Wnt 3a Promotes Proliferation and Suppresses Osteogenic Differentiation of Adult Human Mesenchymal Stem Cells

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Abstract Multipotential adult mesenchymal stem cells (MSCs) are able to differentiate along several known lineages, and lineage commitment is tightly regulated through specific cellular mediators and interactions. Recent observations of a low/high bone-mass phenotype in patients expressing a loss-/gain-of-function mutation in LRP5, a coreceptor of the Wnt family of signaling molecules, suggest the importance of Wnt signaling in bone formation, possibly involving MSCs. To analyze the role of Wnt signaling in mesenchymal osteogenesis, we have profiled the expression of *WNTs* and their receptors, *FRIZZLEDs* (*FZDs*), and several secreted Wnt inhibitors, such as *SFRPs*, and examined the effect of Wnt 3a, as a representative canonical Wnt member, during MSC osteogenesis in vitro. *WNT11*, *FZD6*, *SFRP2*, and *SFRP3* are upregulated during MSC osteogenesis, while *WNT9A* and *FZD7* are downregulated. MSCs also respond to exogenous Wnt 3a, based on increased β-catenin nuclearization and activation of a Wnt-responsive promoter, and the magnitude of this response depends on the MSC differentiation state. Wnt 3a exposure inhibits MSC osteogenic differentiation, with decreased matrix mineralization and reduced alkaline phosphatase mRNA and activity. Wnt 3a treatment of fully osteogenically differentiated MSCs also suppresses osteoblastic marker gene expression. The Wnt 3a effect is accompanied by increased cell number, resulting from both increased proliferation and decreased apoptosis, particularly during expansion of undifferentiated MSCs. The osteo-suppressive effects of Wnt 3a are fully reversible, i.e., treatment prior to osteogenic induction does not compromise subsequent MSC osteogenesis. The results also showed that sFRP3 treatment attenuates some of the observed Wnt 3a effects on MSCs, and that inhibition of canonical Wnt signaling using a dominant negative TCF1 enhances MSC osteogenesis. Interestingly, expression of Wnt 5a, a non-canonical Wnt member, appeared to promote osteogenesis. Taken together, these findings suggest that canonical Wnt signaling functions in maintaining an undifferentiated, proliferating progenitor MSC population, whereas non-canonical Wnts facilitate osteogenic differentiation. Release from canonical Wnt regulation is a prerequisite for MSC differentiation. Thus, loss-/gain-of-function mutations of LRP5 would perturb Wnt signaling and depress/promote bone formation by affecting the progenitor cell pool. Elucidating Wnt regulation of MSC differentiation is important for their potential application in tissue regeneration. J. Cell. Biochem. 93: 1210–1230, 2004. Published 2004 Wiley-Liss, Inc.[†]

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Multipotent adult mesenchymal cells were first reported by Friedenstein and Owen, who isolated from bone marrow a subpopulation of adherent cells capable of differentiating into many lineages [Owen and Friedenstein, 1988]. These multipotential mesenchymal stem cells (MSCs) can be induced to form a number of tissues such as bone, cartilage, muscle, adipose tissue, tendon, hepatocytes, neurons, and cardiomyocytes, as well as give rise to cells for neovascularization. Recent studies have identified a number of tissue sources of MSCs in addition to bone marrow, such as adipose, trabecular bone, and muscle (as reviewed in [Caplan and Bruder, 2001; Tuan et al., 2003]).

The multilineage potential of MSCs strongly indicates that there are specific key mechan-

isms in operation to regulate the differentiation programs of these cells. The importance of these regulatory mechanisms is underscored by the widespread presence of such multipotent cells in a large number of different tissues, possibly contributing to postnatal tissue repair and regeneration. The ease in isolation and availability of MSCs have made them a particularly attractive candidate for use in tissue engineering. Thus, it is important to have a thorough understanding of the specific signals dictating cellular behavior and the specific cues that induce or inhibit differentiation, and/or promote the maintenance of these cells.

Wingless (*wg*) and Frizzled (*fz*) genes were first characterized in *Drosophila*, where they specify embryonic tissue patterning and cell fate determination. These early studies in *Drosophila* have led to the identification of homologous genes in mammals (*WNTs* and *FZDs*) and the study of the function of Wnt and Frizzled proteins [Cabrera et al., 1987; Rijsewijk et al., 1987; Wainwright et al., 1988; McMahon and Moon, 1989; Peifer and Polakis, 2000]. Wnt proteins are a group of secreted, cysteine-rich signaling molecules that are involved in diverse processes during embryogenesis, such as segmentation [Pourquie, 2003], specification of the vertebrate body axis [Parr and McMahon, 1995], central nervous system (CNS) patterning [Parr et al., 1993; McGrew et al., 1995], cell fate and growth [Parr and McMahon, 1994; Taipale and Beachy, 2001], limb development [Dealy et al., 1993; Parr and McMahon, 1995; Kengaku et al., 1998; Hartmann and Tabin, 2000, 2001], and mesenchymal differentiation [Tufan and Tuan, 2001; Fischer et al., 2002a,b; Tufan et al., 2002a,b]. In the adult, Wnts are implicated in oncogenesis and the maintenance of somatic stem cell pools [van de Wetering et al., 2002; Alonso and Fuchs, 2003; Reya et al., 2003].

The cell surface receptors for Wnts consist of the Frizzled class of proteins, which bear structural similarity to G-protein coupled receptors. The family of Wnt co-receptors, the low density lipoprotein-related peptides, LRP5 and LRP6, are known to be involved in canonical Wnt signal transduction by stabilizing the Wnt–Frizzled interaction, and play a role in intracellular signaling through their interaction with Axin [Mao et al., 2001; Cliffe et al., 2003; Schweizer and Varmus, 2003; Tolwinski et al., 2003]. Other regulators of Wnt signaling include the co-receptor, Ror2, and Wnt inhibitors, such as the soluble Frizzled-related proteins (sFRPs). There is more than one downstream signaling cascade that can be activated by a particular Wnt protein. The traditional, canonical pathway involves the stabilization of an intracellular pool of β-catenin, which is usually targeted for degradation, and this conserved β-catenin then translocates to the nucleus and activates the transcription of genes through the TCF/LEF1 family of transcription factors. The other non-canonical Wnt signaling cascades, more thoroughly characterized in *Drosophila* and *Xenopus*, are currently under investigation in mammalian systems and are likely to be mediated via G-proteins, calcium fluxes, and/or MAP kinases [Liu et al., 1999; Sheldahl et al., 1999, 2003].

During vertebrate development, Wnt 3a acts in the apical ectodermal ridge (AER) of the limb bud to maintain cells in the undifferentiated and highly proliferative state [Kengaku et al., 1998; Kawakami et al., 2001]. Wnt 3a has also been shown to function in the adult to expand the skin stem cell population [Alonso and Fuchs, 2003; Jamora et al., 2003], to maintain a pool of reserve undifferentiated cells in the gastrointestinal system [van de Wetering et al., 2002], and can be used to expand a hematopoietic progenitor population [Austin et al., 1997; Van Den Berg et al., 1998; Reya et al., 2003]. We hypothesize here that specific Wnts, such as Wnt 3a, regulate the differentiation program of MSCs by promoting the maintenance of MSCs in an undifferentiated state with a concomitant expansion of the pool of mesenchymal progenitor cells, and thereby play a role in tissue morphogenesis, regeneration, and repair.

Wnt 3a was chosen as the Wnt protein of choice in these experiments for several reasons. There are implications of Wnt involvement in regulating bone maintenance and osteogenesis both during development and in the adult. Wnt 3a is expressed in the primitive streak and tailbud of the developing mouse, and Wnt 3a knockout mice have severe skeletal phenotypes [Takada et al., 1994; Ikeya and Takada, 2001]. Such a functional role for Wnts is consistent with the recent implication of several Wnt-related proteins in adult human skeletal diseases. The human syndrome osteoporosis pseudoglioma syndrome (OPPG), in which patients present with low bone mass, fractures,

and deformation, is linked to mutations in the gene for the Wnt co-receptor, *LRP5* [Gong et al., 2001]. LRP5 mutations are known to affect both the proliferation and osteogenic differentiation of osteoblastic progenitor cells both in vivo and in vitro [Gong et al., 2001; Kato et al., 2002; Little et al., 2002]. There have also been recent reports of gain-of-function mutations in *LRP5*, which lead to a high bone mass phenotype in humans and in a mouse model with a targeted, activating *LRP5* mutation [Babij et al., 2003]. These findings strongly implicate Wnt signaling in developmental bone formation and growth as well as in the maintenance of adult bone function.

In light of the established role of Wnts during limb development, the implicated role of canonical Wnt signaling in the maintenance of somatic stem cell compartments, and the phenotype of both activating and dominant negative mutations in LRP5, we hypothesize that Wnt signaling acts to regulate both the proliferation and differentiation of adult human MSCs. The experiments reported here aim to examine the responsiveness of adult human MSCs to Wnt 3a signaling and the effect of Wnt exposure on the behavior of MSCs undergoing osteogenesis.

Our results show that there is both an increase in MSC proliferation and a reversible suppression of osteogenic differentiation in cultures exposed to Wnt 3a. Interestingly, we have recently identified and isolated multipotential MSCs from adult human trabecular bone [Noth et al., 2002; Tuli et al., 2003a], which may be a candidate target cell for Wnt signaling in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture

Human MSCs. Three sources of MSCs were used: (1) human trabecular bone-derived [Osyczka et al., 2002] (2) commercially available primary marrow-derived MSCs (Cambrex Bio Science), and (3) patient-isolated primary marrow-derived MSCs. All protocols involving human subjects were approved by the Institutional Review Boards of George Washington University and Thomas Jefferson University. All cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (FBS; Atlanta Biologicals). To induce osteogenesis,

cells were plated at a density of 2.5×10^5 cells/ml in 6-well or 24-well tissue culture plates and allowed to adhere for 24 h at 37°C, at which time cells were switched to osteogenic medium (OS) consisting of α-MEM supplemented with 10% FBS, 50 μg/ml L-ascorbate-2-phosphate, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 10 nM 1α,25-(OH)$_2$D$_3$. The medium was changed every 3–4 days.

Wnt 3a treatment. The primary group of cultures were exposed to conditioned medium from cells producing Wnt 3a (see below), and a subset of these cultures were treated with the soluble Wnt inhibitor, Fz8/Fc (R&D Systems), added at 10 ng/ml to the medium. Fz8/Fc is a chimeric protein containing the Wnt-binding cysteine-rich domain (CRD) of mouse Frizzled 8 bound to the Fc fragment of a human IgG, which can act as a competitive Wnt inhibitor, presumably by binding to available Wnt proteins and sequestering them away from the endogenous Frizzled receptors, thereby attenuating the Wnt/Frizzled interaction. The soluble Wnt inhibitor, secreted frizzled related protein 3 (sFRP3) was used at a concentration of 10 ng/ml in select cultures (R&D Systems). Wnt 3a was introduced in the form of conditioned culture medium of a stably transfected L cell line (American Type Culture Collection) producing secreted Wnt 3a, with conditioned medium from L cells containing an empty vector (American Type Culture Collection) as a negative control. Cells were maintained and expanded in DMEM with 10% FBS and 0.4 mg/ml G418. Conditioned medium was prepared as follows. Cultures were plated at 2×10^6 cells/ml in 10 cm dishes, allowed to adhere overnight, and the culture medium was switched to the same osteogenic medium used for MSC culture described above. On culture Day 4, the conditioned medium was collected, filtered, and diluted in a 1:1 ratio with fresh osteogenic medium before its use in MSC cultures.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For all MSC osteogenesis studies, cells were harvested either at Day 12 or Day 24. At this time, cells were rinsed once with phosphate buffered saline (PBS) and total RNA was isolated using Trizol reagent (Invitrogen). RT-PCR was carried out with 500 ng of total RNA using the Superscript One-Step RT-PCR kit

(Invitrogen), according to the manufacturer's protocol starting, with 30–40 cycles of amplification for all gene-specific primer sets, except GAPDH (25 cycles). RT-PCR products were electrophoretically analyzed with ethidium bromide staining or using the Agilent DNA 1000 system for semi-quantitative analysis of relative DNA levels. The gene-specific primers used and their annealing temperatures are given in Table I.

