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ARTICLES

Cross-Talk Between Wnt Signaling Pathways in Human Mesenchymal Stem Cells Leads to Functional Antagonism During Osteogenic Differentiation

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Abstract Wnt signaling is involved in developmental processes and in adult stem cell homeostasis. This study analyzes the role(s) of key Wnt signaling mediators in the maintenance and osteogenesis of mesenchymal stem cells (MSCs). We focus specifically on the involvement of low-density lipoprotein-related protein 5 (LRP5), T-cell factor 1 (TCF1), and Frizzled (Fz) receptors, in the presence or absence of exogenous, prototypical canonical (Wnt3a), and non-canonical (Wnt5a) Wnts. In undifferentiated MSCs, LRP5 and TCF1 mediate canonical Wnt signal transduction, leading to increased proliferation, enhanced synergistically by Wnt3a. However, LRP5 overexpression inhibits osteogenic differentiation, further suppressed by Wnt3a. Wnt5a does not affect cell proliferation but enhances osteogenesis of MSCs. Interestingly, Wnt5a inhibits Wnt3a effects on MSCs, while Wnt3a suppresses Wnt5a-mediated enhancement of osteogenesis. Flow cytometry revealed that LRP5 expression elicits differential changes in Fz receptor profiles in undifferentiated versus osteogenic MSCs. Taken together, these results suggest that Wnt signaling crosstalk and functional antagonism with the LRP5 co-receptor are key signaling regulators of MSC maintenance and differentiation. J. Cell. Biochem. 101: 1109–1124, 2007.

Key words: mesenchymal stem cells; Wnt; LRP5; osteogenesis

Wnt signaling plays a central role in early developmental processes, including morphogenesis, patterning, cell movement, and cell polarity [Cadigan and Nusse, 1997; Veeman et al., 2003], as well as postnatal development and tissue homeostasis [Murdoch et al., 2003; Moon et al., 2004]. Recently, Wnts have been implicated to play a critical role in regulating the differentiation potential of adult human mesenchymal stem cells (MSCs) [Boland et al., 2004; De Boer et al., 2004a,b; Etheridge et al., 2004]. However, the details of the mechanism of Wnt signaling in MSC cell fate determination have yet to be fully elucidated. Determining the signaling mechanism involved in controlling MSC during differentiation is important for the understanding of MSC function and will be critical in designing novel therapeutic strategies utilizing MSCs.

To date, 19 members of the Wnt family have been identified in mammals and, depending on the Wnt, can signal through essentially two classes of Wnt signaling pathways, the canonical and non-canonical Wnt pathways [Du et al., 1995; Kuhl et al., 2000]. The current model of canonical Wnt signaling states that, in the absence of a Wnt ligand, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β), in association with axin and adenomatous polyposis coli (APC), which targets β-catenin for ubiquitinylation and subsequent degradation by proteasomes [Cadigan and Nusse, 1997]. However, when a Wnt ligand binds to its cognate Frizzled (Fz) receptor, as well as to the Wnt co-receptor, low-density lipoprotein-related protein 5 (LRP5),

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the cytoplasmic protein Disheveled (Dvl) is activated. Phosphorylation of β-catenin by GSK-β is inhibited by Dvl, causing β-catenin stabilization and accumulation, before translocation to the nucleus, where it binds with members of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) transcription factors, resulting in the enhanced expression of target genes, such as c-myc and cyclin D1 [He et al., 2004]. Misregulation or aberrant activation of canonical Wnt signaling has been implicated in many forms of human cancers [Polakis, 2000; van Es et al., 2003].

Non-canonical Wnt signaling is distinguished from canonical Wnt signaling in that signal transduction is not mediated through β-catenin, but rather through other signal transduction mediators, such as those that regulate intracellular calcium [Kuhl et al., 2000]. Non-canonical Wnts also play essential roles in development and in postnatal developmental processes. During development, prototypical non-canonical Wnts, such as Wnt5a, fail to signal through β-catenin or induce axis duplication in *Xenopus* embryo, but instead interfere with morphogenetic movements of the convergent extension process that occurs during the process of gastrulation [Du et al., 1995; Kuhl et al., 2000]. In the adult human, Wnt5a inhibits B-cell proliferation through non-canonical Wnt signaling and functions as a tumor suppressor in hematopoietic malignancies [Liang et al., 2003].

Both classes of Wnt ligands transduce signals utilizing membrane Fz receptors. To date, there exist 10 identified Fz receptors in mammals that share a conserved extracellular cysteine-rich domain for Wnt binding [Bhanot et al., 1996b; Rulifson et al., 2000]. However, the affinities between Fz family members and different Wnt ligands vary [Bhanot et al., 1996a; Cadigan and Nusse, 1997], suggesting that the specificity of the signal resides in the ligand–receptor interaction. In addition to Fz receptors, recent evidence demonstrates the involvement of LRP5 as a co-receptor for canonical Wnt signaling [Liu et al., 2005]. Mutations in LRP5 have been shown to result in familial osteoporosis (loss-of-function mutation), high bone density syndromes (gain-of-function mutation), and ocular disorders in the postnatal human [Gong et al., 2001; Boyden et al., 2002; Little et al., 2002]. Theses studies underscore the importance of LRP5 as a key mediator in Wnt signaling and its potential as a therapeutic target in bone mass regulation. However, the exact role of LRP5 in human adult MSCs during the process of osteogenesis, specifically the transit from undifferentiated to differentiated phenotype, is currently poorly understood.

Although MSCs have been isolated and cultured successfully and their phenotypic and functional properties well characterized [Caplan, 1991; Bruder et al., 1997; Pittenger et al., 1999], the mechanisms regulating MSC developmental potential remain to be elucidated. This study aimed to examine how key Wnt mediators might regulate adult MSC maintenance and differentiation. We specifically investigated the effects of important canonical Wnt mediators, such as LRP5, Fz receptors, and transcriptional regulators, such as TCF1, during MSC maintenance and during the process of osteogenesis.

MATERIALS AND METHODS

Human Mesenchymal Stem Cell Harvest and Culture

Human bone marrow cells were harvested from the femoral heads of consenting patients who underwent total hip arthroplasty with IRB approval (George Washington University and National Institutes of Health) ($N = 4$, ages 55–65). Adherent cells were maintained in monolayer cultures in expansion medium (EM), consisting of Dulbecco's-modified essential medium (DMEM) (Gibco/Invitrogen Corp.), 10% fetal bovine serum (FBS, Gibco), and 10% antibiotic solution of penicillin G (167 units/ml) (Sigma), gentamicin (50 μg/ml) (Sigma), and amphotericin B (0.3 μg/ml) (Sigma). Adherent cells were passaged at 1:3 at 80% confluence. Medium was refreshed twice a week. All experiments described were performed on passage 2 MSCs.

