Opposite Spectrum of Activity of Canonical Wnt Signaling in the Osteogenic Context of Undifferentiated and Differentiated Mesenchymal Cells: Implications for Tissue Engineering

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To delineate the competence window in which canonical wingless (Wnt)-signaling can either inhibit or promote osteogenic differentiation, we have analyzed cells with different status, specifically undifferentiated mesenchymal cells, such as adipose-derived stem cells and embryonic calvarial mesenchymal cells, and differentiated mesenchymal cells such as juvenile immature calvarial osteoblasts and adult calvarial osteoblasts. Our analysis indicated that undifferentiated mesenchymal cells and juvenile calvarial osteoblasts are endowed with higher levels of endogenous canonical Wnt signaling compared to fully differentiated adult calvarial osteoblasts, and that different levels of activation inversely correlated with expression levels of several Wnt antagonists. We have observed that activation of canonical Wnt signaling may elicit opposite biological activity in the context of osteogenic differentiation depending on the status of cell, the threshold levels of its activation, and Wnt ligands concentration. The results presented in this study indicate that treatment with Wnt3 and/or expression of constitutively activated β -catenin inhibits osteogenic differentiation of undifferentiated mesenchymal cells, whereas expression of dominant negative transcription factor 4 (Tcf4) and/or secreted frizzled related protein 1 treatment enhances their osteogenic differentiation. Wnt3a treatment also inhibits osteogenesis in juvenile calvarial osteoblasts in a dose-dependent fashion. Conversely, Wnt3a treatment strongly induces osteogenesis in mature calvarial osteoblasts in a dose-dependent manner. Importantly, *in vitro* data correlated with *in vivo* results showing that Wnt3a treatment of calvarial defects, created in juvenile mice, promotes calvarial healing and bone regeneration only at low doses, whereas high doses of Wnt3a impairs tissue regeneration. In contrast, high doses of Wnt3a enhance bony tissue regeneration and calvarial healing in adult mice. Therefore, the knowledge of both endogenous activity of canonical Wnt signaling and appropriate concentrations of Wnt3a treatment may lead to significant improvement for bony tissue engineering, as well as for the efficient implement of adipose-derived stem cells in bone regeneration. Indeed, this study has important potential implications for tissue engineering, specifically for repair of juvenile bone defects.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are an important source for tissue repair and therapy in regenerative medicine. The prospective use of stem cells for regenerative medicine has opened new fields of research. Multipotency is the first requirement for this therapeutic potential. Several studies have demonstrated that this feature is not unique to embryonic stem cells.¹⁻⁴ Multipotent adult stem cells seem to be almost comparable to embryonic stem cells with respect to their ability to differentiate into various tissues *in vitro* and

in vivo, a function known as “stem cell plasticity.” In recent years significant attention has been given to MSCs from fat tissue as adipose-derived stem cells (ASCs), a compartment of multipotent cells capable of differentiating into several mesenchymal lineages: osteoblasts, chondrocytes, adipocytes, and myocytes.^{1,2,4-6} Indeed, the ability of ASCs to differentiate into various tissues *in vitro* and *in vivo*⁷⁻⁹ makes them an appealing cell source for tissue engineering.

Osteoblast, a bone-specific mesenchymal cell type, is defined by its three functions. It is responsible for bone formation, that is, the synthesis and secretion of most proteins

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of the bone extracellular matrix (ECM), and also expresses genes that are necessary and sufficient to induce mineralization of this ECM.^{10–12} The major event that triggers osteogenesis and bone remodeling is the transition of MSCs into differentiating osteoblast cells and monocyte/macrophage precursors into differentiating osteoclasts.^{12–16} Imbalance in differentiation and function of these two cell types will result in skeletal diseases such as osteoporosis, Paget's disease, rheumatoid arthritis, osteopetrosis, periodontal disease, and bone cancer metastases.^{17–19} Osteoblast and osteoclast commitment and differentiation are controlled by complex activities involving signal transduction and transcriptional regulation of gene expression.^{11–13,20}

Significant progress has been made over the past decade in our understanding of the molecular framework that controls osteogenic differentiation. A large number of morphogens, signaling molecules, and transcriptional regulators have been implicated in regulating bone development. Among them is the wingless (Wnt)/ β -catenin signaling, known also as canonical Wnt signaling.^{18,21,22}

Wnts are secreted proteins that are essential for a wide array of developmental and physiological processes. In the canonical pathway, Wnts signal across the plasma membrane by interacting with serpentine receptors of the Frizzled family and coreceptors, members of the low-density-lipoprotein-related protein (LRP) family, leading to inactivation of axin-glycogen synthase kinase (GSK)3 β complex, which otherwise phosphorylates and directs degradation of β -catenin. Stabilized β -catenin translocates into the nucleus and forms a complex with T cell factor transcription factor to activate Wnt target genes.^{18,21} Growing lines of evidence indicate that Wnt signaling plays a critical role in stem/progenitor self-renewal in adult tissues,²³ in which these cells serve as reservoirs for tissue renewal in response to trauma, disease, and aging. Moreover, several lines of evidence have demonstrated the importance of canonical Wnt signaling in promoting osteogenesis *in vitro* and *in vivo*.^{24–26} The appropriate manipulation of Wnt ligand expression might lead to significant improvement in the efficiency of tissue engineering and enhance the therapeutic value of these stem cells for the restoration of bone defects.