Assays for Osteogenesis

Osteogenic cultures were stained with alizarin red to detect matrix mineralization [Bodine et al., 1996]. Cells were rinsed with PBS, fixed in 60% isopropanol, and stained in a 1% alizarin red solution (Rowley Biochemical Institute) for 3 min. Alkaline phosphatase activity was also detected histochemically in cultures fixed with 2% paraformaldehyde/methanol using the

TABLE I. Gene-Specific Primers for RT-PCR Analysis

Genes	Anneal temp (°C)	Prod size (bp)	Sequence	Genbank accession number
GAPDH	54	554	(F) CCA GAA CAT CAT CCC TGC CTC TAC (R) GGT CTC TCT CTT CCT CTT GTG C	J04038
Frizzled 1	60	309	(F) TAC CTC AAC TAC CAC TTC CTG GGG (R) CTC GGC GAA CTT GTC ATT ACA CAC	AB017363
Frizzled 2	60	252	(F) CCT CAA GGT GCC ATC CTA TCT CAG (R) GTG TAG CAG CCC GAC AGA AAA ATG	AB017364
Frizzled 3	55	330	(F) AAG GCT TCC ACA GTG ACA CAA GG (R) AGA GGA GAG AAA CCC CAA CTA CCA C	AB039723
Frizzled 4	56	215	(F) GGA AAT GGT TGG GTG AAG CCT G (R) TTT TTG ATG CTG GGG TCG GG	NM_012193
Frizzled 5	60	334	(F) TCG TCGC CCA TTC TGA AGG AGT C (R) TAG TGG ATG TGG TTG TGC TCG C	AB043702
Frizzled 6	54	346	(F) TCT CTG CTG TCT TCT GGG TTG G (R) TCC GCT TTC ACC TCT CTC ATT G	NM_003506
Frizzled 7	59	214	(F) ACA GAC TTA GCC ACA GCA GCA GGG (R) TTT CCA AAT CAC CCC TCG CC	AB017365
Frizzled 8	58	310	(F) TGG AGT GGG GTT ACC TGT TG (R) AGC GGC TTC TTG TAG TCC TC	NM_031866
Frizzled 9	60	396	(F) TTC TTC TCC ACC GCC TTC AC (R) CAG GAT GAC GAT GGT CTT GAG	NM_003508.1
Frizzled 10	58	328	(F) TCG CCT GCT ACT TTT ACG AAC G (R) TGG TGA GTT TTC TGG GGA TGC	AB027464
LRP5	55	295	(F) GCT GGA ATG GAT GGT TCA AGT CG (R) CAG AAT GGA TTT CAC GCA GAC CC	NM_002335.1
LRP6	60	300	(F) AAT ACT GAT GGC ACT GGG AGA CGA G (R) AAT AGG GCA AGC ACA GCG AAG G	NM_002336
Ror2	60	301	(F) TCC TTC TGC CAC TTC GTG TTT CC (R) TGC TTG CCG TTC CTC TGT AAT CC	NM_004560
SFRP2	59	467	(F) GGA GAC CAA GAG CAA GAC CAT TTA C (R) CGG GTG GGC TTT TCC TTT AG	AF311912
SFRP3	54	302	(F) GCA AGC AGT GAA CGC TGT AAA TG (R) GAG CCT TCC ACC AAG AGT AAT CTG	NM_001463
Alk Phos	51	453	(F) TGG AGC TTC AGA AGC TCA ACA CCA (R) ATC TCG TTG TCT GAG TAC CAG TCC	NM_000478
Osteocalcin	51	297	(F) GCC GTA GAA GCG CCG ATA GGC (R) ATG AGA GCC CTC ACA CTC CTC	NM_000711
BSP	51	257	(F) CGA AGA CAA CAA CCT CTC CAA ATG (R) ACC ATC ATA GCC ATC GTA GCC TTG	NM_004967
Runx2	57	530	(F) CCG CAC GAC AAC CGC ACC AT (R) CGC TCC GGC CCA CAA ATC TC	NM_004348
Collagen 1	57	335	(F) GGT GTA AGC GGT GGT GGT TAT (R) GCT GGG ATG TTT TCA GGT TGG	NM_000089
Wnt 1	60	302	(F) CGA TGG TGG GGT ATT GTG AAC G (R) GCG GAG GTG ATA GCG AAG ATA AAC	NM_005430
Wnt 3	58	218	(F) CTA CGA GAA CTC CCC CAA CTT TTG (R) AGA TGC GAA TAC ACT CCT GGC AG	NM_030753
Wnt 5a	60	567	(F) ACA CCT CTT TCC AAA CAG GCC (R) GGA TTG TTA AAC TCA ACT CTC	NM_003392
Wnt 9a/14	60	376	(F) ACA ACA ACC TCG TGG GTG TG (R) CAG ATG CTC TCG CAG TTC TTC	NM_003395
Wnt 10b	60	200	(F) GAA TGC GAA TCC ACA ACA ACA G (R) TTG CGG TTG TGG GTA TCA ATG AA	NM_003394.2
Wnt 11	60	295	(F) CTA TTT GCT TGA CCT GGA GAG AGG (R) CCC CAC TTC ACT GTT GTG TAG ACG	NM_004626
mWnt 3a	60	372	(F) GGA ATG GTC TCT CGG GAG TTT G (R) TTC GGG GTT AGG TTC GCA GAA G	X56842

Leukocyte Alkaline Phosphatase Kit (Sigma) according to the manufacturer's protocol.

Protein Isolation and Western Blot Analysis

Osteogenic cultures were harvested at Day 12 and proteins were extracted at 4°C and fractionated by centrifugation into the cytosolic and nuclear fractions (27). For Western Analysis, protein samples were separated by SDS–polyacrylamide gel electrophoresis (6%) and electrotransferred onto nitrocellulose membranes (Bio-Rad). The blots were immunoprobed with antibodies against β-catenin (Santa Cruz) or β-actin (Santa Cruz) as a control for protein loading. Alkaline phosphatase conjugated anti-rabbit or anti-mouse (Sigma) antibodies were used as secondary antibodies and the blots were developed using the Zymed Alkaline Phosphatase Kit (Zymed).

Immunostaining

Cultures were fixed in 4% paraformaldehyde and predigested with 300 U/ml hyaluronidase (Sigma) in 50 mM Tris-HCl, pH 8.0, 30 mM sodium acetate, and 0.5 mg/ml BSA for 15 min at 37°C. The cultures were incubated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. Primary antibodies included monoclonal antibodies to osteocalcin and bone sialoprotein (BSP), kindly provided by Dr. Larry Fisher (NIDCR, NIH) at 1:500 dilution. Secondary antibodies were detected using an HRP broad-spectrum kit, Histostain SP for DAB (Zymed), according to the manufacturer's protocol.

Transfection and Luciferase Assay

An optimized version of the TOPFLASH reporter plasmid (pGL3-OT) [Shih et al., 2000] (kindly supplied by Dr. Bert Vogelstein, Johns Hopkins University) was used to assay for Wnt-responsive gene activation. The negative control consisted of a modified version of the FOPFLASH plasmid construct containing mutated TCF binding sites (pGL3-OF). MSCs in the growth phase were cotransfected with a Renilla luciferase plasmid attached to the CMV promoter (Promega) using a Nucleofection protocol (AMAXA). Luciferase activity was assayed using the Promega Dual Luciferase Assay System according to the manufacturer's protocol. For the Wnt transfection experiments, a dominant negative TCF, dnTCF1 (a kind gift of Dr. Hans Clevers), Wnt 3a (Upstate), Wnt 5a

(Upstate), or a pcDNA3 control (Invitrogen) were transfected into the MSCs prior to their placement into the various culture conditions.

Retroviral Infection

Amphotropic Phoenix packaging cells were transfected with 40 μg of the pBABE construct (both kindly provided by Dr. Garry Nolan, UCSF) containing the gene of interest, Wnt 3a (kindly provided by Dr. Roel Nusse, Stanford University), using the calcium-phosphate method. Culture medium was removed after 24 h. The cells were washed gently with PBS, and 5 ml volume of fresh medium per 10 cm culture plate was added and allowed to incubate for an additional 48 h. The virus-containing medium was then removed from the Phoenix cells, filtered, and at 5 ml per 10 cm culture plate was combined with Polybrene (8 μg/ml) and applied to subconfluent, proliferating MSCs, obtained by splitting cultures in a ratio of 1:20 or 1:40 1 or 2 days prior to infection. After 2 additional days of culturing, fresh medium (6 ml) was added to each culture and MSCs were grown to near confluence without changing the medium (approximately four additional days). The cultures were either used directly or split for further expansion. A LacZ-containing pBABE construct was used to determine the level of infection on the basis of β-galactosidase activity (Promega).

Cell Counts and Flow Cytometry

Cell counts. MSCs in culture were released using 0.25% trypsin–EDTA. Trypan blue was added to the suspension and the number of viable, trypan blue negative cells were counted using a hemocytometer.

Flow cytometry. MSCs were released with 0.25% trypsin–EDTA, fixed in chilled 70% ethanol, labeled with a mixture of 2 μg/ml propidium iodide in the presence of RNase A (25 μg/ml), and then examined by flow cytometry (FACS Calibur, Becton-Dickinson).

Statistical analysis. Cell counts and FACS data were analyzed using a paired t-test, and $P < 0.05$ considered statistically significant.