Misexpression Studies

To study directly the effects of candidate canonical Wnt mediators, the following plasmid constructs were transfected (4 μg per 500,000 cells) into MSCs using a nucleofection protocol (amaxa Biosystems; [Haleem-Smith et al., 2005]): wild type LRP5 (LRP5 WT) (provided by Dr. Bjorn R. Olsen, Harvard Medical School); inhibitory, truncated secreted LRP5 (LRP5 TS) (Dr. Bjorn R. Olsen); dominant negative TCF1 (dnTCF1) (provided by Dr. Hans Clever,

Netherlands Institute for Developmental Biology); TCF1 (Upstate Cell Signaling Solutions); or pcDNA3/pUSEAmp (empty vector, EV, control; Upstate Cell Signaling Solutions), where appropriate. LRP5 TS lacks the intracellular domain of the wild-type LRP5 construct and, therefore, competitively blocks Wnt/Fz signaling in a dominant-negative fashion. Each transfected group was assayed for their proliferative potential, colony forming unit-fibroblast (CFU-F), and -osteoblast (CFU-O) development with or without additional Wnt treatment. Transfection efficiency was determined by dsRED expression. Briefly, MSCs were transfected with 2 μg of dsRED plasmid (BD Biosciences) and the percent positive dsRED was determined by flow cytometry on Day 4, 10, 20, and 35 post-transfection.

Exogenously Derived Wnt-Conditioned Media

Representative canonical and non-canonical Wnts (Wnt3a and Wnt5a, respectively) were added to cultures of adult human MSCs maintained as undifferentiated cells in EM and under osteogenic conditions (αMEM-supplemented with 10% FBS, 10 nM dexamethasone, 50 μg/ml L-ascorbic acid, and 3.5 mM β-glycerophosphate (all from Sigma). Wnt3a and Wnt5a proteins were applied as conditioned medium collection from mouse L-cells that were stably transfected with either Wnt3a or Wnt5a plasmid (L-cells, Cat#: CRL-2648; L-Wnt3a cells, Cat#: CRL-2647; and L-Wnt5a cells, Cat#: CRL-2814; American Type Culture Collection). Control-conditioned medium (CM), Wnt3a CM, and Wnt5a CM were prepared according to the supplier's protocol. Two rounds of conditioned media were collected, pooled, and diluted 50% with DMEM for final use in experiments. This dose of Wnt CM was chosen based on a dose–response experiment, which involved exposing MSCs transfected with the TOP-FLASH reporter plasmid (details below) to 100%, 50%, 20%, and 10% of each CM stock (CM, Wnt3a, and Wnt5a), the difference made up with DMEM, for 4 days. On Day 4, the luciferase activity (details below) was determined.

Luciferase Reporter Assays

An optimized version of the TOP-FLASH luciferase reported plasmid (pLG3-OT) (provided by Dr. Bert Vogelstein, Johns Hopkins University) was used to assay canonical Wnt-responsive transcriptional activity. The plasmid contains three copies of the wild type TCF-4 binding sites cloned into the pGL3-OT plasmid (Promega). LRP5 WT, LRP TS, dnTCF1, and EV groups were co-transfected with Renilla luciferase plasmid (1 μg) attached to the CMV promoter (Promega) and pLG3-OT (2 μg) by nucleofection (amaxa Biosystems). To study osteogenic transcriptional activity, parallel experiments were performed using the Runx2 responsive luciferase reporter construct (6xOSE-2) (a gift from Dr. Gerard Karsenty, Baylor College of Medicine), and co-transfection with LRP5 WT, LRP5 TS, dnTCF1, and EV. Reporter luciferase activities were assayed in cultures maintained in both undifferentiated and osteogenic conditions using the Dual Lucifease Assay System (Promega), normalized to Renilla luciferase activity, and expressed relative to that of cultures in control CM conditions.

Immunohistochemistry of LRP5/6

MSCs from the transfected groups were cultured on glass coverslips (Cat#: 12-548-B, Fisher Scientific). After 4 days, cultures were washed in phosphate buffered saline (PBS), fixed in 4% paraformaldyhe (FD Neuro Technologies), permeabilized with 0.1% Triton-X 100 solution, and then blocked with non-specific goat serum (2% in PBS) prior to the addition of 2 μg/ml LRP5/6 monoclonal antibody (Cat#: 3801-100, BioVision). Immunolabeling was detected with goat anti-mouse IgG-conjugated to TRITC (1:250 dilution, Cat#: T-5393, Sigma), and staining was observed using epifluorescence. Negative controls consisted of incubations with secondary antibodies only.

Cell Proliferation Assay

To assay cell proliferation, cells were seeded at 1,000 cell/cm^2 in 12 well-plates. The MTS assay (Promega) was performed on Days 4, 7, and 14. Cell numbers were determined based on A_{490} using a standard curve calibrated using known cell numbers.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of cyclin D1 mRNA transcripts in MSC cultures assayed for proliferation was examined by semi-quantitative RT-PCR at Days 7, 14, and 21, the expression of LRP5 mRNA after 4 days post-transfection in misexpression and overexpression experiments and the expression Runx2 after 4 days

post-6xOSE-2 luciferase experiments. The primers (5′ to 3′) used were: (1) human (h) cyclin D1 (forward, TCC TGT GCT GCG AAG TGG AAAC; reverse, AAA TCG TGC GGG GTC ATTG); (2) hLRP5 (forward, GCT GGA ATG GAT GGT TCA AGT CG; reverse, CAG AAT GGA TTT CAC GCA GAC CC); (3) human Runx2 (forward, CCG CAC GAC AAC CGC ACC AT; reverse, CGC TCC GGC CCA CAA ATC TC), and (4) hGAPDH (forward, CCA GAA CAT CAT CCC TGC CTC TAC; reverse, GGT CTC TCT CTT CCT CTT GTG C). Reaction conditions were: 94°C for 3 min, 94°C for 30° s, 55°C for 60 s, and 60°C for 60 s; 30 cycles of amplification; and final incubation at 72°C for 5 min. The amplified products from 1 μg total RNA per sample were resolved on 1.5% Tris-acetic acid-EDTA (TAE; pH 8.0) agarose gel and visualized with ethidium bromide. All amplified products were detected as single bands of the expected sizes.