Emerging data suggest that Wnt signaling plays an essential role in normal bone biology and deregulation of this process contributes to bone disease.^{18,22,26–35}

This study demonstrated that Wnt/ β -catenin signaling represents a mechanism for either inhibiting or inducing osteogenesis, depending on the differentiation status of mesenchymal cells, as well as, threshold levels of Wnt ligands. We provide *in vitro* and *in vivo* evidence suggesting that strong activation of canonical Wnt3a signaling as well as treatment with high concentrations of Wnt3a ligand are not beneficial for engineering bony tissue from a mesenchymal cell and/or immature osteoblasts.

Materials and Methods

Cell primary cultures and osteogenic differentiation

Mouse ASCs (mASCs), embryonic-stage day 16 calvarial mesenchymal cells (E16), postnatal day 1 frontal (FpN1) and parietal (PpN1) bone-derived osteoblast, as well as postnatal day 60 frontal (FpN60) and parietal (PpN60) bone-derived osteoblast primary cultures were prepared and grown as previously described.^{36,37} For differentiation con-

ditions, mASCs were cultured in the osteogenic differentiation medium prepared with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin plus 5 mM-glycerophosphate, 100 mg/mL ascorbic acid, and 0.1 M all-trans-retinoic acid (Sigma-Aldrich). For all other cells the osteogenic differentiation medium was α -minimum essential medium containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin plus 5 mM-glycerophosphate and 100 μ g/mL ascorbic acid. Osteogenic differentiation in the presence of Wnt3a was performed by adding 25, 100, and 200 ng/mL of recombinant mouse Wnt3a protein (R&D Systems) to the osteogenic medium. The medium with or without Wnt3a was changed every 3 days. Osteogenic differentiation was carried out until day 21. Only first- and second-passage cells were used for all experiments.

Alkaline phosphatase activity and mineralization assay

Alkaline phosphatase enzymatic activity was performed using Sigma-Aldrich alkaline phosphatase assay (104-LS) kit as previously described.⁴ Briefly, cells harvested at day 10 of differentiation were washed twice with phosphate-buffered saline (PBS), incubated in 0.01% sodium dodecyl sulfate in PBS, and then sonicated for 30 s on ice three times. Alkaline phosphatase enzymatic activity in cell lysates was determined by measuring levels of *p*-nitrophenol formed during hydrolysis of *p*-nitrophenylphosphate substrate. The reaction was stopped by adding 1 mL of 0.05 N NaOH and the optical density was read at 420 nm. Concentration of alkaline phosphatase was determined by comparison to a standardized *p*-nitrophenol curve. All values were normalized against protein concentrations using a BCA protein assay kit (Pierce Biotechnology). Samples were run in triplicate.

Alizarin red staining was performed as previously described.⁵ All values were normalized against protein concentration obtained from triplicate wells.

Transfections and infections

Subconfluent Ψ -2 packaging cells were transfected with 10 μ g of a retroviral expression vector encoding mouse dominant negative transcription factor 4 (Tcf4), S33Y β -catenin,³⁸ or with a Neo empty control vector as previously described.^{36,37} Stable transfected clones were isolated after 2 weeks of selection with 1 mg/mL G418 (Life Technologies) and retroviruses were generated after expansion. Retroviruses were used to infect ASCs and frontal and parietal osteoblasts for stable expression of DnTcf4, catenin S33Y, or Neo control. Infections were performed as previously described.⁵

RNA isolation and reverse transcription-polymerase chain reaction analysis

Procedures for tissue harvesting, RNA isolation, and reverse transcription were previously described.⁵ Primer sequence and quantitative reverse transcription-polymerase chain reaction (QRT-PCR) conditions for the glyceraldehyde 3-phosphate dehydrogenase, runt-related transcription factor 2 (*Runx2*), *Alk Phos*, *osteocalcin axin2*, *c-myc*, and *cyclin D1* genes have been previously described.^{36,37} Other primers are listed in Table 1. The results are presented as mean $\pm$ standard deviation of three independent experiments.

TABLE 1. PRIMER SEQUENCES AND ANNEALING TEMPERATURE CONDITIONS FOR PCR

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
<i>Dkk1</i>	AATGTATCACACCAAAGGACAAGAAG	GATCTTGGACCAGAAGTGTCTAGCA	60
<i>Dkk2</i>	AGGAGCCGGGGGAAACAAGAGA	GAGGACGCCCCGCCGACACT	61.2
<i>Dkk3</i>	AGGCCCCGGATGAGTACGAAGATG	GGGAGCGGCGCAAGACAAAAG	60.6
<i>sFrp1</i>	CGCCATGACCCCGCCCAATACCA	CCCCACCCTCACCCCAAGCCACAC	60
<i>sFrp2</i>	TCGCTCTTCGCCCCTGTCTGTCTC	CTCGCCGCCCTGCTTCTGTCC	60
<i>Sost</i>	CCGGGTGGGATGGGGGTCTT	GGGGGCCAGGAGTGTGATTTCT	60
<i>Twist1</i>	GACCTCGGGGCCCTCCACAC	GCCGCCTCCGCCCGCAGATT	60
<i>Wif-1</i>	GCTCTGGAGCATCCTACCTT	AATCATGTGTAAAGGGGGCC	55

sFrp, secreted frizzled related protein; *DKK*, Dickkopfs; *Sost*, sclerostin; *Wif*, WNT inhibitory factor.