RESULTS

The initial experiments described here addressed the responsiveness of adult human MSCs to Wnt 3a signaling and the effect of Wnt exposure on the course of MSC osteogenesis. To

assess Wnt responsiveness, mRNA expression of all known human *FRIZZLED* genes was analyzed by RT-PCR in undifferentiated MSCs and MSCs induced to undergo osteogenesis for 12 days (Fig. 1A). Many of the *FRIZZLED* genes were expressed in MSC cultures, although at varying levels. While the level of expression of most *FRIZZLED* genes was unchanged during differentiation, a significant change was seen in *FZD6*, whose levels were upregulated upon osteogenic differentiation, and in *FZD7*, whose levels were downregulated with differentiation. Similarly, the mRNA expression of several Wnt-related coreceptors (LRP5, LRP6, and Ror2) or inhibitors (sFRP2 and sFRP3) were examined as a function of MSC differentiation state (Fig. 1B). The expression of the noncanonical Wnt coreceptor, *ROR2* [Oishi et al., 2003], was dramatically increased upon osteogenic differentiation of MSCs, and the secreted Wnt inhibitors *SFRP2* and *SFRP3* were also upregulated

with osteogenic differentiation of MSCs. As shown in Figure 1C, it was observed that 7 of the 19 currently identified Wnt family members were expressed by MSCs, with the expression of some being dependent upon cellular differentiation state. Specifically, the expression of *WNT11* increased, while that of *WNT9A* decreased upon osteogenic differentiation of MSCs. To examine other downstream Wnt effectors, MSCs undergoing osteogenesis were exposed to control or Wnt 3a conditioned medium, and as shown in Figure 1D, the levels of both nuclear and cytoplasmic β-catenin were markedly increased in osteogenic MSC cultures exposed to Wnt 3a. Another means to assess the responsiveness of MSCs to Wnt signaling was by analyzing the activity of the TCF-responsive reporter, pGL3-OT, with the mutated construct, pGL3-OF, as a negative control. As shown in Figure 2, exposure of undifferentiated MSCs to Wnt 3a resulted in approximately a 26-fold
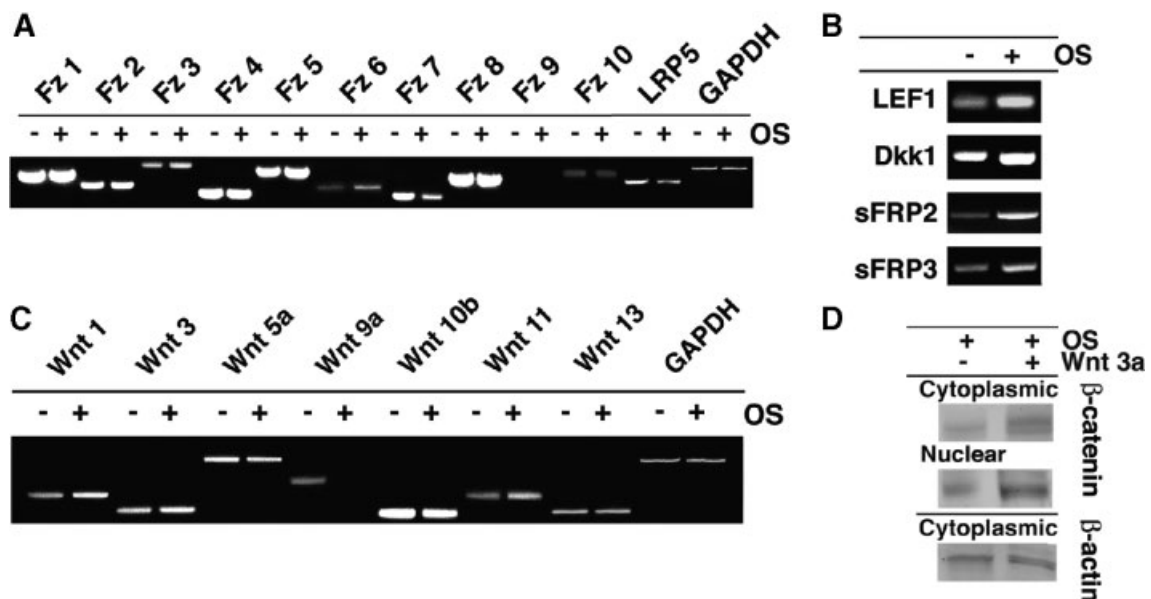

Fig. 1. MSCs express Wnt receptors and related molecules during osteogenic differentiation and are capable of responding to Wnt signaling using the canonical pathway. **A**: *FRIZZLED* expression. MSCs were maintained in the undifferentiated state or induced to undergo osteogenesis (OS) for 12 days. By RT-PCR analysis, it was clear that many of the Wnt receptors, members of the Frizzled family of proteins, were expressed by MSCs both during maintenance in the undifferentiated state and during osteogenic differentiation. The mRNA expression level of *FZD7* was downregulated during osteogenesis, while *FZD6* was upregulated in MSCs undergoing osteogenic differentiation. **B**: Expression of Wnt coreceptors and secreted inhibitors. RT-PCR analysis showed that MSCs expressed the Wnt coreceptors, *LRP5*, *LRP6*, and *Ror2*, and the secreted Wnt inhibitors, *SFRP2*

and *SFRP3*, and that the level of gene expression of Ror2 and the sFRPs was increased during osteogenic induction. **C**: Wnt expression. After 12 days of culture in either expansion medium or in an osteoinductive medium cocktail (OS), MSCs were analyzed by RT-PCR for the expression of Wnt family members. There was mRNA expression of 7 out of the 19 Wnt members by the MSCs (others not shown). There was a noticeable decrease in the levels of *WNT9A* upon osteogenic differentiation while there was an increase in *WNT11* during the differentiation process. **D**: β-Catenin nuclearization. Osteogenic cultures of MSCs examined by Western blot analysis for β-catenin protein showed that MSCs express β-catenin protein, which increases in level with Wnt 3a treatment, particularly in the nuclear pool.
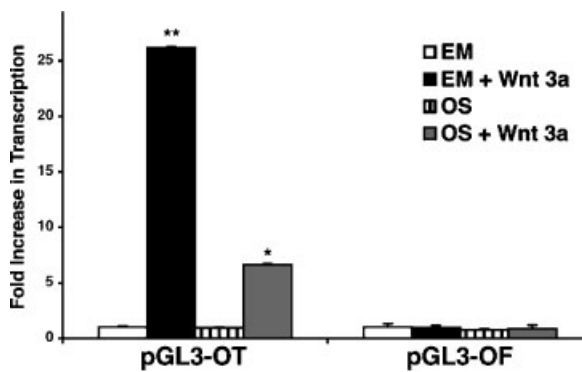
Fig. 2. Wnt signaling activation. MSCs were transfected with the Wnt-responsive reporter, pGL3-OT, or the mutated control reporter, pGL3-OF, and exposed to medium with or without Wnt 3a. Undifferentiated MSCs exposed to Wnt 3a showed over a 25-fold increase in transcriptional activity, while those exposed to Wnt 3a during osteogenesis showed only about a sixfold increase in transcription, while pGL3-OF controls did not show any differential Wnt responsiveness (*, $P < 0.05$; **, $P < 0.01$).

increase in transcriptional activity of pGL3-OT as compared to control undifferentiated cultures, clearly indicating the responsiveness of MSCs to canonical Wnt signaling via the β-catenin/TCF pathway. Interestingly, when MSCs were osteogenically differentiated while exposed to Wnt 3a, the transcriptional activity in response to Wnt signaling was significantly suppressed and resulted in a more modest increase over control.

To characterize the effect of Wnt 3a signaling on the process of MSC osteogenic differentiation, MSCs were exposed either to Wnt 3a-containing or control L cell conditioned medium during the 12-day course of MSC osteogenic differentiation. At Day 12, cells were stained for mineralization using alizarin red or histochemically for alkaline phosphatase activity (Fig. 3A). As seen in Figure 3A,B, the level of gene expression of several bone-related proteins was also examined at an mRNA (RT-PCR) and protein level (immunohistochemistry). As measured by mineralization, alkaline phosphatase activity, and immunohistochemistry, the various markers of osteogenesis that normally appeared during differentiation were severely compromised in the Wnt 3a treated cultures. These inhibitory Wnt effects were somewhat attenuated in the presence of the soluble Wnt inhibitor, Fz8/Fc. By RT-PCR, the normal osteogenic induction of bone-related mRNA expression was also inhibited upon Wnt 3a treatment. Specifically, the mRNA expression

of both BSP and alkaline phosphatase (AP) was suppressed in the presence of Wnt 3a, and these inhibitory effects were again attenuated in the presence of the competitive Wnt inhibitor, Fz8/Fc. The inhibitory effect of Wnt 3a on osteogenesis was also observed to be dose-dependent, on the basis of alkaline phosphatase activity (Fig. 3C). Taken together, these results strongly suggested that exposure of MSCs to Wnt 3a during the course of osteogenesis suppresses differentiation, and that this inhibitory effect is Wnt specific as demonstrated by the counteractive effect of Fz8/Fc.

In the course of these studies, it was visually apparent that osteogenic cultures exposed to Wnt 3a showed altered cell numbers as compared to their controls. Cell counts of trypan blue negative cells confirmed that exposure of MSC cultures undergoing osteogenesis to Wnt 3a led to an apparent increase in cell number as compared to controls (Fig. 4A). To address whether this was due to an increase in cellular proliferation or a decrease in apoptosis, cells were staged with respect to the cell cycle using flow cytometry. It was observed that Wnt 3a treatment of MSCs undergoing osteogenesis led to both an increase in proliferation and a decrease in apoptosis (Fig. 4B). We next assessed whether this Wnt effect could also be observed in undifferentiated MSCs. In cells undergoing expansion (EM), the differences in cell number between Wnt-treated and control cultures were even more apparent than in the MSCs undergoing osteogenesis, with a more than 2.5 fold increase in cell number in Wnt 3a-treated cultures by culture Day 9 (Fig. 4C). Under these conditions, there was a statistically significant increase in proliferation and a decrease in apoptosis in MSC cultures exposed to Wnt 3a as compared to control cultures (Fig. 4D), similar to but more prominent than the effect seen previously in MSCs undergoing osteogenesis. The dominant effect of Wnt 3a under normal circumstances thus appeared to be an increase in MSC proliferation, but under more stressful conditions, such as nutrient starvation, the more prominent effect appeared to be a decrease in apoptosis (unpublished observation). These results imply a dual role for Wnt 3a in both cellular proliferation and in cell survival.