CFU-F and CFU-O Assay

Cell conditions were grown under CFU-F and CFU-O assay conditions and the developmental potential of MSCs seeded at 1,000 cell/cm^2 were assessed according to methods described previously [Baksh et al., 2003]. CFU-F development was assessed by Giemsa staining (Sigma) and CFU-O were visualized by alkaline phosphatase (ALP) staining at Day 21, according to the manufacturer's protocol (Sigma kit 86-R).

Flow Cytometry

The expression profiles of a subset of Fz receptors on MSCs were quantified by flow cytometry. MSCs were incubated with saturating concentrations (10 μg/ml) of mouse antibodies to human Fz proteins (Fz1, MAB11201; Fz3, MAB1001; Fz4, MAB194; Fz6, MAB1526; and Fz7, MAB1981; all from R&D Systems). These Fz receptors were chosen in part because of the commercial availability of antibodies. Cells labeled with the primary antibodies to Fz1, Fz3, Fz4, and Fz7 were subsequently stained with Alexa Fluor 488 goat anti-mouse IgG (1:1,000, Cat#: A21121, Molecular Probes), and cells labeled with Fz6 antibodies were stained with Alexa Fluor 488 goat anti-rat IgG (Cat#: A11006, Molecular Probes) to visualize positive expressing cells. The cell suspension was washed twice with phosphate-buffered saline (PBS) and resuspended in PBS + 2% FBS for analysis on a flow cytometer (FACSCalibur, BD Biosciences) using the CellQuest software. Positive staining was determined based on fluorescence emission, which exceeded negative control samples (secondary antibody staining only).

Wnt Signaling Pathway Gene Expression Analysis

A Wnt signaling pathway-specific gene microarray (Cat.#: EHS-043, SuperArray Biosciences, Frederick, MD) was used to detect changes in 113 candidate genes associated with the Wnt signaling pathway. RNA was harvested using Trizol Reagent (Invitrogen) from MSCs transfected with LRP5 WT, LRP5 TS and EV, which were grown in the presence of Control, Wnt3a, and Wnt5a CM, under basal, undifferentiated conditions, for 4 days. A total of 3 μg of RNA from each group was converted to cDNA, and cRNA labeling, amplification, hybridization was performed overnight and detection of gene expression was determined the next day, all according to the manufacturer's protocol (SuperArray). A total of 4 μg of cRNA was hybridized to each microarray gene chip. Relative changes in gene expression was analyzed and quantified based on densiometry using the GEarray expression analysis suite software (SuperArray) ($N = 2$). RT-PCR was used to confirm the enhancement in gene expression related to the overexpression of LRP5.

Data Analysis

Data are presented as mean values ± SD for at least three independent experiments (different patient samples) performed in triplicate, unless stated otherwise. Statistical significance is determined by ANOVA and set at $P < 0.05$.

RESULTS

Wnt5a Suppresses Wnt3a-Mediated TOP-FLASH Reporter Activity in Undifferentiated MSCs

First, we confirmed that the bone marrow-derived adherent populations used in these studies expressed the cell surface receptors CD44, CD105, CD106, and Stro-1 (data not shown) and hence, we referred to these cells as MSCs. To examine the early effects of candidate Wnt mediators, namely LRP5 and TCF1, on canonical Wnt transcriptional activity, MSCs were transfected with the TOP-FLASH reporter construct and cultured in Wnt3a, Wnt5,

or both Wnt3a and Wnt5a (referred to as Wnt3a/5a hereon) CM under undifferentiated or osteogenic conditions. The Wnt3a/5a CM group was included to determine how these two functionally different Wnts might interact to affect the developmental potential of MSCs. In order to maximize the differential effects of Wnt3a and Wnt5a, we determined that a dose of 50% of control CM, and Wnt3a and Wnt5a CM from L-cells was a sufficient dose to use in our medium formulation for all studies described, as this concentration elicited TOP-FLASH reporter activity levels similar to that seen in stock media (100%) (Fig. 1). According to the manufacturer's protocol (ATCC), the final conditioned media collection would result in ~100–200 ng/ml of Wnt3a and Wnt5a protein and thus, 50% of the stock media would translate into ~50–100 ng/ml of Wnt3a and Wnt5a protein in the final media formulation.

We then confirmed that in undifferentiated MSCs, treatment with Wnt3a enhanced TOP-FLASH reporter activity, while Wnt5a had no apparent effect on TOP-FLASH activity relative to the EV control group (Fig. 2A). Interestingly, when MSCs were grown in Wnt3a/5a CM, ~50% reduction in reporter activity was observed, relative to the Wnt3a-alone treatment group (Fig. 2A). The overexpression of LRP5 (LRP5 WT) resulted in an even greater increase in TOP-FLASH reporter activity in the presence of Wnt3a, relative to control conditions; similar results were observed when MSCs were transfected with TCF1 (data not shown). Even in this treatment group (LRP5 WT), Wnt5a alone showed no effect on TOP-FLASH transcriptional activity, suggesting that Wnt5a is unlikely to be a functional activating ligand of LRP5 for canonical signaling. Interestingly, Wnt5a retained the ability to significantly abrogate the effects of Wnt3a in this transfected group. Functional blocking of LRP5 (LRP5 TS transfection) also suppressed the effects of Wnt3a to enhance TOP-FLASH reporter activity. Furthermore, co-transfection of MSCs with both dnTCF1 and LRP5 TS also dramatically suppressed the Wnt3a effects.

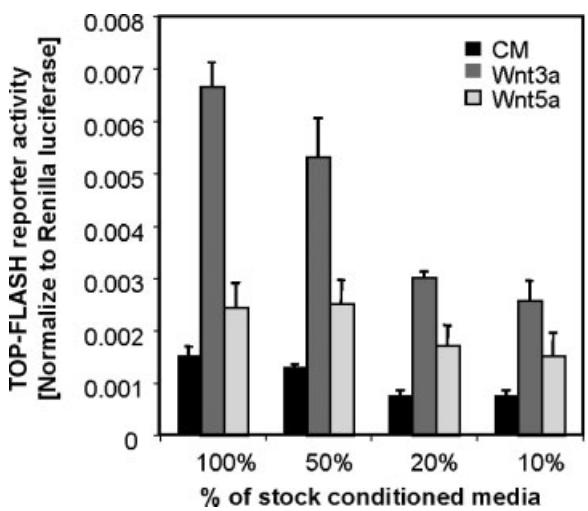


Fig. 1. Dose effect of L-cell-conditioned medium on TOP-FLASH reporter activity in human MSCs. Conditioned medium was collected from Wnt3a, Wnt5a, and control L-secreting cells (ATCC) and diluted to 50%, 20%, and 10% with DMEM. MSCs were transfected with the TOP-FLASH reporter plasmid and grown in the presence of these different concentrations of Wnt-conditioned media. After 4 days, luciferase activity was determined. Bars represent the mean ± SD (N = 2). Comparable TOP-FLASH activation was seen at 50% and 100% in Wnt3a-conditioned medium.