Statistical analysis

The results are presented as mean $\pm$ standard deviation of two or three independent experiments. Statistical differences between the means were examined by Student's *t*-test, and significance was set a *p*-value of <0.05 .

Western blot analysis and immunofluorescence

Nuclear β -catenin was detected on nuclear fractions isolated from the different cell types. Nuclear fractions were prepared as previously described.^{37,39} Each nuclear fraction (80 μ g) was resolved by 12% Tris-HCl sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred to an Immobilon-P membrane (Millipore). Membranes were probed with mouse anti- β -catenin antibody 1:200 (sc-7963; Santa Cruz Biotechnology). To control for equal loading and transfer of the samples, the membrane was also re-probed with mouse monoclonal anti-Lamin B1-nuclear envelop marker antibody (119D5-F1) dilution 1:200 (abcam.com). The densitometric results of each band was normalized to their respective loading controls (Lamin B1) and presented as percentage increase. For immunofluorescence, cells were fixed with Methanol for 5 min at -20°C and then with Acetone for 2 min at -20°C . Cells were washed five times with cold PBS and then blocked with 2% normal goat serum for 30 min at room temperature before incubation with primary antibody mouse-anti- β -catenin (1:100, sc-7963; Santa Cruz Biotechnology) overnight at 4°C followed by a fluorescein-conjugated donkey anti-mouse secondary antibody (1:800, Alexa-fluor 488; Molecular Probes, Invitrogen) for 1 h at room temperature. Immunostaining with a primary normal (irrelevant) mouse immunoglobulin G (sc-2025) (1:100, sc-2027; Santa Cruz Biotechnology) was performed as negative control followed by incubation with anti-mouse fluorescein-isothiocyanate-conjugated antibody. Nuclear counterstaining was performed using Vectashield H-1200 mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories), and a Zeiss Axioplan microscope equipped with an Axiocam HRc digital camera was used for imaging.

Animals

All experiments using animals were performed in accordance with Stanford University Animal Care and Use Committee guidelines. CD-1 and FRB mice were purchased from Charles River Laboratories.

Animal surgery: Creation of calvarial defects

CD-1 postnatal (pN7) and pN60 mice underwent calvarial defect procedures. Each treatment group contained at least seven animals ($n=7$). After deeply anesthetizing the mice and removing the overlying pericranium, 2 mm calvarial defects were created with a trephine drill on the right frontal and the contra lateral left parietal bone with meticulous care to avoid damage the underlying dura mater. In a first set of experiments, parietal bone defects were treated with a 1.5-mm-diameter collagen sponge (Helistat, Integra Life-sciences Cooperation) soaked with recombinant mWnt3a (R&D Systems) either at concentrations of 50, 100, and 200 ng for pN7 mice or at concentrations of 100 and 800 ng for pN60 mice. Control groups included a collagen sponge soaked with PBS (PBS), whereas frontal bone defects remained untreated (empty). A second set of experiments was performed with the distinction that frontal defects were treated with 50 and 100 ng Wnt3a for pN7 mice and 100 and 800 ng for pN60 mice, whereas parietal defects remained untreated.

Imaging

Micro-computed tomography (MicroCT) was performed using a high-resolution MicroCAT IITM (ImTek) small animal imaging system with the following settings: X-ray voltage of 80 kVp, anode current of 500 μ A, and an exposure time of 500 ms for each of the 360 rotational steps. The two-dimensional projection images were used to reconstruct tomograms with a Feldkamp algorithm, using a commercial software package (Cobra EXXIM; EXXIM Computing), resulting into a resolution of 80 μ m. The duration of each scan was 9.5 min. Three-dimensional reconstructions were generated by MicroView software (GE Healthcare). Each mouse was scanned with a CT-phantom. To set the correct threshold, three skulls with easily recognizable calvarial regeneration characteristics were harvested and stained with Alizarin Red whole mount staining. In MicroView, CT thresholds for these mice were manually adjusted to match the characteristics seen in the whole mount stain. By plotting these values on a linear curve obtained by the values of air, water, and hydroxyapatite from the phantom, the required threshold for regenerating calvarial bone was determined (equivalent to 510 Hounsfield Units). After normalizing each scan with the phantom values thereafter, the precise threshold was calculated for each scan. To evaluate bone regeneration, skulls were oriented in the axial plane with the zygomatic bones as a guiding structure. Mice were imaged at postoperative day 1 and then at week 2, 6, and 12.

After standardizing the images, percentage healing of the defects was evaluated with the magic wand tool of the imaging software Adobe Photoshop (Adobe Systems). Percentage healing was determined by dividing the rest defect area by the mean of the defect size 1 day postoperatively. For statistical analysis, normal distribution within a group was verified. Thereafter, each treatment group was compared to the PBS control group at each time point using Student's *t*-test. A *p*-value <0.05 was considered statistically significant. Student's *t*-test was used for statistical analyses. A *p*-value <0.05 was considered statistically significant.

Histology

Immediately after the 12 week scans, calvaria were harvested, fixed in 10% neutral buffered formalin overnight, and decalcified in 19% ethylenediaminetetraacetic acid at 4°C.