The next question was whether Wnt 3a would have an effect on osteogenesis once differentiation had already begun. The following experi-
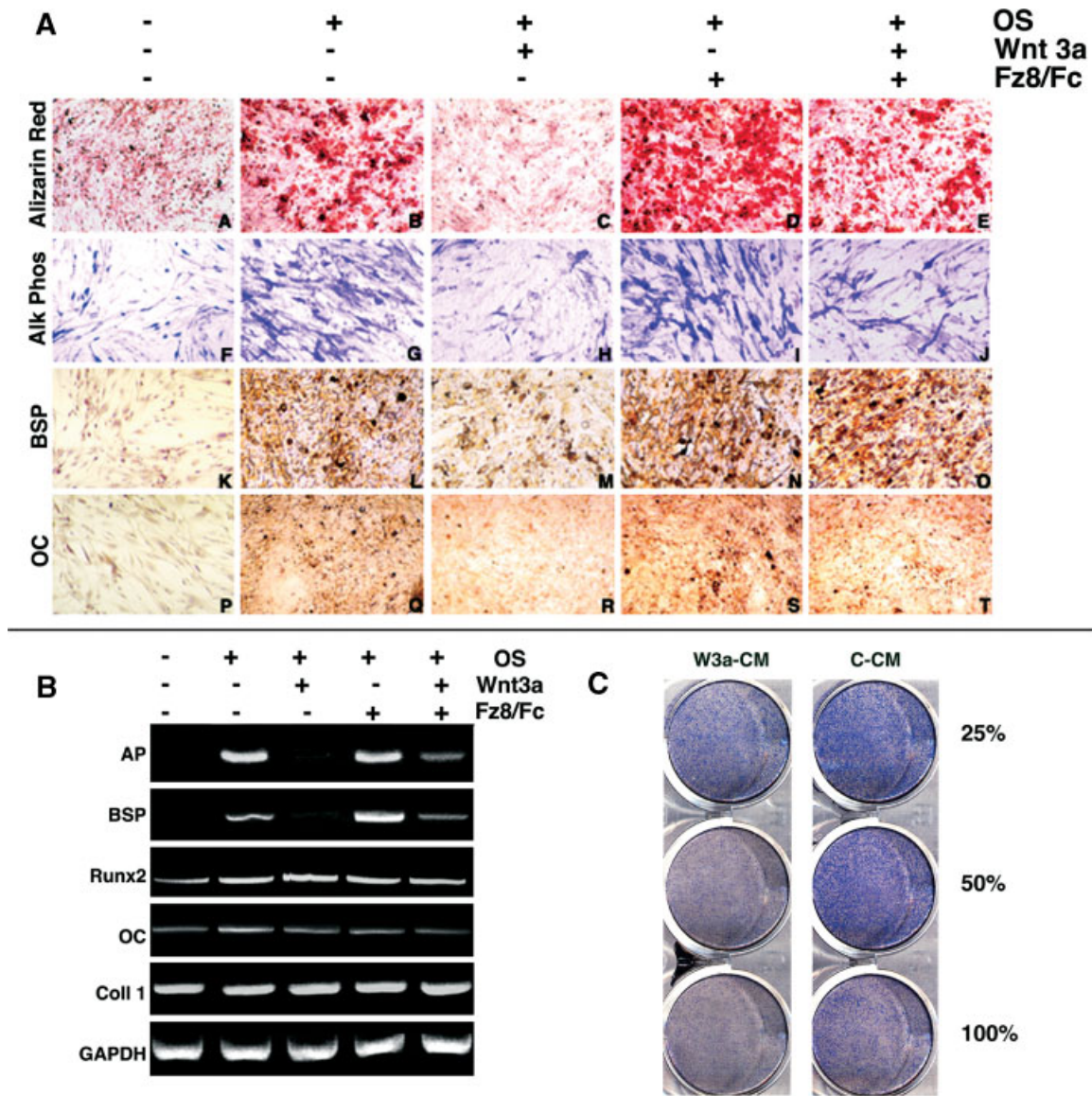
Fig. 3. Wnt 3a inhibits MSC osteogenic differentiation. MSC cultures undergoing osteogenesis (OS) were treated with Wnt 3a as described in Figure 1, and a soluble Wnt inhibitor, Fz8/Fc, was included to perturb Wnt activity. Addition of Fz8/Fc to these cultures partially restored the osteogenic activities suppressed by the presence of Wnt 3a. **A:** Mineralization, alkaline phosphatase activity, and immunostaining. During osteogenic induction of MSCs mineralized nodules were produced, and there was an increase in alkaline phosphatase activity. Exposure of MSCs to Wnt 3a during osteogenesis inhibited mineralization and caused a decrease in alkaline phosphatase activity. These effects were visibly attenuated in Wnt cultures by the addition of a soluble Wnt inhibitor, Fz8/Fc. A similar pattern was seen immunohisto-chemically, with an increase in protein expression during osteogenesis and a decrease in bone sialoprotein (BSP) and osteocalcin (OC) protein deposition in the presence of Wnt 3a. The Wnt 3a effects on protein expression were again attenuated in the presence of Fz8/Fc. **B:** Gene expression analysis. RT-PCR analysis showed that during osteogenesis Wnt 3a treatment severely inhibited the induction of some bone-related genes, specifically alkaline phosphatase (AP) and BSP. This Wnt-mediated inhibition was again rescued by the addition of Fz8/Fc. There was constitutive expression of several other bone-related mRNA including OC, Runx2, and collagen type I (Coll 1), which was not affected by Wnt 3a treatment of cultures. **C:** Wnt 3a Dose Response. MSCs undergoing osteogenic differentiation were exposed to varying levels of supplementation with Wnt 3a conditioned medium (W3a-CM) and stained for alkaline phosphatase at Day 12. Control, L cell-conditioned medium (C CM) was used as the control. When the osteogenic medium (OS) was completely replaced with W3a-CM (100%), an overall suppression of alkaline phosphatase activity was seen; at 50% dilution of OS with W3a CM, there was a demonstrable Wnt 3a-mediated suppression of alkaline phosphatase activity. In comparison, control MSCs cultured with C-CM supplementation showed suppressed osteogenesis (substantially less than with W3a-CM), only at 100% replacement of OS (presumably due to lack of osteogenic supplements). To preserve both the effect of the osteogenic supplements and the Wnt 3a conditioned medium, a 50:50 mix of OS and W3a-CM was used for all subsequent cultures for the analysis of Wnt 3a effects.
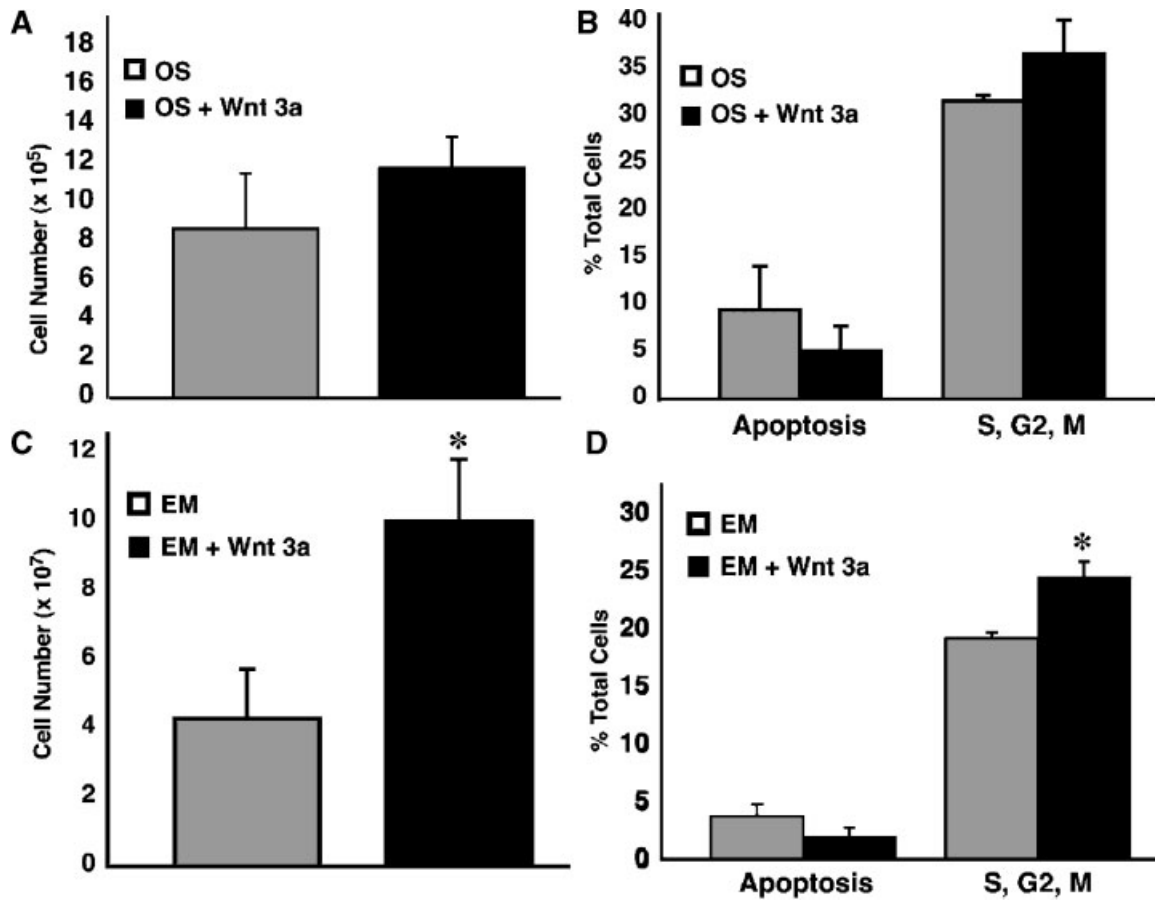
Fig. 4. Wnt 3a increases proliferation of MSCs undergoing osteogenesis and undifferentiated MSCs. **A**, **B**: MSCs undergoing osteogenesis (OS); (**C**, **D**) undifferentiated MSCs maintained in expansion medium (EM). A, C: Cell counts. Upon exposure to Wnt 3a (L cell conditioned medium), both osteogenic and undifferentiated MSCs showed increased cell numbers. The effect was more pronounced in undifferentiated MSCs under- going culture expansion as compared to controls. B, D: FACS analysis. Both increased proliferation as well as decreased apoptosis, indicated by S, G2, and M cell cycle stages, were seen in both osteogenic and undifferentiated MSC cultures upon Wnt 3a treatment. The effect was again more significant in MSCs undergoing culture expansion. *, $P < 0.05$.

mental scheme was undertaken (Fig. 5A), in which MSCs were first treated with osteogenic medium for 12 days, at which point selected cultures were assayed for mineralization and alkaline phosphatase activity to verify that osteogenic induction had begun. At this point, Day 12, the remaining cells were treated with either control or Wnt 3a conditioned medium for another 12 days. At the final time point, Day 24 in total, the cultures were assayed for markers of osteogenesis, such as mineralization, alkaline phosphatase activity, and bone-related mRNA expression. There was no obvious effect of Wnt 3a on the previously initiated mineralization, but there was a significant decrease in alkaline phosphatase activity in MSCs exposed to Wnt 3a for the final 12 days of osteogenic culture (Fig. 5B). At an mRNA level, the expression

level of several bone-related markers, specifi- cally BSP and alkaline phosphatase, was again decreased in cultures exposed to Wnt 3a, despite the fact that these cultures had been osteogeni- cally differentiated for 12 days prior to Wnt 3a exposure (Fig. 5C).

