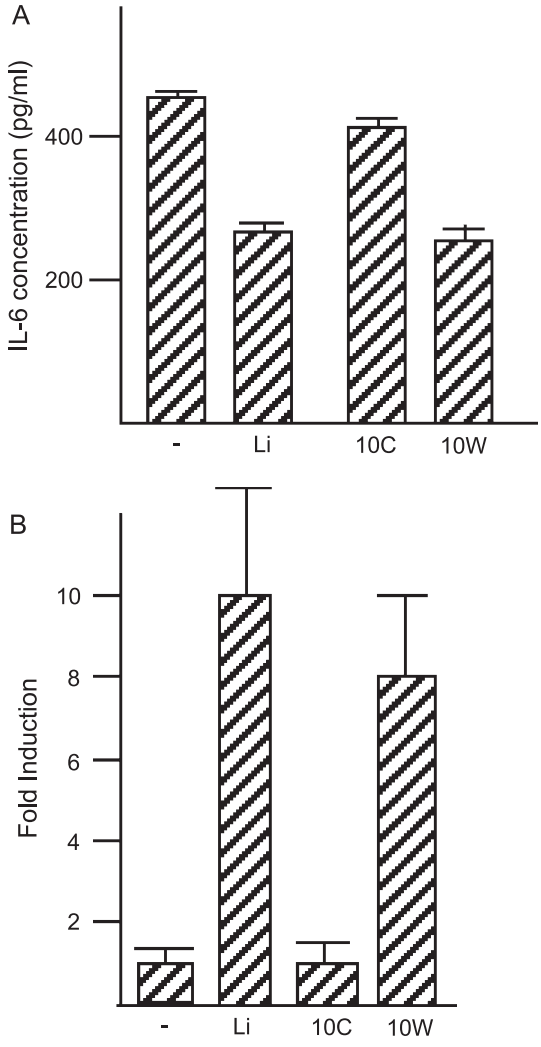

Fig. 3. Wnt signaling regulates IL-6 and S100A4 expression in hMSCs. (A) IL-6 concentration in medium conditioned for 4 days by hMSCs of donor 4. hMSCs were grown in basic medium (−), basic medium supplemented with 4 mM LiCl (Li), basic medium supplemented with 10% control-conditioned medium (10 c), or basic medium supplemented with 10% Wnt3A-conditioned medium (10 w). IL-6 levels were determined by ELISA. Each experiment was performed in triplicate and error bars indicate the standard deviation. Data was analyzed using Student's *t* test and a statistically significant difference was found between − and Li, and 10 c and 10 w, respectively ($P < 0.01$). (B) S100A4 mRNA expression in hMSCs determined by quantitative PCR. Cells were grown in basic medium (−), and basic medium supplemented with either 4 mM lithium (Li), 10% control-conditioned medium (control), or 10% Wnt3A-conditioned medium (Wnt). Values represent the average S100A4 expression level in cells from three different donors. The bar indicates the standard error of mean. S100A4 level in lithium-treated cells is expressed relative to the level in cells grown in basic medium; similarly S100A4 levels in Wnt3A-treated cells are normalized to control-treated cells. A statistical significant difference was found between − and Li and between 10 c and 10 w using Student's *t* test ($P < 0.05$).

regulator of mineralization S100A4 is upregulated by both Wnt3A and lithium.

Discussion

Wnt signaling inhibits osteogenic differentiation of hMSCs

Dexamethasone-induced osteogenesis in hMSCs is characterized by an increase in ALP expression after 4 days, followed by matrix deposition, matrix maturation, and mineralization at later stages [8,22,32]. Bone-specific ALP is a member of a family of three ALP proteins required for phosphate homeostasis [14,35], and inhibition is expected to affect mineralization. Although Wnt overactivation did not overtly affect matrix deposition, ALP expression was inhibited. Moreover, micro-array analysis revealed that Wnt signaling downregulates transglutaminase, which cross-links proteins in the extracellular matrix. Downregulation of these proteins suggests that Wnt signaling inhibits expression of genes directly involved in the formation of a mineralized bone matrix. Interestingly, micro-array analysis further revealed that lithium and Wnt3A downregulate ID2 gene expression. ID2 is a direct target gene of the BMP signal transduction cascade [21,27,31], which suggests that Wnt signaling can also interfere with early events of osteogenic differentiation. Because we performed the micro-array experiment after 4 days of exposure to Wnt3A or lithium, the effect of Wnt signaling on ID2 expression might be secondary. Future research has to delineate the direct targets of Wnt signaling and the subsequent events that lead to inhibition of osteogenesis.