Samples were then processed for paraffin embedding and cut in 10 µm sections. Sections of the defect region were stained with Pentachrome as previously described.³⁷ Sections were examined with a Carl Zeiss Axioplan 2 (Zeiss) microscope at 10× magnification. Images were captured by AxioVision (Zeiss) and evaluated by Adobe Photoshop (Adobe Systems).

Results

Comparative analysis of endogenous activation of canonical Wnt signaling in different cell types

To evaluate the extent of activation of canonical Wnt signaling in mASC, E16 calvarial mesenchymal cells, and early juvenile (FpN1 and PpN1) and adult (FpN60 and PpN60) frontal and parietal bone-derived osteoblasts, we first analyzed the expression level of three down-stream targets of the

FIG. 1. (A) QRT-PCR showing the expression profiles of three downstream targets of canonical Wnt signaling, *axin2*, *myc*, and *cyclin D1*. Quantified mRNA values were normalized by the amounts of glyceraldehyde 3-phosphate dehydrogenase mRNA, and results are given as fold induction (**p* < 0.05). (B) Immunoblotting analysis of β-catenin performed on nuclear fractions, revealed higher levels of nuclear β-catenin in ASCs, E16, and FpN1 cells. Membranes were stripped and reprobed with anti-Lamin B1 antibody to assess for equal loading and transfer of nuclear proteins fraction. Histogram represents the densitometric analysis of electrophoresis bands, and the relative intensities of bands were normalized to their respective loading control and set as 100%. The results are presented as the mean ± standard deviation of three independent experiments. (C) Indirect immunofluorescence staining detected the most intense nuclear staining in ASCs, E16, and FpN1. As negative control normal primary (irrelevant) mouse immunoglobulin G was used. Nuclear counterstaining was performed with DAPI. QRT-PCR, quantitative reverse transcription-polymerase chain reaction; mASCs, mouse adipose-derived stem cells; E16, embryonic-stage day 16 calvarial mesenchymal cells; FpN1, postnatal day 1 frontal bones-derived osteoblast; PpN1, postnatal day 1 parietal bone-derived osteoblast; FpN60, postnatal day 60 frontal bone-derived osteoblast; PpN60, postnatal day 60 parietal bone-derived osteoblast; DAPI, 4',6-diamidino-2-phenylindole; Wnt, wingless. Color images available online at www.liebertonline.com/ten.

canonical Wnt signaling, *axin2*, *cyclin D1*, and *c-myc* (Fig. 1A). Real-time QRT-PCR analysis revealed significant differences in the expression level of these genes, with higher expression in mASCs, E16 cells, and FpN1 osteoblasts, and lower expression in PpN1, FpN60, and PpN60 osteoblasts. However, in PpN1 osteoblasts the expression level of the three genes was higher than that in FpN60 and PpN60 osteoblasts. Differences in the activation of canonical Wnt signaling observed among the various cells analyzed were further confirmed by immunoblotting analysis of nuclear β -catenin (Fig. 1B). mASCs, E16 cells, and FpN1 osteoblasts were characterized by elevated amount of nuclear β -catenin as result of an enhanced activation of canonical Wnt signaling. Conversely, less nuclear β -catenin was detected in the other cells (Fig. 1B). Further, immunofluorescence performed using anti β -catenin antibody also revealed differences in nuclear staining for β -catenin (Fig. 1C). The most intense staining was observed in mASCs, and E16 cells (in these cells, in addition to nuclear staining, membranes staining was also detected). FpN1 osteoblasts also stained positive for nuclear β -catenin, whereas in PpN1 osteoblasts cytoplasmic staining was also observed. FpN60 osteoblasts showed fewer cells with nuclear staining, which was barely detected in PpN60 osteoblasts. Taken together, these data demonstrated that the degree of endogenous activation of canonical Wnt signaling is largely different between mesenchymal undifferentiated and differentiated cells.

Opposite effect of exogenous added Wnt3a on osteogenesis of undifferentiated and differentiated cells

ASCs undergo osteogenic differentiation mimicking embryonic calvarial mesenchymal cells.⁴⁰ We have previously demonstrated that enhanced activation of canonical Wnt/ β -catenin signaling is responsible for the greater osteogenic potential of neural-crest-derived frontal osteoblasts.³⁷ Moreover, our previous study showed that treatment with Wnt3a protein (50 ng/mL) enhanced osteogenic differentiation of both juvenile and adult osteoblasts harvested from parietal bones.³⁷ Herein, we sought to investigate the effect of Wnt3a treatment on undifferentiated cells that are endowed with enhanced endogenous activation of canonical Wnt signaling, such as mASCs, E16 cells, and FpN1 osteoblasts, or on differentiated cells with a less activated endogenous canonical Wnt signaling (e.g., PpN1 and PpN60 osteoblasts). To this aim, a differentiation assay was performed in the absence or presence of different concentrations of exogenous Wnt3a. As shown in Figure 2, addition of Wnt3a had a profound different effect depending on both the status of cell and the concentration of protein employed in the assay. As assessed by alkaline phosphatase enzymatic activity and mineralization of ECM, using Alizarin red staining and its quantification (Fig. 2A–C), Wnt3a treatment inhibited osteogenic differentiation in a dose-dependent manner in mASCs, E16 cells, and FpN1 osteoblasts, with higher doses inhibiting osteogenesis. Conversely, treatment with increasing concentrations of Wnt3a induced osteogenic differentiation of FpN60 and PpN60 osteoblasts. In PpN1 osteoblasts at low doses Wnt3a enhanced osteogenesis, whereas high doses were inhibitory. In mASCs and E16 cells, Wnt3a significantly inhibited osteogenesis already with the lowest concentration of 25 ng/mL, whereas in FpN1 osteoblasts at a concentration of