In order to address the reversibility of the Wnt effects on osteogenesis, the following expe- rimental conditions were established (Fig. 6A). MSC cultures were exposed to either L cell conditioned expansion medium (EM) or osteo- genic medium (OS) in the presence or absence of Wnt 3a for 12 days at which point the cultures were released by trypsinization and replated for an additional 12 days of osteogenic culturing without Wnt 3a. The various cultures showed little differences in their ability to undergo osteogenesis, as assayed by mineralization,
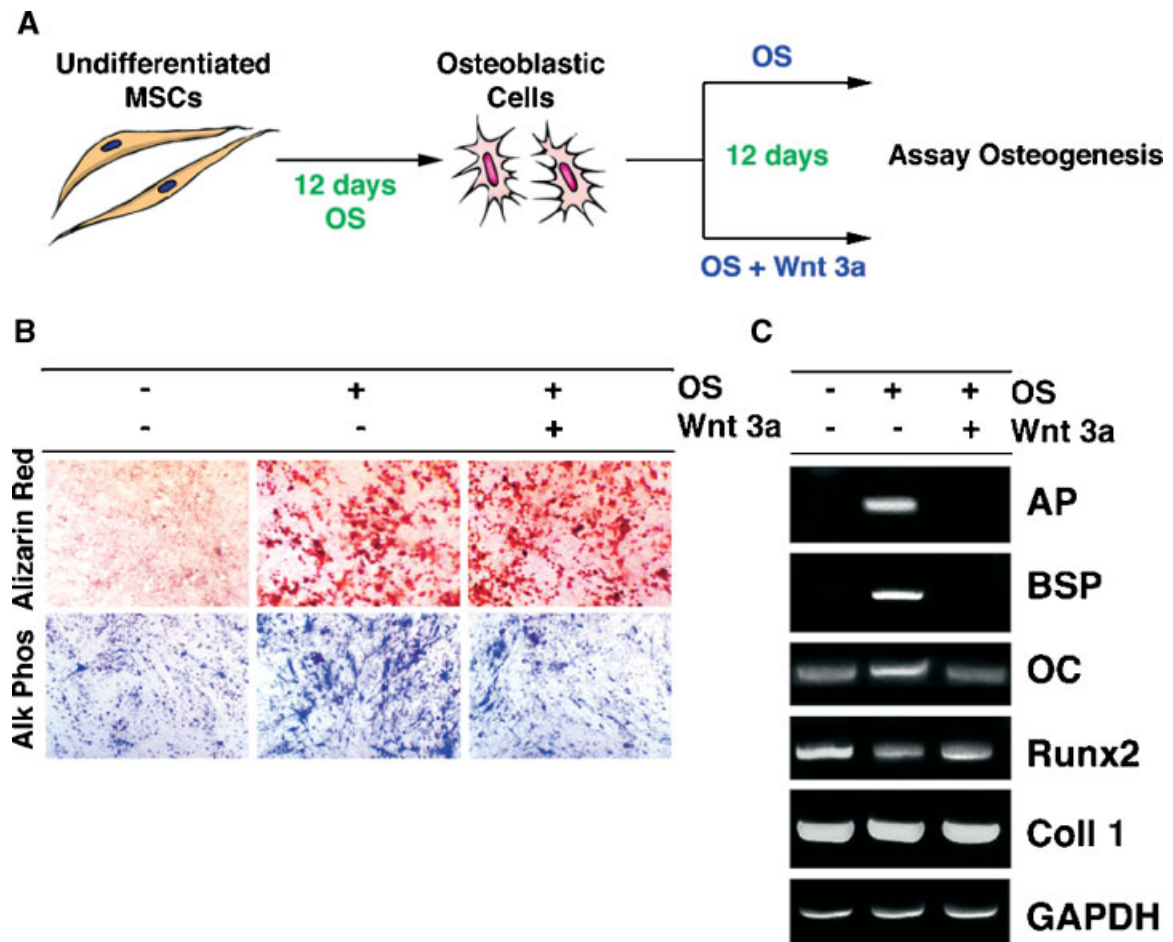
Fig. 5. Wnts 3a alters the profile of osteogenically differentiated MSCs. **A**: Experimental scheme. Adult human MSCs were placed into osteogenic conditions (OS) for 12 days of differentiation, at which time point osteogenesis was verified in a subset of cultures. The remaining MSCs were then grown for another 12 days (24 days total) either in the presence or absence of Wnt 3a to determine if Wnt 3a would have an effect on previously differentiated osteogenic MSCs. **B**: Mineralization and alkaline phosphatase activity. There was no apparent difference between the levels of mineralization in control and Wnt 3a treated cultures, whereas alkaline phosphatase activity was decreased with Wnt treatment as compared to controls. **C**: Gene expression analysis. RT-PCR analysis again showed decrease mRNA expression of both alkaline phosphatase (AP) and BSP upon Wnt 3a treatment without remarkable changes in the expression levels of osteocalcin (OC), Runx2, and collagen type I (Coll 1).

alkaline phosphatase activity, and the expression of bone-related mRNA (Fig. 6B and C). Similar patterns of mineralization, alkaline phosphatase expression, and mRNA expression were seen, even if the cultures had been exposed to Wnt 3a during the 12 days of expansion or osteogenesis prior to the final 12 days of osteogenic induction. This set of experiments supports the hypothesis that canonical Wnt signaling may play a reversible role in expanding a progenitor population of cells while suppressing their differentiation until other signals intervene and begin to promote terminal differentiation of these progenitor cells.

The observation of an increase in sFRP3 in MSCs undergoing osteogenesis (Fig. 1B) led us to examine the effect of Wnt 3a on MSC osteogenesis in the presence or absence of sFRP3. As seen in Figure 7A, MSCs exposed to Wnt 3a for 12 days show decreased mRNA levels of both AP and BSP. Cultures that underwent differentiation in the presence of Wnt 3a and sFRP3 showed decreased response to Wnt 3a treatment, i.e., reduced suppression of AP and BSP expression. Similarly, in MSC cultures osteogenically differentiated for 12 days and then exposed to either control or Wnt 3a conditioned medium for another 12 days (24 days total), the addition of sFRP3 again attenuated the suppressive effects of Wnt 3a on AP and BSP mRNA expression (Fig. 7B). Finally, the levels of β-catenin protein, seen to increase with
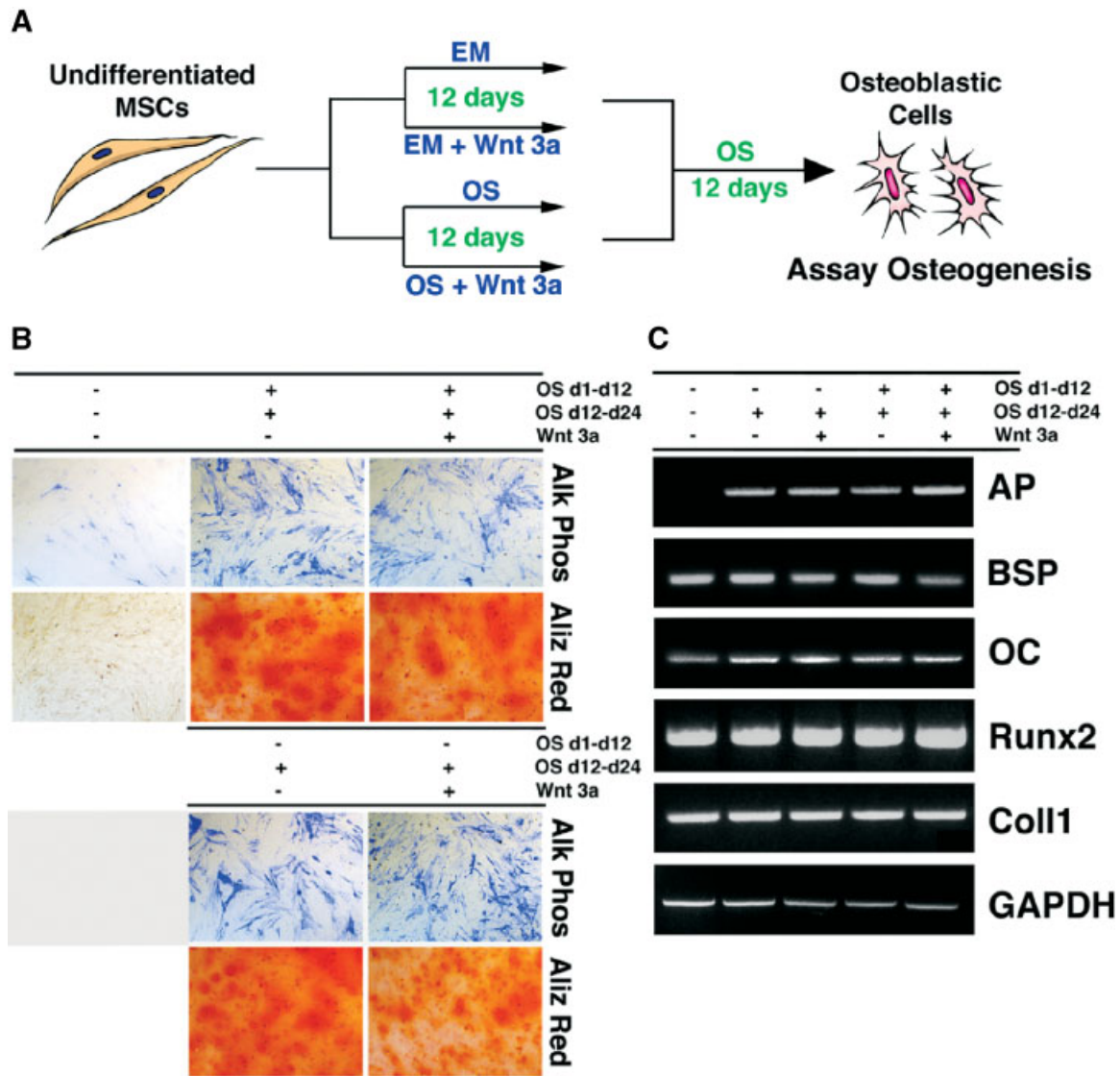
Fig. 6. Wnt 3a inhibitory effects on osteogenesis are reversible upon Wnt removal. **A**: Experimental scheme. MSCs were grown for 12 days in either expansion medium (EM) or osteogenic supplements (OS) in the presence or absence of Wnt 3a. At Day 12, the cells were trypsinized, counted, and replated for an additional 12 days under osteogenic conditions. **B**: Mineralization and alkaline phosphatase activity. There was no apparent difference in the ability of MSCs previously exposed to either control or Wnt 3a conditioned medium to undergo osteogenesis, regardless of whether exposure occurred during expansion (EM) or during osteogenic induction (OS). **C**: RT-PCR gene expression analysis. At an mRNA level, there was also no apparent difference in osteogenic capabilities of cultures exposed to Wnt 3a for 12 days prior to a subsequent 12 days of osteogenesis during which no exogenous Wnt 3a was administered.

Wnt 3a treatment of MSCs, were also slightly decreased in the presence of sFRP3 (Fig. 7C).