Under conditions promoting osteogenic differentiation, Wnt responsive activity was predominantly at basal levels in the various transfected groups (Fig. 2B). The only significant but mild Wnt responsive transcriptional activity was observed in the LRP5 WT group with Wnt3a supplementation. We confirmed on the basis of dsRED expression that the transfection efficiency associated with these short-term studies was 68 ± 4% (data not shown).

Next, using the Wnt signaling pathway-specific gene microarray, we analyzed the upregulation of LRP5 gene expression due to transfection in all Wnt treatment groups, relative to that in EV-transfected MSCs (Fig. 2C). RT-PCR was subsequently performed to confirm the overexpression of LRP5 observed on the gene microarray (Fig. 2D). Interestingly, transfection with LRP5 TS decreased the level of LRP5 gene expression detected in MSCs grown in CM, Wnt3a, and Wnt5a CM (Fig. 2C). We also verified, on the basis of immunostaining, that transfection of MSCs with LRP5 WT or LRP TS in MSCs resulted in the increase or slight reduction, respectively, of expression of membrane-associated LRP5, relative to EV control conditions (Fig. 2E). Expression of transfected LRP5 (both WT and TS) was unaffected by the presence of Wnt3a or Wnt5a protein in the conditioned medium (Fig. 2E). Taken together, these results suggest that transfection with a truncated secreted LRP5 expression vector affects both gene and protein expression of LRP5.