100 ng/mL. Increasing concentration of Wnt3a further inhibited the osteogenic differentiation of these cells. In contrast, increasing concentration of Wnt3a enhanced osteogenesis in FpN60 and PpN60 osteoblasts. Further, in PpN1 osteoblasts at a concentration of 25 ng/mL, Wnt3 had a slight stimulatory effect on osteogenic differentiation, while the highest (200 ng/mL) concentration inhibited osteogenesis. The osteogenic differentiation was also scored at molecular level by analyzing the expression profile of osteogenic markers by real-time quantitative PCR. In all untreated cells, upregulation of *Runx2* at an early time point of osteogenic differentiation was observed (day 3) followed by a downregulation at later time points, whereas in Wnt3a-treated mASCs, E16 cells, and FpN1 osteoblasts *Runx2* expression was sustained at all time points analyzed (Fig. 2D). A relatively sustained upregulation of *Runx2* was also observed in PpN1 osteoblasts treated with the highest dose of Wnt3a (200 ng/mL). By day 10, in Wnt3a-treated mASCs, E16 cells, and FpN1 osteoblasts, we observed a lack of upregulation of *Alk phos*, an intermediate marker of osteogenic differentiation, as well as of the late marker *osteocalcin* at day 21. Conversely, Wnt3a treatment on FpN60 and PpN60 osteoblasts resulted in higher expression levels of all osteogenic markers, at appropriate times, compared to untreated cells. Similarly, to what observed with alkaline phosphatase enzymatic assay and mineralization of ECM, in PpN1 osteoblasts Wnt3a induced higher expression of osteogenic markers only at low doses, whereas the highest dose (200 ng/mL) was inhibitory (Fig. 2D). Thus, the above data suggested that activation of canonical Wnt signaling may either induce or inhibit osteogenesis depending on the status of target cell, the extend of endogenous activation of Wnt signaling, and concentration of Wnt ligands treatment.

Expression profile of antagonists of canonical Wnt signaling

The canonical Wnt pathway is regulated by a large number of antagonists, including the Dkk family, secreted frizzled related proteins (sFrps), sclerostin, and others. The initial comparative analysis of mASCs, E16 cells, and juvenile and adult frontal and parietal osteoblasts revealed a differential endogenous activation of canonical signaling (see Fig. 1), suggesting that perhaps the cells express different threshold levels of Wnt antagonists. To verify this hypothesis we performed a QRT-PCR analysis to profile the expression pattern of Wnt antagonists in the cells, either at the basal level or during their osteogenic differentiation. This analysis revealed at day 0 a differential expression pattern of several Wnt inhibitors among the different cell types. Moreover, a unique expression pattern was observed in all cells undergoing osteogenic differentiation. As shown in Figure 3, at day 0, mASCs, E16 cells, and FpN1 osteoblasts expressed overall lower levels of inhibitors than PpN1, FpN60, and PpN60 osteoblasts. By day 3 of osteogenic differentiation, expression of sFrp1–3, Dkk1–3, Wif, and sclerostin was dramatically upregulated, and remained steady by day 10 in all cell types. Thus, inhibitory components of Wnt signaling are tightly and coordinately regulated during osteogenic differentiation.

Differential effects of constitutive activation and/or inhibition of canonical Wnt signaling

Because of inhibitory effect elicited by Wnt3a on osteogenesis of mASCs, E16 cells, and FpN1 osteoblasts, we next

FIG. 2. Effect of Wnt3a on osteogenic potential of different cell types. (A) Alkaline phosphatase assay at day 10 detected decreased levels of enzymatic activity in ASCs, E16, and FpN1 cells treated with different doses of Wnt3a, whereas Wnt3a treatment increased the enzymatic activity in FpN60 and PpN60 osteoblasts. Low doses of Wnt3a increased the enzymatic activity in PpN1 osteoblasts, whereas higher doses decreased the activity. (B, C) Alizarin red staining and its quantification mirrored the profile observed for alkaline phosphatase activity. (D) Time-course QRT-PCR for osteogenic markers further confirmed the osteogenic inhibitory and/or inducing activity of Wnt3a on ASCs, E16, FpN1, PpN1, FpN60, and PpN60 cells. Quantified mRNA values were normalized as above ($*p < 0.05$). Oc, osteocalcin; *Runx2*, runt-related transcription factor 2; OD, optical density. Color images available online at www.liebertonline.com/ten.