To further confirm and analyze the observed Wnt 3a effects on MSC osteogenesis, well characterized, primary, bone marrow-derived MSCs were used for the following experiments with retroviral-mediated Wnt 3a expression. By altering the experimental approach, we hoped to address the true nature of the adult human MSC response to canonical Wnt signaling with-

out the confounding factors of cell source and the use of conditioned medium. The MSCs purchased from Cambrex had been thoroughly characterized by the supplier as CD105, CD166, CD29, and CD44 positive, and negative for CD14, CD34, and CD45. They had also been tested for their ability to differentiate into the osteogenic, chondrogenic, and adipogenic lineages. These MSCs were retrovirally infected with the retrovirus pBABE expressing Wnt 3a
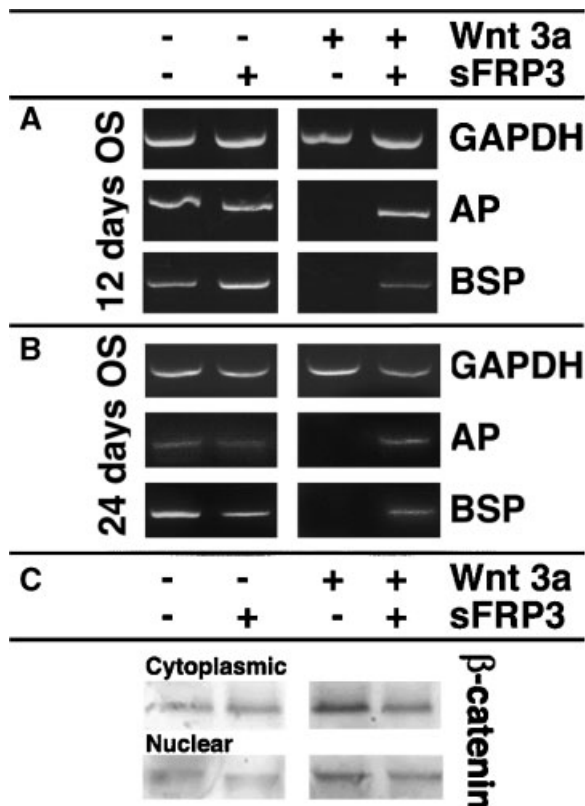
Fig. 7. sFRP3 rescues Wnt 3a-mediated suppression of osteogenic gene expression. Gene expression of AP and BSP was analyzed by RT-PCR in (**A**) MSCs undergoing osteogenesis for 12 days and exposed to either control or Wnt 3a conditioned medium in the presence or absence of 10 ng/ml of sFRP3, and (**B**) MSCs osteogenically differentiated for 12 days at which point they were exposed to either control or Wnt 3a conditioned medium for 12 additional days in the presence or absence of 10 ng/ml sFRP3. sFRP3 rescues Wnt 3a-mediated suppression of AP and BSP expression. **C**: Effect on β-catenin. MSCs were osteogenically differentiated in control or Wnt 3a treated cultures in the presence or absence of sFRP3. The Wnt 3a-mediated increase in β-catenin protein levels were partially attenuated in the presence of sFRP3.

or with an empty pBABE vector as a control. It was first verified by RT-PCR that the retrovirally infected cells were, indeed, producing Wnt 3a, and that β-catenin protein levels were increased in MSCs expressing Wnt 3a (Fig. 8B and C). Retrovirally infected MSCs were then placed into osteogenic culture for 12 days. At Day 12, the levels of mineralization, alkaline phosphatase activity, and bone-related mRNA expression were examined. In the Wnt 3a expressing cultures, the familiar suppression of osteogenesis by Wnt 3a was seen (Fig. 8A). The cultures expressing Wnt 3a showed decreased levels of mineralization, alkaline phosphatase activity and bone-specific mRNA

expression as compared to the empty vector controls (Fig. 8C). As in the earlier experiments, the mRNA expression of both alkaline phosphatase and BSP appeared to be specifically targeted by Wnt 3a signaling. In a similar manner, the previous observation of increases in total cell numbers with Wnt 3a treatment was also seen under these experimental conditions. Undifferentiated MSCs expressing Wnt 3a had substantially higher cell counts as compared to control cultures when grown in the presence of serum (Fig. 8D), and there was a visible increase in cell numbers and proliferation during in vitro expansion in cultures expressing Wnt 3a (Fig. 8E). The Wnt 3a-mediated increase in cell number was not seen in MSCs cultures in serum-free medium (SFM), indicating that other proteins (i.e., substances present in serum) are necessary for Wnt activity.

To test further the hypothesis that canonical Wnt signaling acted to suppress the osteogenic differentiation of MSCs, we next examined the effect of inhibiting the activation of the canonical cascade using a dominant negative TCF1 (dnTCF1). Transfection of MSCs with dnTCF1 effectively suppressed the Wnt 3a-mediated activation of a Wnt-responsive reporter, pGL3-OT (Fig. 9A). We next analyzed the effect of inhibition of Wnt signaling on osteogenesis in primary bone-marrow derived MSCs transfected with either an empty vector control or a Wnt 3a-expression construct. MSCs expressing dnTCF1 demonstrated increased alkaline phosphatase activity (Fig. 9C) and an increase in the mRNA expression levels of AP and OC (Fig. 9B) as compared to empty vector controls. These results confirmed suppression of osteogenic differentiation by canonical Wnt signaling.

Finally, to examine the potential interaction between the canonical and non-canonical Wnt pathways during MSC differentiation, MSCs were transfected with either a control vector or Wnt expression constructs. The first experiment addressed the activation of the canonical Wnt signaling cascade in the presence of either Wnt 3a or Wnt 5a. Primary, marrow-derived MSCs were co-transfected with either an empty plasmid control, Wnt 3a, or Wnt 5a in combination with the canonical Wnt-responsive reporter, pGL3-OT. There was a statistically significant increase in transcription in the MSCs expressing Wnt 3a, while there was a much lower level of activation in the Wnt 5a cultures (Fig. 10A). Next, the effect of Wnt 5a on
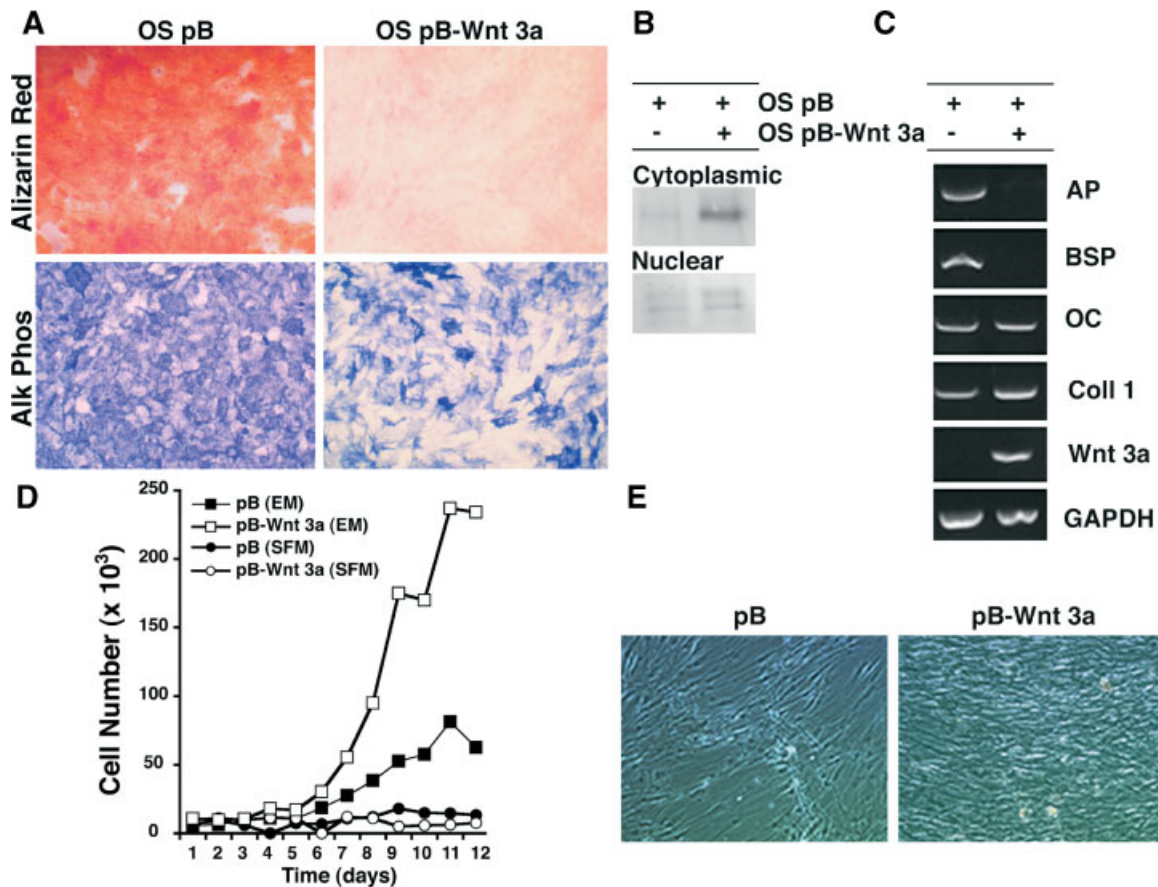
Fig. 8. Retrovirally expressed Wnt 3a inhibits MSC osteogenesis. Commercially obtained, primary bone marrow-derived MSCs (Cambrex) were infected with the retrovirus pBABE containing either an expression construct for Wnt 3a (pB-Wnt 3a) or an empty vector control (pB) and induced to undergo osteogenesis. **A**: Mineralization and alkaline phosphatase activity. As seen by alizarin red staining for mineralization and by alkaline phosphatase histochemistry, there were substantial decreases in both osteogenic mineral deposition and alkaline phosphatase activity in the presence of Wnt 3a expression (OS pB-Wnt 3a) as compared to control cultures (OS pB). **B**: Western blot analysis. Retrovirally mediated Wnt 3a expression also resulted in increased protein levels of β-catenin in both the cytoplasmic and nuclear pools. **C**: RT-PCR gene expression analysis. At an mRNA level, MSCs expressing Wnt 3a (OS pB-Wnt3a) showed downregulation of both alkaline phosphatase (AP) and BSP without remarkable effects on osteocalcin (OC) and collagen type I (Coll 1) as observed in similar cultures treated with Wnt 3a derived from transfected L cells (Fig. 3). **D**: Cell number. Compared to control cultures, the retrovirally infected MSCs expressing Wnt 3a showed drastic differences in cell number during maintenance and expansion of MSCs (pB-Wnt3a (EM) vs. pB (EM)). This stimulatory effect of Wnt 3a was not apparent in MSC cultures grown in serum-free medium (SFM), implying that Wnt interaction with other nutrients/growth factors is necessary for the stimulation of proliferation. **E**: Phase contrast microscopic examination. There were visibly apparent differences in cell density seen between the control and Wnt 3a producing cultures undergoing in vitro expansion when plated at the same initial density.