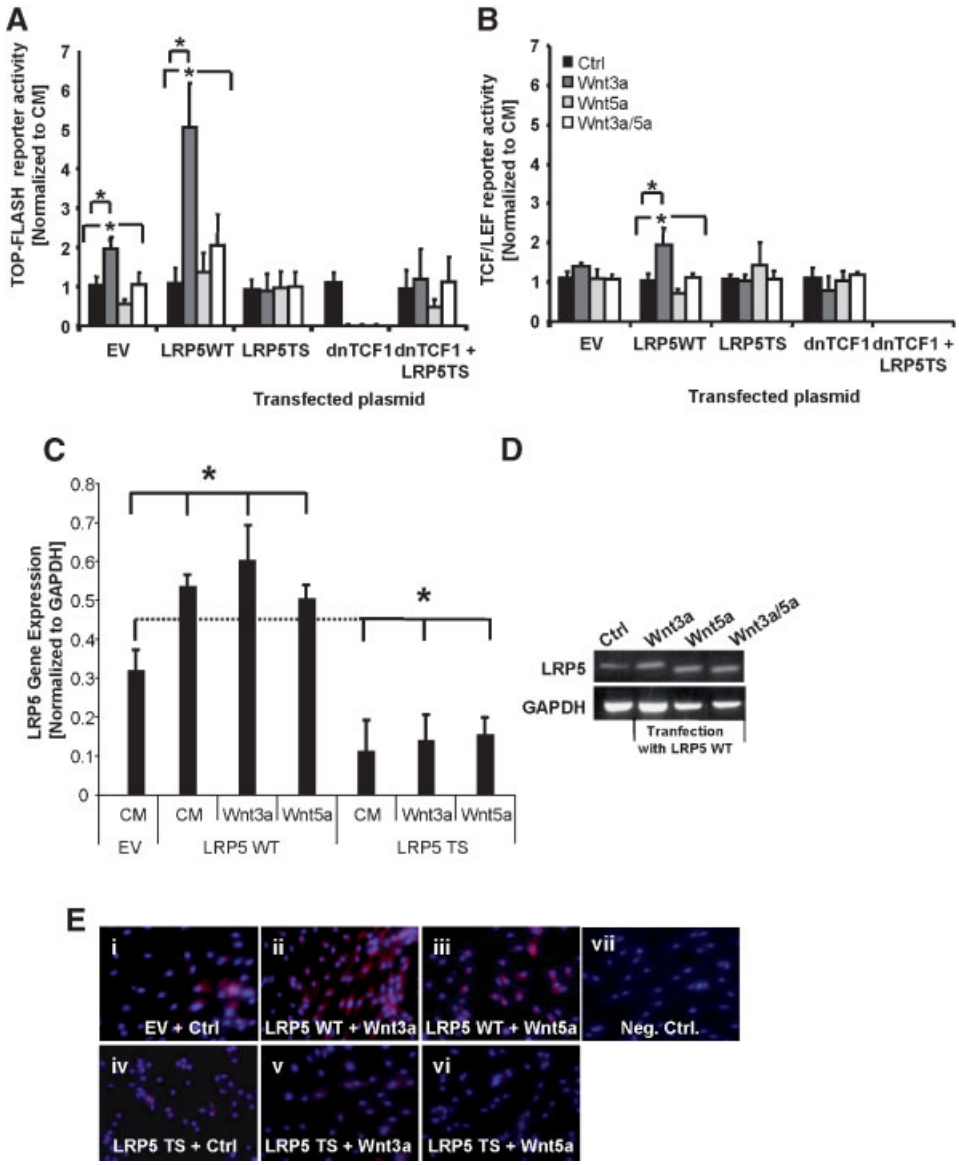


Fig. 2. Effects of LRP5 and TCF1 expression in human MSCs on TOP-FLASH transcriptional activity in the presence and absence of Wnt3a and Wnt5a. Day 4 TOP-FLASH reporter activity in MSCs transfected with empty vector, LRP5 WT, LRP TS, dnTCF1 or dnTCF, and LRP5 TS, and subsequently grown in Ctrl, Wnt3a, Wnt5a, or Wnt3a/5a CM under (**A**) undifferentiated and (**B**) osteogenic conditions. Each TOP-FLASH luciferase value was normalized to its internal transfection control values as determined by Renilla luciferase activity, and further normalized to Ctrl CM values. MSCs transfected with LRP5 WT and then cultured in Wnt3a-supplemented medium showed a significant enhancement in Wnt responsive activity in undifferentiated conditions, while basal level Wnt transcriptional activity was observed in osteogenic conditions. Each bar represents the mean ± SD from n = 4 bone marrow samples, performed in triplicate. $^*P < 0.05$. **C**, **D**: Relative changes in LRP5 gene expression in MSCs transfected with LRP5 WT and LRP5 TS. C: The Wnt signaling pathway gene array was used to determine the changes in LRP5 gene expression following transfection with LRP5 WT and LRP5 TS plasmids. Following transfection, MSCs were grown in CM, Wnt3a and Wnt5a-conditioned media. Analysis was performed on Day 4 cultured cells. D: RT-PCR was used to confirm LRP5 gene expression in LRP5 WT-transfected MSCs grown in Wnt3a, Wnt5a, and Wnt3a/5a-conditioned media. MSCs transfected with EV and grown in Ctrl CM conditions was used as a control for the endogeneous expression of LRP5. Results showed that over-expression of LRP5 WT significantly enhanced LRP5 expression, while transfection with LRP5 TS significantly decreased LRP5 relative to EV-transfected MSCs grown in CM. $^*P < 0.05$. **E**: LRP5 level in MSCs transfected with wild type (WT) or truncated secreted form (TS) LRP5 was assessed by immunofluorescence staining followed by nuclear counterstaining with Hoechst dye. (i) Empty vector (EV)-transfected MSCs cultured in control medium (Ctrl); (ii & iii) MSCs transfected with LRP5 WT cultured in Wnt3a CM and Wnt5a CM, respectively; (iv, v, & vi) MSCs transfected with LRP5 TS cultured in control medium (Ctrl), Wnt3a CM, and Wnt5a CM, respectively; and (vii) representative negative control incubated with secondary antibodies only.

Wnt3a Synergizes With LRP5 to Enhance Cell Proliferation

We next determined the effects of modulating LRP5 level on MSC proliferation, in response to canonical and non-canonical Wnts. MTS assay analysis revealed that LRP5 WT cells, in the absence of any Wnt ligand, resulted in increased cell numbers by Day 14, relative to the other transfected groups (Fig. 3A). Cell proliferation was further enhanced by the overexpression of LRP5 WT in the presence of Wnt3a as early as Day 7 and exceeded cell numbers relative to CM conditions (Fig. 3B). Interference of LRP5 (transfection with LRP5 TS) or TCF1 (transfection with dnTCF1) resulted in significant reduction in cell proliferation (Fig. 3A,B), similar to that observed upon exposure to Wnt5a (Fig. 3C). Interestingly, the presence of Wnt3a enhanced the ability of TCF1-transfected cells to proliferate (Fig. 3B), while the absence of any Wnt ligand had no effect on cell proliferation (Fig. 3A). Furthermore, Wnt5a alone had no significant effect on cell proliferation (Fig. 3C); however, the presence of Wnt5a in combination with Wnt3a dampened the ability of Wnt3a to enhance cell proliferation (Fig. 3D).

Cyclin D1 and other genes (e.g., c-myc and Wnt1 inducible signaling pathway protein 1, WISP-1) are transcriptional targets of the β-catenin/TCF complex [Tetsu and McCormick, 1999]. As shown in Figure 4A, RT-PCR analysis revealed elevated mRNA expression of cyclin D1 in EV-transfected MSCs treated with Wnt3a by Day 7. No cyclin D1 gene expression was detected by RT-PCR in Wnt5a-treated MSCs, while a significant decrease in cyclin D1 expression was detected when MSCs were

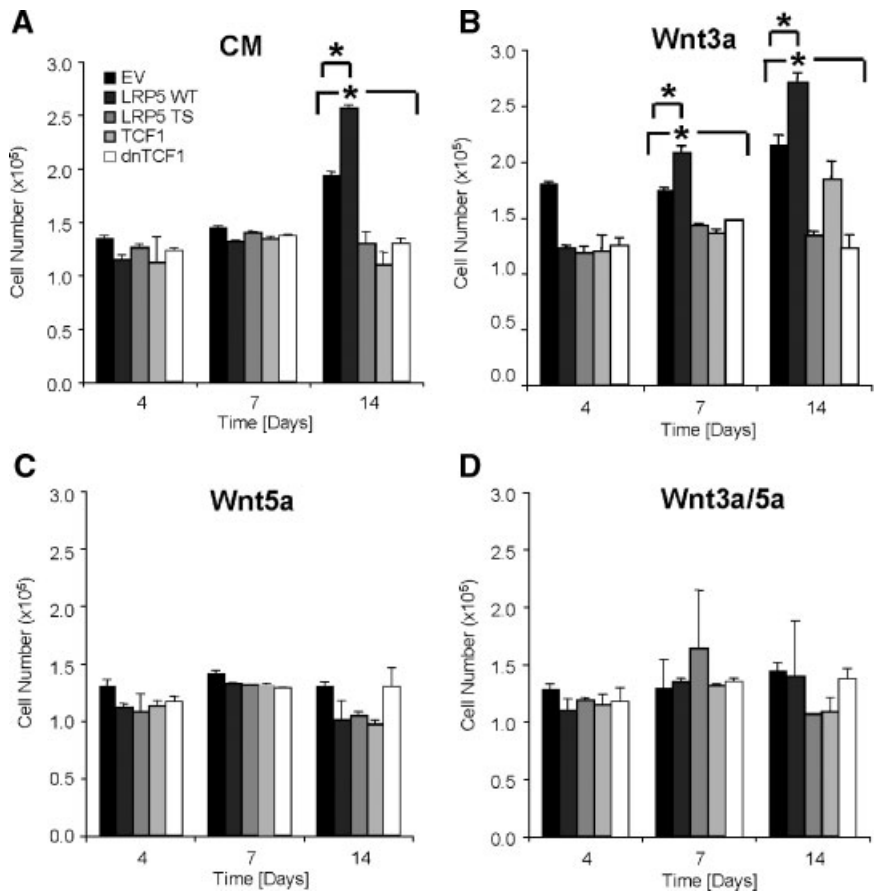


Fig. 3. Effects of LRP5 and TCF1 on MSCs proliferation in the presence and absence of Wnt3a and Wnt5a. Bone marrow-derived MSCs transfected with LRP5 WT, LRP5 TS, dnTCF1, and empty vector were cultured in (**A**) control CM and (**B**) Wnt3a and (**C**) Wnt5a, and (**D**) Wn3a/5a CM. At 4, 7, and 14 days, cell proliferation was assessed by the MTS assay. MSCs cultured in Wnt3a CM showed enhanced cell proliferation in control conditions, and was further enhanced with LRP5 WT transfection. However, dnTCF1 transfection did not result in any significant cell proliferation in the absence or presence of Wnt3a. Each bar represents the mean ± SD of n = 3 experiments, preformed in triplicate. $^*P < 0.05$.