FIG. 3. Expression profile of Wnt inhibitors during osteogenic differentiation. QRT-PCR analysis demonstrated lower basal endogenous levels (day 0) of inhibitors in mASCs, E16 cells, and FpN1 osteoblasts. During the osteogenic differentiation, an upregulation of members of sFrps, Dkks, Wif, and sclerostin was detected in all cells. Quantified mRNA values were normalized as above. sFrps, secreted frizzled related proteins; Dkks, Dickkopfs; Wif, WNT inhibitory factor. Color images available online at www.liebertonline.com/ten.

investigated whether a constitutive enhanced activation of canonical Wnt signaling could impact in an irreversible manner the osteogenic differentiation of these cells. To address this question the different cell types were stable transduced with a retroviral plasmid harboring a β -catenin mutant (pSy33) that accumulates in the nucleus and consti-

tutively activates TCF-mediated transcription activated β -catenin.³⁸ A Neo empty retroviral vector (pNeo) was used as negative control. Subsequently, the retrovirally infected cells were tested for their osteogenic potential. As shown in Figure 4A–C, in pSy33ASCs, pSy33E16 cells, and pSy33FpN1 osteoblasts the osteogenic differentiation was dramatically

inhibited, whereas in pSy33PpN1, pSy33FpN60, and pSy33PpN60 osteoblasts enhanced osteogenic differentiation was observed compared to control pNeo cells. Alkaline phosphatase enzymatic activity was significantly reduced in pSy33ASCs, pSy33E16 cells, and pSy33FpN1 (Fig. 4A), and Alizarin red staining did not detect mineralization of ECM in these cells (Fig. 4B, C). In contrast, elevated levels of alkaline phosphatase activity and ECM calcium deposition were detected in pSy33PpN1, pSy33FpN60, and pSy33PpN60 osteoblasts. As counterpart, we also investigated the effect of constitutive inactivation of canonical Wnt signaling in the cells by stable transducing them with a DnTcf4 retroviral plasmid (pDnTcf4).³⁸ Interestingly, this analysis revealed that inactivation of canonical Wnt signaling increased osteogenic differentiation in pDnTcf4 mASCs, pDnTcf4 E16 cells, and pDnTcf4 FpN1 osteoblasts compared to Neo control cells, whereas a partial inhibition of osteogenic differentiation was observed in PpN1, FpN60, and PpN60 osteoblasts transduced with the DnTcf4 (Fig. 4A–C). Importantly, QRT-PCR analysis of three downstream targets of canonical Wnt signaling confirmed the enhanced activation of canonical Wnt signaling achieved in pSy33-transduced cells, and the partial inhibition of signaling in cells transduced with pDnTcf4 (Fig. 4D). Moreover, the results observed in DnTcf4 ASCs and DnTcf4 E16 cells were further supported by sFrp1 protein treatment of ASCs, E16 cells, or PpN60 osteoblasts during osteogenic differentiation. As shown in Figure 4E, at concentrations of 0.1 and 1 ng/mL sFrps, an antagonist of canonical Wnt signaling increased the osteogenic differentiation of ASCs and E16 cells, but inhibited osteogenesis of PpN60 osteoblasts.

In vivo effect of Wnt3a treatment upon calvaria injury

Taken together, our *in vitro* data suggested that activation of canonical Wnt signaling may trigger different effects in the context of osteogenic differentiation depending on the state of cell, and that Wnt3a may induce or inhibit osteogenic differentiation in a dose-dependent manner. Therefore, we investigated the effect of different doses of Wnt3a on calvarial defects created in juvenile and adult mice. To this end, 2 mm calvarial defects were first created in the frontal and parietal bones of pN7 juvenile mice (Fig. 5A). Treatment of calvarial defects with Wnt3a resulted in different effects dependent on the dose. After 12 weeks, parietal defects in pN7 mice treated with 50 ng of Wnt3a protein showed 83.1% healing, and 72.2% healing when treated with 100 ng, as compared to 23.6% healing in the control group. Healing rate

in parietal defects treated with 50 ng of Wnt3a was therefore similar to the high endogenous healing capacity of the untreated frontal bone at pN7 (85.0%). Interestingly, increasing Wnt3a dose to 200 ng resulted in a dramatic impaired healing of the parietal bone (17.6% healing) (Fig. 5A). The MicroCT scans and corresponding Pentachrome staining of the defect areas show complete healing in pN7 mice treated with 50 and 100 ng mWnt3a. In contrast, the parietal defect treated with 200 ng Wnt3a revealed poor healing.

In pN60 mice, conversely to what observed in pN7 mice, increasing the dose of Wnt3a further enhanced the healing capacity of parietal defects (76.8% healing with 100 ng of Wnt3a and 92.6% with 800 ng of Wnt3a), whereas control parietal defects treated with collagen soaked with PBS showed 17.3% healing. Pentachrome staining revealed that regenerated bone was thicker in defects treated with 800 ng (Fig. 5B).

A dose-dependent effect was also observed after treatment of frontal defects with Wnt3a. At week 12, frontal defects in pN7 mice treated with 50 ng of Wnt3a showed similar healing rates as the PBS controls (85.2% and 82.3%), whereas a dose of 100 ng resulted in decreased healing (44.9%). Moreover, treatment with increasing doses of Wnt3 (200 and 800 ng) further decreased bone repair (data not shown). Conversely, in adult mice treatment of frontal bone defects with 800 ng of Wnt3a resulted in increased healing (92.2%) as compared to treatment with 100 ng of Wnt3a (44.2%) or PBS (59.7%) at week 12 (Fig. 5C). MicroCT revealed increased healing of defects compared to parietal bone defects in pN7 and pN60 mice throughout the course of the investigation. Histology analysis performed on coronal sections using pentachrome staining revealed the presence of osseous regenerate bridging at the injury site with new bone formation centrally in the frontal bone defects after 12 weeks, consistent with successful bone healing. Thus, these *in vivo* results correlated with the previous *in vitro* observation.