cellular proliferation was examined, and there was no overwhelming difference between control and Wnt 5a cultures, although there was a very modest decrease in proliferation seen in all of the cultures (Fig. 10B). Then, MSCs were transfected with either a control vector or Wnt 5a and osteogenically differentiated for 12 days. There was a noticeable increase in alkaline phosphatase staining in the Wnt 5a cultures, although there were no obvious effects on the process of mineralization at this time point (Fig. 10C). By RT-PCR analysis of this representative sample, it was clear that the presence of Wnt 5a led to an increase in AP and OC expression in osteogenic cultures, while the levels of BSP and Runx2 were not significantly affected (Fig. 10D). These observations, taken together, strongly suggest functional involvement of Wnt signaling in MSC proliferation and osteogenic differentiation.
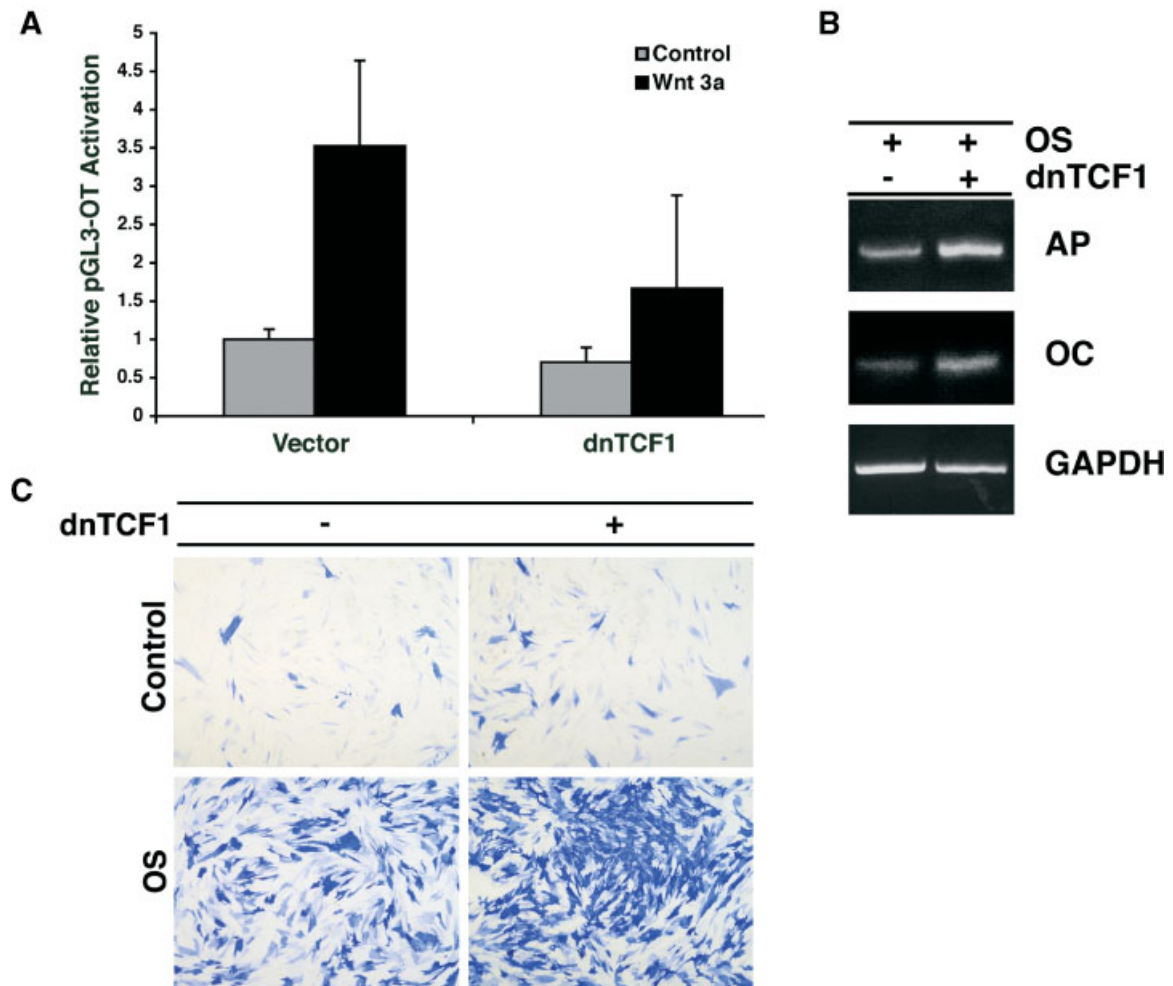
Fig. 9. Suppression of canonical Wnt signaling enhances MSC osteogenesis. Canonical Wnt signaling was suppressed by transfection with the dominant negative TCF construct, dnTCF1. **A**: Wnt-responsive reporter activation. MSCs co-transfected with pGL3-OT and dominant negative TCF1 (dnTCF1) showed a reduced ability to respond to Wnt 3a-stimulated reporter activation as compared to cultures transfected with an empty vector control. **B**: RT-PCR analysis. MSCs expressing dnTCF1 expressed higher mRNA levels of AP and OC upon osteogenic differentiation as compared to empty vector controls. **C**: Alkaline phosphatase activity. MSCs expressing dnTCF1 demonstrated an increase in alkaline phosphatase activity upon osteogenic differentiation in vitro as compared to empty vector controls.

DISCUSSION

We have shown here that adult human MSCs express a number of Wnts, their receptors, their co-receptors, and several secreted Wnt inhibitors in both the undifferentiated state and during osteogenic differentiation. The mRNA levels of several *WNTs* and *FRIZZLEDs* were differentially expressed during osteogenesis, with *WNT9A* and *FZD7* being downregulated, while *WNT11* and *FZD6* were upregulated. In the literature, there are reports of several downstream signaling pathways capable of being activated by Wnt proteins. The canonical Wnt signaling pathway involves the LRPs, β-catenin, and activation of transcription through the LEF/TCF family of transcription factors [Mao et al., 2001; Brantjes et al., 2002], while the alternative Wnt pathways are thought to involve G-proteins, MAP kinases, and calcium-dependent enzymes [Sheldahl et al., 1999, 2003; Yamanaka et al., 2002]. Based upon the available literature, Wnt 9a (downregulated during osteogenesis) is likely to signal in a manner similar to the canonical Wnt 1 [Hartmann and Tabin, 2001], while Wnt 11 (upregulated during osteogenesis) is thought to signal through the non-canonical Wnt pathways [Marlow et al., 2002; Pandur et al., 2002]. We have also demonstrated that MSCs are also capable of
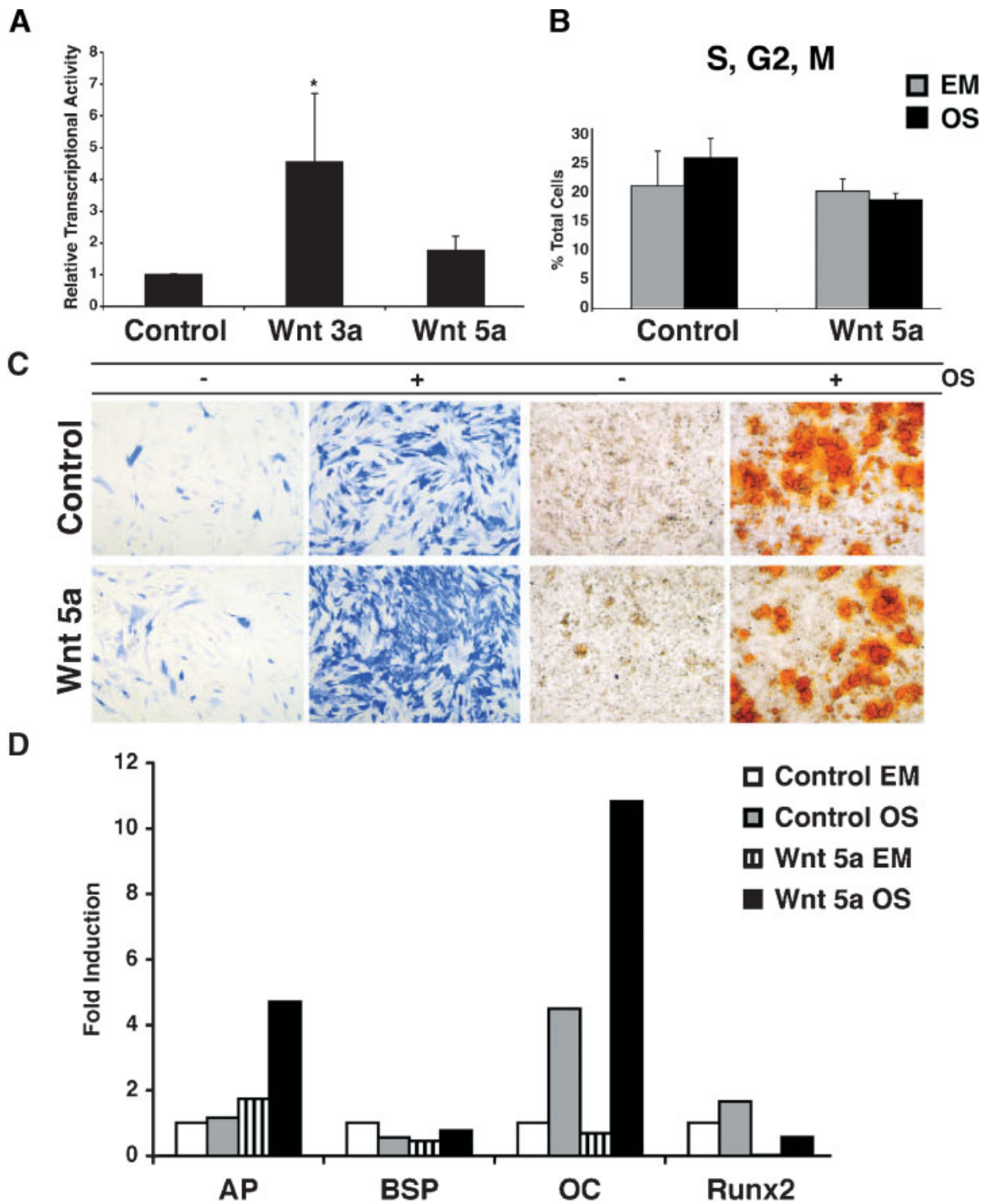
Fig. 10. Wnt 5a enhances MSC osteogenesis, but does not activate canonical Wnt signaling. **A**: TCF-responsive transcriptional activation. MSCs transfected with Wnt 3a showed a statistically significant increase in TCF-responsive transcriptional activity (*$P < 0.05$) as assayed by the Wnt-responsive reporter, pGL3-OT, while MSCs transfected with Wnt 5a showed a much lower level of activation. **B**: FACS analysis. MSCs expressing Wnt 5a did not show increases in proliferation as compared to control MSCs. **C**: Alkaline phosphatase activity and mineralization. MSCs transfected with Wnt 5a displayed increased alkaline phosphatase activity as compared to empty vector controls, while the level of mineralization was not dramatically different between the two groups at this time point. **D**: Semi-quantitative RT-PCR. The mRNA expression of AP and OC was increased in the osteogenic cultures transfected with Wnt 5a, while BSP and Runx2 mRNA expression was not significantly affected by Wnt 5a expression.

responding to canonical Wnt signaling through intracellular β-catenin and the TCF family of transcriptional activators with the magnitude of transcriptional activation being dependent upon the differentiation state of MSCs. These differences in transcriptional activation are probably a result of crosstalk between the canonical Wnt signaling cascade and other signaling pathways that also act in the maintenance and differentiation of MSCs, such as noncanonical Wnt signaling and members of the TGF-β family of proteins [Fischer et al., 2002b; Tuli et al., 2003b].

We have shown that exposure of MSCs to Wnt 3a during the process of differentiation suppresses osteogenesis. This suppression of osteogenic markers was seen even in MSCs previously committed to the osteogenic lineage and subsequently exposed to Wnt 3a. The Wnt inhibition of MSC osteogenesis was accompanied by increases in cell proliferation and decreases in apoptosis. Similar to the pattern of activation of Wnt-responsive transcription, exposure of undifferentiated MSCs to Wnt 3a had a more dramatic effect on cellular behavior (i.e., increased proliferation and decreased apoptosis) as compared to osteogenic cultures. A recent microarray study of human MSC osteogenesis has demonstrated a downregulation of key proteins involved in proliferation upon osteogenic differentiation, such as the Wnt-responsive gene myc [Qi et al., 2003], and there is a naturally occurring transition from a more proliferative to a less proliferative phenotype during osteogenic differentiation [Bianco et al., 1993]. These Wnt 3a effects on MSC proliferation and differentiation were observed in MSCs derived from various sources and with two different means of Wnt 3a exposure, indicating that MSCs are likely to be genuine target cells for Wnt signaling. The observed Wnt 3a effects were also shown to be fully reversible (i.e., with no apparent difference in the ability of MSCs to undergo osteogenesis even after being previously treated with Wnt 3a for 12 days) when exposure occurred either during expansion or osteogenic induction.