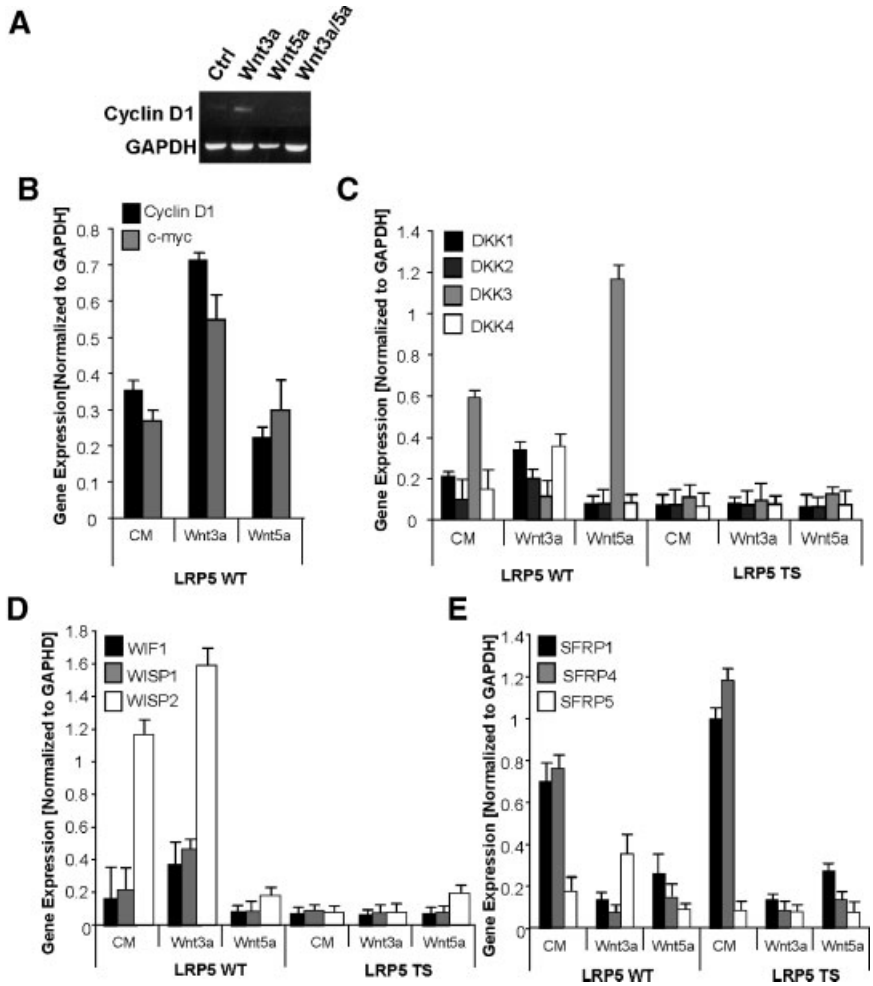


Fig. 4. Changes in Wnt signaling gene expression as a function of Wnt exposure. **A**: RT-PCR analysis of cyclin D1 mRNA expression in MSCs grown in control, Wnt3a, Wnt5a, and Wnt3a/5a CM showed that cyclin D1 expression was detected only in Wnt3a-transfected MSCs (Day 7 results shown). Microarray-based relative changes in (**B**) cyclin D1 and c-myc gene expression in MSCs transfected with LRP5 WT plasmid; and in (**C**) DKK1-4, (**D**) WIF1, WISP1-2, and (**E**) SFRP1-4 gene expression, respectively, in MSCs transfected with LRP5 WT and LRP5 TS (Day 4 results shown). Values represent mean of two experiments. Microarray analysis showed the upregulation of cyclin D1 and WISP-2 in Wnt3a-exposed LRP5 WT transfected MSCs, while enhanced expression of DKK3 was observed in Wnt5a-exposed LRP5 WT-transfected MSCs relative to LRP5 WT-transfected MSCs maintained in Control CM.

cultured with Wnt3a/5a CM, relative to Wnt3a conditions.

The Wnt signaling gene microarray was also used to analyze the expression pattern of cyclin D1 and c-myc in MSCs transfected with LRP5 WT, which were subsequently grown in control CM, Wnt3a, and Wnt5a CM for 4 days (Fig. 4B). The analysis revealed Wnt3a enhanced cyclin D1 and c-myc gene expression in MSCs, compared to contol and Wnt5a (Fig. 4B). Furthermore, we observed differences in the gene expression of DKK1, 2, 3, 4, WIF1, WISP1, WISP2, secreted frizzled related protein (SFRP)1, 4, and 5, as a function of Wnt exposure and in the absence and/or presence of functional LRP5 (Fig. 4C–E). Specifically, MSCs transfected with LRP5 WT and exposed to Wnt5a showed a significant increase in DKK3 (Dikkopf-3) gene expression relative to the other wild-type groups (Fig. 4C). Exposure to Wnt3a resulted in elevated gene expression levels of WISP1 in LRP5 WT-transfected MSCs (Fig. 4D). Misexpression of LRP5 (LRP5 TS) showed no change in DKKs and WISPs gene expression in MSCs exposed to CM versus Wnt conditions (Fig. 4C,D). However, LRP5 TS-transfected MSCs, exposed to CM, showed elevated levels of SFRP1 and SFRP4, compared to the other groups (Fig. 4E).

Suppression of Canonical Wnt Signaling Enhances Wnt5a Effects on Promotion of Osteogenesis

MSCs derived from each transfection group were grown in the presence of supplements that induced osteogenic differentiation. ALP activity was assessed on Day 21, and the extent of mineralization was examined by von Kossa staining after 5 weeks of osteogenic culture. Elevated ALP staining was observed in MSCs cultured in Wnt5a CM (Fig. 5C), relative to control CM (Fig. 5A). However, the presence of Wnt3a suppressed ALP activity (Fig. 5B), and inhibited the effects of Wn5a to enhance ALP activity (Fig. 5D). Interestingly, cells transfected with LRP5 TS or dnTCF1, and cultured in Wnt3a CM, showed increased ALP activity (Fig. 5J,N), compared to EV and LRP5 WT cells cultured in Wnt3a CM (Fig. 5B,F), while LRP5 WT-transfected cultures had fewer detectable ALP-positive cells (Fig. 5F). Co-transfection of dnTCF1 and LRP5 TS resulted in even greater numbers of ALP-positive cells, even when cultured in the presence of Wnt3a (Fig. 5R), relative to EV or LRP5 WT-Wnt3a conditions. Inhibition of LRP5 via LRP TS transfection in MSCs resulted in a significant increase in ALP activity in the presence of Wnt5a (Fig. 5K), compared to LRP5 WT conditions.