Discussion

Wnt/ β -catenin signaling, also referred as canonical Wnt signaling, is central to bone development and homeostasis in adulthood. Moreover, canonical Wnt signaling plays an important role in bone repair.^{21,22,35} In this study we defined the differential spectrum of activity of canonical Wnt signaling and showed that it may elicit opposite biological activity in the context of osteogenic differentiation depending on the status of cell (undifferentiated vs. differentiated), as well as on the threshold levels of its activation and Wnt

FIG. 4. Effect of constitutive activation and inhibition of canonical Wnt signaling. (A) Alkaline phosphatase assay showing decreased levels of enzymatic activity in ASCs, E16, and FpN1 cells transduced with pSy33 as compared to pNeo controls, whereas increased levels of alkaline phosphatase activity are detected in pSy33PpN1, pSy33FpN60, and pSy33PpN60 cells. Conversely, expression of DnTcf4 increased alkaline phosphatase activity in ASCs, E16, and FpN1 cells, whereas decreasing it in PpN1, FpN60, and PpN60 cells. (B) Alizarin red staining and its quantification (C) detected very poor mineralization of extracellular matrix in ASCs, E16, and FpN1 cells transduced with pSy33, whereas the amount of mineralization was increased in pSy33-transduced PpN1, FpN60, and PpN60 cells. Conversely, expression of DnTcf4 increased mineralization in ASCs, E16, and FpN1 cells, whereas decreasing it in the other cells. (D) QRT-PCR analysis of *c-myc*, *cyclin D1*, and *axin2* confirmed the enhanced activation of canonical Wnt signaling in cells transduced with pSy33, and the downregulation of signaling in cells transduced with pDnTcf4. (E) Treatment with sFrp1 (0.1 and 1 ng/mL) protein increased osteogenesis in ASCs and E16 cells, as indicated by alkaline phosphatase enzymatic activity and Alizarin red staining of extracellular matrix. sFrp1 treatment inhibited osteogenesis in PpN60 osteoblasts. pSy33, retroviral plasmid harboring a β -catenin mutant; pNeo, Neo empty retroviral vector; DnTcf4, dominant negative Tcf4. Color images available online at www.liebertonline.com/ten.

FIG. 5. *In vivo* effect of Wnt3a treatment upon calvarial injury. (A) Two-millimeter calvarial defects in the frontal and parietal bones of pN7 mice. Parietal defects were treated with 50, 100, and 200 ng Wnt3a, or PBS as control, while frontal defects were untreated (empty). Pentachrome staining showing bone regeneration at 12 weeks (12w) in the area of defects. Black arrows mark the edges of defect. Red arrows indicate the area of defect from where coronal sections for Pentachrome staining were obtained. Micro-computed tomography performed at 2, 6, and 12 weeks after osteotomy. Histograms represent quantification of bone regeneration ($*p \leq 0.05$). (B) Two-millimeter calvarial defects in the frontal and parietal bones of pN60 mice. Parietal defects were treated with 100 and 800 ng Wnt3a or PBS as control, and frontal defects were untreated (empty). Pentachrome staining revealed the bony tissue regeneration in the area of defects. Micro-computed tomography and quantification of bone regeneration were performed as above ($*p \leq 0.05$). Black and red arrows indicate as above. (C) Wnt3a treatment of frontal bone defects in pN7 and pN60 mice. Two-millimeter frontal bone defects of pN7 mice were treated with 50 and 100 ng Wnt3a or PBS as negative control, while parietal were untreated. Frontal defects of pN60 mice treated with 100 and 800 ng Wnt3a. Histograms represent the quantification of bone regeneration; 150 μ m in 20 $\times$ magnification panels, 50 μ m in 40 $\times$ magnification panels. PBS, phosphate-buffered saline. Color images available online at www.liebertonline.com/ten.

ligands concentration. Several observations emerged from the current study. First is the existence of a differential activation of canonical Wnt signaling between an undifferentiated mesenchymal cell and osteoblast, which is merely a differentiated mesenchymal cell. We provided evidence showing that either ASCs or embryonic calvarial mesenchymal cells are characterized by the highest endogenous activation of canonical Wnt signaling compared to juvenile and adult osteoblasts. Thus, differences in activation of canonical Wnt signaling reflect the different status of cells, and enhanced endogenous activation on canonical Wnt signaling is a signature of undifferentiated mesenchymal cells. However, our analysis indicated that also FpN1 osteoblasts are endowed with high endogenous activation of canonical Wnt signaling although the activation is lower than that observed for ASCs and E16 cells. The enhanced activation of canonical Wnt signaling in FpN1 osteoblasts reflects the neural-crest tissue origin of frontal bone as previously reported.³⁷