Wnts are secreted proteins that act as morphogens; that is, a protein can elicit differential responses in the same cell type depending on its concentration [4]. In this study, we show that low levels of lithium and Wnt3A inhibit dex-induced osteogenesis in hMSCs, whereas previous studies have shown that high levels of Wnt signaling can induce ALP activity. For instance, we have shown that 40 mM, but not 4 mM lithium, induces ALP expression in hMSCs [10], and Bain et al. [1] showed similar results in C3H10T1/2 cells. Moreover, Gong et al. [17] show that a stabilized, and therefore more active form of the Wnt effector molecule β-catenin, can induce ALP in C3H10T1/2 cells, whereas wild type β-catenin failed to do so. Based on these data, we propose that Wnt signaling has a dose-dependent effect on osteogenic differentiation. At low levels, Wnt signaling blocks differentiation and stimulates osteoprogenitor proliferation, whereas at high levels, Wnt signaling stimulates osteogenic differentiation. To further test dose-dependent effects of Wnt signaling on osteogenesis, experiments have to be designed in which the level of Wnt stimulation is carefully controlled, for instance by using defined concentrations of recombinant Wnt3A or in mouse models with defined molecular defects in the Wnt signaling pathway [25].

Wnt downregulates differentiation-specific markers in proliferating hMSCs

Our micro-array experiments demonstrate that proliferating, undifferentiated hMSCs express many cell lineage-specific transcripts. Previously, Tremain et al. [43] elegantly showed expression of multiple transcripts specific for bone, cartilage, and hematopoiesis supporting stromal cells as well as nonmesenchymal cell types such as neurons and endothelial cells in a single undifferentiated hMSC colony. In our analysis of genes that were regulated by both lithium and Wnt3A, we observed downregulation of many genes that are typical for differentiated cells such as transglutaminase (osteogenesis), collagen type X (chondrogenesis), and stromal cell-derived factor (SDF-1, hematopoiesis supporting stromal cells [28]). In all cases, the lineage-specific genes were downregulated. Interestingly, genes that negatively regulated either osteogenesis (S100A4 [12]) or the hematopoiesis supporting function (SLIT2 [15]) were upregulated. S100A4 is an intracellular calcium-binding protein expressed by osteoblastic cells. S100A4 is expressed during early stages of MC3T3 osteogenesis and inhibits mineralization. S100A4 is upregulated by Wnt3A and lithium in our experiments, which suggests that Wnt signaling brings hMSCs into a more primitive, less differentiated state. Similarly, SDF-1 is a cytokine involved in chemotaxis of hematopoietic cells and a marker for the hematopoiesis supporting function of bone marrow stromal cells [28]. Both Wnt3A and lithium downregulate SDF-1, whereas SLIT-2, a secreted antagonist of SDF-1 [15], is upregulated. This demonstrates that exposing undifferentiated hMSCs to Wnt signaling results in inhibition of the hematopoiesis supporting function of hMSCs as described before [45]. An exception to the rule that Wnt signaling downregulates hMSC differentiation is the upregulation of SM22, a smooth muscle specific gene [42], by both lithium and Wnt3A (Table 3), suggesting that Wnt signaling induces smooth muscle cell differentiation in hMSCs. This is consistent with SM22 upregulation by Wnt signaling as reported in C3H10T1/2 cells [1] and the role that Wnt signaling plays in smooth muscle cell development [9,40]. Furthermore, the profile of inhibition and stimulation of mesenchymal differentiation that emerges from our study is strikingly similar to the differentiation profile of embryonic stem cells in which Wnt signaling is constitutively active [25]. We previously studied mouse embryonic stem cells in which Wnt signaling was constitutively activated by targeted inactivation of the APC gene. Whereas wild-type ES cells form tissue of all different germ layers when injected subcutaneously into immune-deficient mice, APC mutant ES cells fail to differentiate into the osteogenic and chondrogenic lineage whereas abundant smooth muscle differentiation was observed [25].

In conclusion, Wnt signaling inhibits dexamethasone-induced osteogenesis in hMSCs, and in future bone tissue engineering studies, we will try to limit release of lithium or

Wnt3A from calcium phosphate scaffolds to the proliferative phase of osteogenesis.

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