Next, the extent of mineralization in the various treatment groups (as described in Fig. 5) was quantified by plating cells at limiting dilution in CFU-O assays. After 5 weeks of culture, the number of von Kossa-positive colonies was counted. When maintained in Wnt5a CM, a significant fold-increase in CFU-O numbers was seen in cultures of LRP5 TS-transfected cells, while LRP5 WT-transfected cells displayed a significant reduction in CFU-O numbers (Fig. 5U). In comparison, minimal von Kossa staining was seen in LRP5 WT-transfected cultures, with significantly less detectable CFU-O (Fig. 5U).

The transfection efficiencies associated with these long-term osteogenic studies was determined to be $57 \pm 3\%$, $27 \pm 2\%$, and $23 \pm 1.7\%$ at Days 10, 20, and 35 (5 weeks), respectively (data not shown).

Wnt5a Enhances Osteocalcin Transcriptional Activity

To assess the specific action of Wnt5a on MSC osteogenesis, we characterized Wnt5a effects on osteogenesis-associated gene expression using the 6xOSE-2 construct, a reporter which contains six Runx-binding elements in tandem sequence upstream of the OCN promoter driving luciferase [Kahler and Westendorf, 2003]. In these experiments, MSCs were co-transfected with the 6xOSE-2 reporter and LRP5 WT, LRP5 TS, dnTCF, or EV constructs. Consistent with osteogenic induction, all cultures showed significantly higher reporter activity under osteogenic conditions. Specifically, Wnt5a treatment enhanced reporter activity in basal conditions (Fig. 6A), but higher activity was observed in osteogenic conditions (Fig. 6B); however, Wnt3a treatment maintained reporter levels similar to control conditions. Overexpression of LRP5 (LRP5 WT) in MSCs, grown in either undifferentiated or osteogenic conditions, significantly reduced OCN reporter activity. Upon exposure to Wnt5a/Wnt3a, OCN reporter activity was further reduced in the LRP5 WT group (Fig. 6A,B). The enhancement of OCN reporter activity by Wnt5a was unaffected by the interference of LRP5 (transfection with LRP5 TS). These results are also supported by the detection of Runx2 mRNA transcript in Wnt5a treatment groups of MSCs transfected with LRP5 WT (data not shown) and LRP5 TS grown in basal (Fig. 6C) and osteogenic (Fig. 6D) media. Interestingly, MSCs treated with Wnt5a had a higher detectable level of Runx2 expression under osteogenic conditions relative to control, Wnt3a (no Runx2 detection), and Wnt3a/5a conditions (Fig. 6D).

Differential Expression of Frizzled Receptors on MSCs Undergoing Osteogenesis

To further examine the cellular characteristics that contribute to the Wnt-related effects, MSCs that were transfected with LRP5 WT, LRP5 TS, dnTCF1, or EV plasmids, and then grown in basal medium and osteogenic medium, were analyzed for their membrane expression of the Fz receptors, Fz1, Fz3, Fz4, Fz6, and Fz7, using flow cytometry. The percentage of Fz1, Fz3, Fz4, Fz6, and Fz7-positive cells in each group was determined in both basal and osteogenic conditions and was normalized to values obtained from EV-transfected control MSCs (Fig. 7A,B). The relative changes in Fz receptors expression, based on statistical significance, for each treatment group are presented in Figure 7C. The results showed that Fz4 and Fz1 were highly expressed on the cell