To delineate the competence window in which canonical Wnt signaling can either inhibit or promote osteogenic differentiation, we analyzed cells with a different status, specifically undifferentiated mesenchymal cells such as ASCs and embryonic calvarial mesenchymal cells, and juvenile and adult calvarial osteoblasts. A second observation emerged from our investigation was that undifferentiated cells, such as ASCs and E16 cells, as well as the neural-crest-derived immature FpN1 osteoblasts, are the most susceptible to inhibitory effects elicited by treatment with Wnt3a. Moreover, our study demonstrated that Wnt3a acts as a morphogen and that the Wnt3a ligand may elicit differential responses in the same cell type depending on its concentration, but also depending on active canonical Wnt signaling background of the target cell. Our observation that Wnt3a treatment inhibits osteogenic differentiation in mesenchymal cells (e.g., ASCs and E16) is consistent with previous *in vitro* studies performed on other multipotent mesenchymal cells.^{41–44} In addition, our study provides also *in vivo* evidence complementing *in vitro* results. It must be pointed out that *in vitro* Wnt3a treatment represents a constant and continuous treatment of cells, compared to *in vivo* treatment where calvarial bones are initially exposed to doses of Wnt3a protein, which gradually decreased overtime. Nevertheless, the effect observed *in vivo* mirrored the results obtained *in vitro*.

Regulation of Wnt signaling pathway may occur at different levels, including extracellular components of the signaling cascade.^{35,45} sFrps are most thoroughly studied regulators of Wnt signaling, working at the extracellular environment.⁴⁵ Sclerostin also is an extracellular antagonist working by binding LRP-5/6 co-receptors.⁴⁶ Wif1, an evolutionary conserved protein, is another member of the secretory Wnt modulators that directly bind to Wnt proteins.⁴⁵ In addition, Dkk family proteins inhibit Wnt signaling by binding to LRP5 coreceptor.⁴⁷ Our analysis indicated that the enhanced activation of canonical Wnt signaling observed in undifferentiated cells correlates with lower endogenous levels of Wnt antagonists. Interestingly, the expression profile analysis of several antagonists of canonical Wnt signaling clearly revealed that during the osteogenic differentiation there is, overall, an upregulation of inhibitors of canonical Wnt signaling among all cell type analyzed. Our data are in agreement with previous studies indicating, for example, increased expression of sFrps, sclerostin, and Dkk1 during

osteogenesis.^{41,42,48} The upregulation of antagonists of canonical Wnt signaling occurring during osteogenesis is suggestive of a requirement of gradient activity of Wnt signaling during this differentiation process. Increased expression of inhibitors during osteogenic differentiation may represent a system to tune the activity of Wnt signaling and create the proper balance between cell proliferation and differentiation.

Collectively, our data suggest that higher levels of endogenous Wnt signaling are requested for maintenance of the undifferentiated state of cell, while low levels of activation are permissive for their differentiation along the osteogenic lineage. This hypothesis finds support in the results obtained from ASCs, E16 cells, and FpN1 osteoblasts either expressing DnTcf4 or treated with sFrp1 protein, showing that in both cases the cells differentiated better than Neo control cells. Conversely, the same cells, when expressing a constitutively activated β -catenin, failed to differentiate along the osteogenic lineage, likely when treated with Wnt3a protein. A complete opposite effects was elicited by increased activation of canonical Wnt signaling on differentiated cells. We found that treatment with exogenous Wnt3a increased alkaline phosphatase enzymatic activity and mineralization of ECM in FpN60 and PpN60 osteoblasts in a dose-dependent fashion. Specifically, increased concentrations of Wnt3a correlated with increased osteogenic induction of FpN60 and PpN60 osteoblasts, while for PpN1 osteoblasts, only a low concentration of Wnt3a was stimulatory. A similar scenario was observed in Sy33-transduced FpN60 and PpN60.

To further test the dose-dependent effects of Wnt signaling on osteogenesis, we performed calvarial healing experiments using different concentrations of recombinant Wnt3a protein. Consistent with the *in vitro* results, increasing dose of Wnt3a protein had an inhibitory effect on bone formation and healing of calvarial defects in juvenile mice. The inhibition was more dramatic in frontal bone, which is known to be endowed with enhanced activation of canonical Wnt signaling.³⁷ Conversely, in adult mice increasing concentration of Wnt3a promoted a better healing of defect. The latter observation may reflect not only the less endogenous activation of canonical Wnt signaling, but also the presence of fewer osteoprogenitor cells in adult bone.⁴⁹

All together, our data indicate that enhanced activation of canonical Wnt signaling inhibits osteogenic differentiation of undifferentiated mesenchymal cells, whereas increasing the mineralization of differentiated osteoblasts. More importantly, from this study emerges evidence that a high dose of Wnt3 treatment may impair *in vivo* bony tissue regeneration in immature calvarial bones. Indeed, the knowledge of the competence window in which canonical Wnt signaling can either inhibit or promote osteogenic differentiation, and the determination of appropriate dose concentrations on Wnt ligands for treatment may further improve bone tissue engineering protocols.

Acknowledgments

This work was supported by the Hagey Family Program in Pediatric Stem Cell Research and Regenerative Medicine, The Oak Foundation, and NIH R21DE019274 to M.T.L., and the German Research Foundation (DFG BE 4169-1) to B.B.

Disclosure Statement

No competing financial interests exist.

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Received: March 04, 2010

Accepted: May 21, 2010

Online Publication Date: June 28, 2010