The expression of the noncanonical Wnt coreceptor, ROR2, and the secreted Wnt inhibitors, SFRP2 and SFRP3, were also upregulated during osteogenic differentiation of MSCs. Recently, mutations in the Wnt antagonist, sFRP1, have been associated with increased levels of trabecular bone formation. sFRP1 null mice display increased trabecular bone mineral density and volume as compared to wild type controls. This increase in trabecular bone mass and mineralization occurs in both sexes, at multiple skeletal sites, and during the first year of life. The proposed mechanism involves a reduction in osteoblast and osteocyte apoptosis in vivo [Bodine et al., 2004]. To gain additional insight into the regulation of Wnt responsiveness, sFRP3 (shown to be upregulated during osteogenesis) was added to MSC cultures in the presence or absence of Wnt 3a to examine its effect on the observed Wnt 3a-mediated suppression of osteogenic markers. In both MSCs undergoing osteogenesis and osteogenically differentiated MSCs later exposed to Wnt 3a, sFRP3 attenuated the suppressive effects of Wnt 3a on Wnt target genes, AP and BSP (Fig. 7). This observation further supports the hypothesis that the upregulation of Wnt inhibitors during MSC osteogenesis also plays a role in attenuating canonical Wnt signals to regulate MSC osteogenesis. Consistent with this hypothesis, we have also shown that specific inhibition of canonical Wnt signaling using a dominant negative TCF1 results in an enhancement of MSC osteogenesis (Fig. 9).

In keeping with the observed suppressive role of canonical Wnt signaling on MSC differentiation is the downregulation of Wnt 9a, which is reported to play a role in suppression of chondrogenesis and in stimulation and formation of the joint cavity [Hartmann and Tabin, 2001]. In a recent microarray study focusing on candidate genes involved in the maintenance of a pluripotent undifferentiated phenotype, FZD7 was found to be highly expressed by undifferentiated embryonic stem cells [Sperger et al., 2003]. In light of our observed downregulation of FZD7 upon osteogenic differentiation and the Wnt 3a effects on progenitor cell maintenance and proliferation, it is interesting to consider the potential role of Frizzled 7-mediated canonical Wnt signaling on in vivo progenitor cell pools. The MSC expression profile also revealed an upregulation of WNT11 during osteogenesis. Interestingly, this noncanonical Wnt 11 has been implicated in human embryonic skeletal development [Lako et al., 1998], and is known to be upregulated at an mRNA level during adult human MSC chondrogenesis [Sekiya et al., 2002]. Unlike the canonical Wnts, Wnt 11 has been categorized as one of the few identified pro-differentiation Wnts, acting through a pathway

independent of β-catenin and involving PKC and jun-terminal kinase (JNK) [Eisenberg et al., 1997; Eisenberg and Eisenberg, 1999; Pandur et al., 2002].

Recent investigations have strongly suggested that there may be antagonism between the various downstream Wnt signaling pathways, with one pathway regulating the activity of the other [Topol et al., 2003; Westfall et al., 2003; Yang et al., 2003]. In accordance with this model, our observation of the upregulation of both a "non-canonical" *WNT11* [Heisenberg et al., 2000] and *FZD6* [Sheldahl et al., 1999; Golan et al., 2004] concurrent with the downregulation of *WNT9A* and *FZD7*, known to signal in the more traditional, β-catenin related Wnt pathway [Sheldahl et al., 1999; Sumanas et al., 2000], could be an example of this type of cross-regulation during normal osteogenic differentiation of MSCs. In support of this proposed "switch" from canonical to noncanonical Wnt signaling during the course of osteogenic differentiation is our observation here that exposure of MSCs to noncanonical Wnt signaling during osteogenic induction, by the overexpression of Wnt 5a, results in enhancement of osteogenic differentiation (Fig. 10).

Recent studies have also demonstrated the importance of the canonical Wnt co-receptor, LRP5, in the postnatal maintenance of bone [Gong et al., 2001; Kato et al., 2002]. Activating LRP5 mutations lead to patients with increased bone density [Boyden et al., 2002; Little et al., 2002; Babij et al., 2003; Van Wesenbeeck et al., 2003], while loss-of-function mutations in LRP5 result in OPPG, in which patients show decreased bone density and destructive bone changes as they age [Gong et al., 2001]. Certain cancers of the musculoskeletal system are also thought to involve alterations in Wnt signaling. Recently, LRP5 has been established as a marker for osteosarcoma, since increased LRP5 expression has not only been found in samples from osteosarcomas, but the expression of LRP5 was correlated with higher grade osteosarcomas (demonstrating a more undifferentiated phenotype) and decreased event-free survival (153). Interestingly, we observe here that canonical Wnt signaling inhibits MSC osteogenic differentiation in a fully reversible manner, and this inhibition of differentiation is accompanied by stimulatory effects on cellular proliferation. Preliminary studies support the hypothesis that the observed Wnt 3a effects are mediated by LRP5, since expression of a truncated, soluble LRP5 protein can partially attenuate the Wnt 3a suppression of osteogenesis (unpublished observation).

Based on these observations, we propose a model for the role(s) of Wnt signaling in MSC differentiation and maintenance. In this model, MSCs exist in two functional compartments: (1) the progenitor cell compartment in which they proliferate and expand, and (2) the differentiation compartment where they undergo lineage commitment and phenotypic changes to become a specific differentiated cell type. We propose that the in vivo role of canonical Wnts, such as Wnt 3a (used in these studies) or Wnt 9a (downregulated during MSC osteogenesis), is in the maintenance of an undifferentiated, proliferating progenitor cell population from which progenitor cells are then released in order to differentiate. Additionally, we propose that non-canonical Wnt signaling, e.g., by Wnt 5a or Wnt 11 (seen to be upregulated during normal MSC osteogenesis and chondrogenesis [Sekiya et al., 2002]), is involved in regulating and promoting the differentiation process. This model predicts that enhancement of canonical Wnt signaling leads to an increase in the progenitor population of cells. These progenitors would eventually be released from suppression of differentiation by specific regulation of Wnt signaling, spatial or temporal control of Wnt signals, and/or crosstalk with other signaling pathways (e.g., noncanonical Wnts, BMPs). The progenitors would then be available to respond to environmental signals normally regulating lineage commitment (i.e., promoting osteogenesis, chondrogenesis, adipogenesis). The terminal cell fate decision may be mediated by a variety of signals and may be localized or controlled by crosstalk between various signaling pathways and cell types. In accordance with this model, stimulation of canonical Wnt signals as represented by activating mutations of LRP5 could, indeed, promote bone formation as an indirect result of expanding the progenitor cell compartment. On the other hand, mutations resulting in the inactivation of LRP5 could lead to a low bone-mass phenotype as a result of a targeted effect on the self-renewing progenitors, again as suggested by this model.

The recent identification of the orphan receptor tyrosine kinase, Ror2, as a coreceptor for non-canonical Wnt signaling via Wnt 5a [Oishi et al., 2003] is very interesting in light of our

observed upregulation of *ROR2* upon osteogenic induction of MSCs. Also of interest is the fact that mutations in Ror2 are associated with human skeletal dysplasias, such as Robinow Syndrome [Afzal et al., 2000; van Bokhoven et al., 2000], and that both Ror2 and Wnt 5a knockout mice display similar skeletal dysmorphogeneses [Oishi et al., 2003]. We also have demonstrated that unlike the suppressive effects of Wnt 3a on osteogenesis, Wnt 5a expression during osteogenic induction of MSCs actually results in an enhancement of MSC osteogenesis. Since MSC cultures demonstrate an upregulation of noncanonical Wnt 11 during the process of in vitro osteogenesis and Wnt 11 has been demonstrated to be capable of inhibiting canonical Wnt signaling through various intracellular pathways [Maye et al., 2004], we hypothesize that selective changes in Wnt signaling can regulate the transition of MSCs from a less differentiated to a more differentiated state, and that alterations in Wnt signaling may act in vivo to both maintain a pool of reserve progenitor cells and to modulate the transition of progenitors into specific differentiated cell types.

Several recent studies of canonical Wnt effects on both osteogenesis and chondrogenesis in various in vitro systems [Gong et al., 2001; Fischer et al., 2002b; Bain et al., 2003; Rawadi et al., 2003] have reported results that are apparently inconsistent with our observations in human MSCs. Many of these studies have used various murine cell lines (e.g., C3H/10T1/2, MC3T3-E1, and ST2 cells), and the variability in the cell sources (from embryonic to postnatal) and species (from murine to human), are likely to influence the downstream responses of the cells to signaling molecules, such as Wnts. In our laboratory, experiments run concurrently with those presented here, but using murine C3H/10T1/2 cells, have also yielded very different results than those seen in human MSCs [Derfoul et al., 2004]. Many reports have shown an increase in alkaline phosphatase activity and expression in C3H/10T1/2 cells in response to Wnt 3a with further increases in differentiation markers upon the addition of BMP-2 [Gong et al., 2001; Fischer et al., 2002b; Bain et al., 2003; Rawadi et al., 2003]. These results support a role for Wnt signaling in mesenchymal differentiation, although in none of these studies did the addition of Wnt 3a (or activated β-catenin) alter the expression of osteocalcin, a marker of differentiated osteoblasts, unless BMP-2 was added also. There are also known interactions between the BMP and Wnt pathways [Jamora et al., 2003], and there may be a temporally specified role for the various Wnts during mesenchymal differentiation. The interesting observation here is that, although the effects of Wnt 3a treatment on AP expression and activity in C3H/10T1/2 cells and adult human MSCs are different (i.e. activation vs. suppression), the target being regulated, AP, remains the same. It is well known that TCFs can act both as transcriptional activators as well as transcriptional repressors, and the difference appears to lie in the cofactors present within a given cell. It is conceivable that the cellular context is different between the embryonic mouse cell lines and adult human stem cells, and therefore different modulating cofactors may be present that result in a Wnt-mediated activation of AP in embryonic cells and a repression of AP in these adult human cells. Also noteworthy is that many of these recent studies have focused on the effect of Wnts in the context of BMP stimulation, and it is very likely that Wnts may act in priming cells for BMP signaling or in titrating the response or timing of cellular responses to BMPs. Also of interest is a recent publication that demonstrates an inhibitory action of mouse Frizzled 1 (but not human Frizzled 1) on canonical Wnt signaling in C2C12 and Cos-7 cells, which leads to repression of Wnt 3a- or BMP-2-induced alkaline phosphatase activity [Roman-Roman et al., 2003]. These results again emphasize the subtle, but critical, interspecies and/or developmental differences that can influence outcomes in these highly specific signaling cascades. Our study represents the first look at the direct response of adult human MSCs to canonical Wnt signaling in the absence of other growth factors, such as BMPs. Therefore, some of the apparent discrepancies between our observations and those in the literature may be due to differences in the cellular background (embryonic vs. adult), species (murine vs. human), and/or the specific experimental conditions and stimuli used.

Our ongoing studies aim to further examine the timing and location of in vivo Wnt signaling, identify the specific Wnts and Frizzleds involved in MSC maintenance, lineage commitment, and differentiation, elucidate the downstream mediators of the various Wnt signaling

pathways, and analyze the nature of crosstalk with other signaling pathways. Such information should enhance our understanding of the process of adult tissue regeneration to facilitate lineage commitment in vivo, and provide a rational foundation for cell-based tissue repair in the adult human.

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