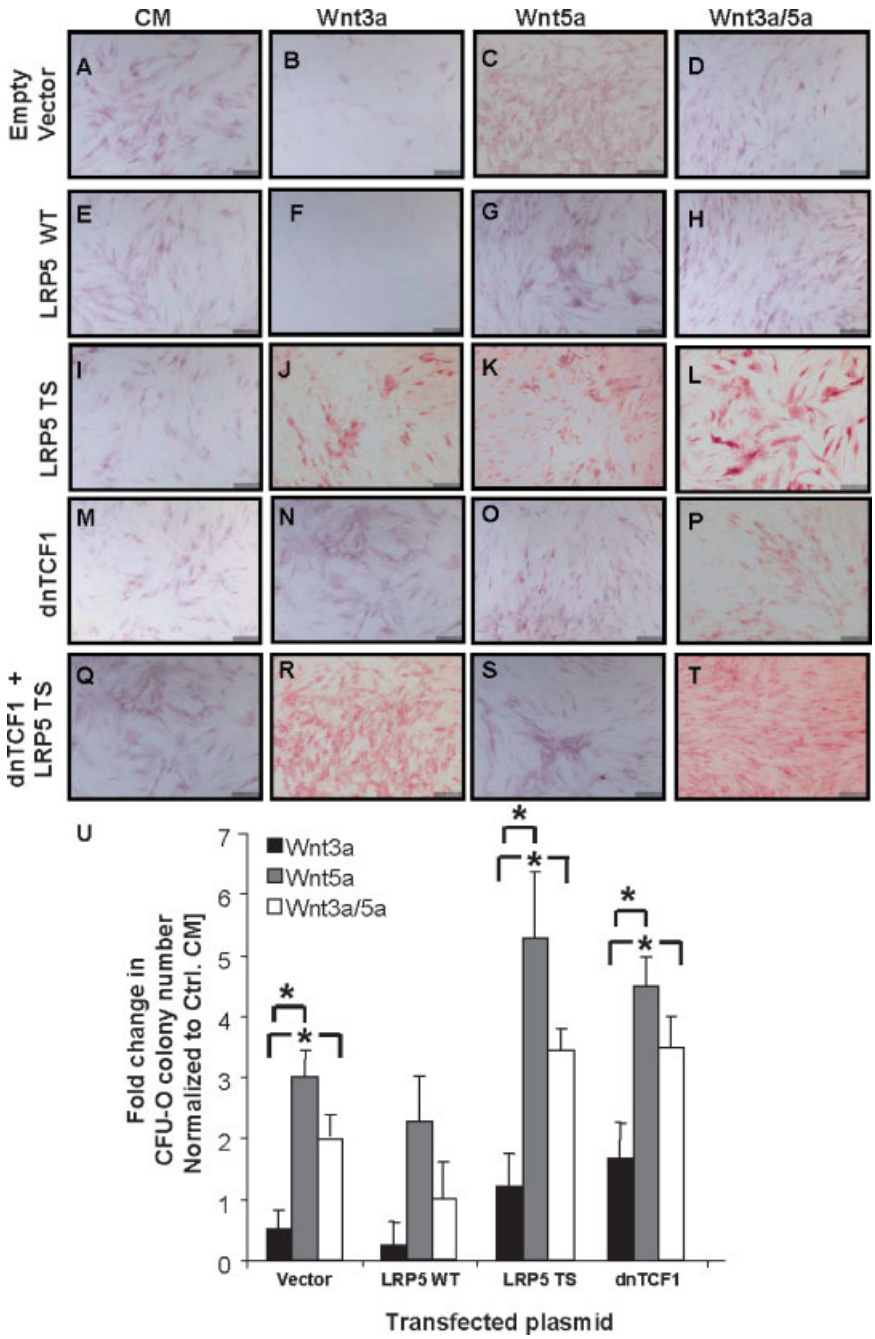


Fig. 5. Effects of LRP5 expression on MSCs osteogenesis, as analyzed by ALP staining. The effects of Wnt3a and Wnt5a exposure on osteogenic differentiation of MSCs transfected with (**A**–**D**) empty vector, (**E**–**H**) LRP5 WT, (**I**–**L**) LRP5 TS, (**M**–**P**) dnTCF1, or (**Q**–**T**) combined dnTCF1 and LRP5 TS. Day 21 ALP staining is shown in representative cultures. Cultures were stained for ALP expression, with Fast red violet LB base, and positive cells appeared red. ALP staining was significantly enhanced in MSCs cultured in Wnt5a-supplemented osteogenic medium, while Wnt3a exposure resulted in less ALP staining. Overexpression of LRP5 WT enhanced the ability of Wnt3a to inhibit ALP expression, while inhibition of LRP5 (LRP5 TS) enhanced the ability of Wnt5a to increase ALP expression in MSCs. Scale bar = 50 μm. **U**: Quantitation of CFU-O, on the basis of bone nodule number, generated from MSCs transfected with empty vector, dnTCF1, LRP5 WT, or LRP5 TS and subsequently grown in Wnt3a, Wnt5a, and Wnt3a/5a CM under osteogenic conditions. Wnt5a-supplemented osteogenic medium increased the number of bone nodules detected in culture, relative to the other conditions. Bars represent the fold change in colony number normalized to control CM conditions. N = 3, performed in triplicate. Values are mean ± SD. $^*P < 0.05$.

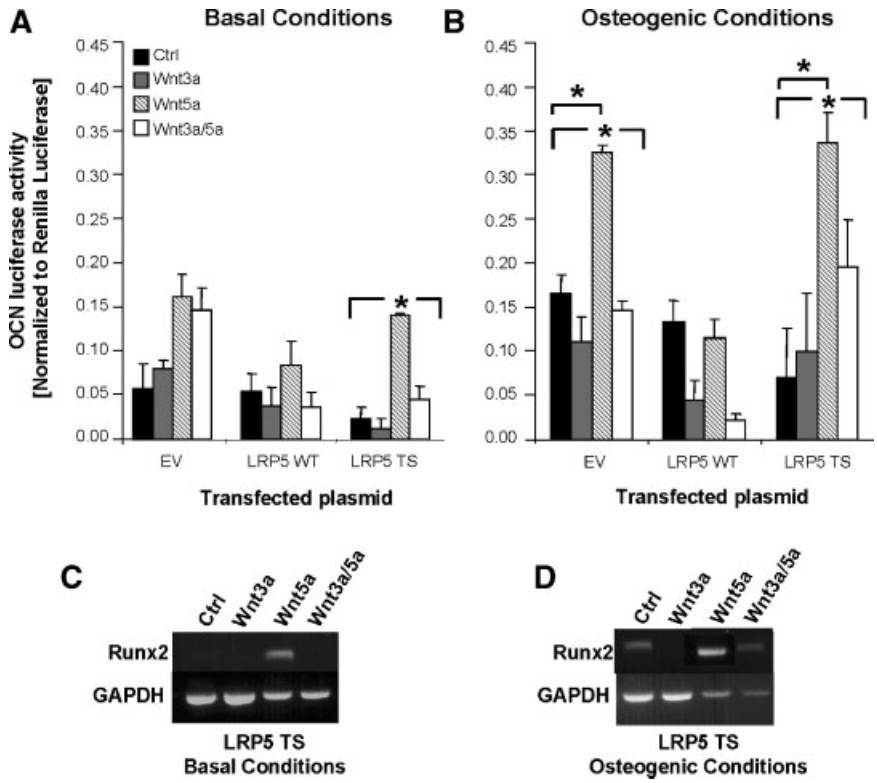
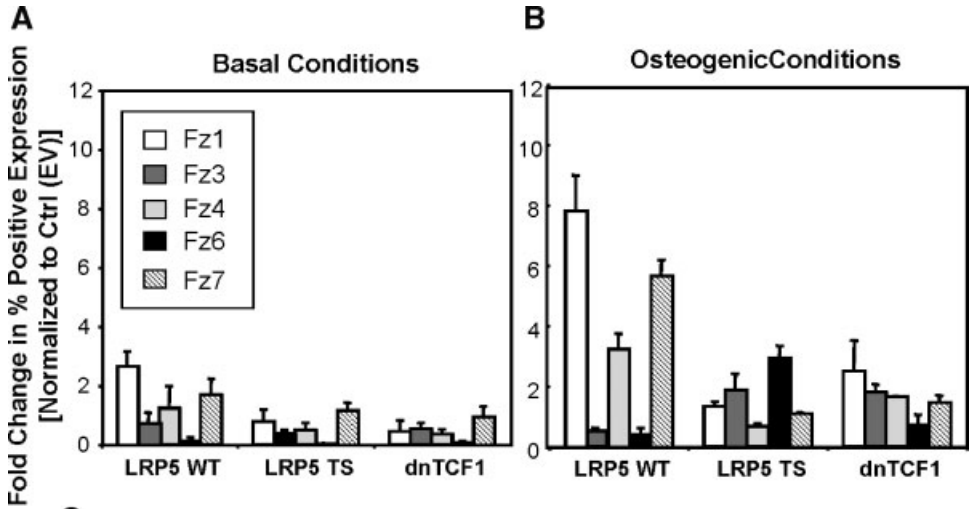


Fig. 6. Effects of LRP5 on OCN reporter activity as a function of Wnt3a and Wnt5a exposure. OCN reporter (6xOSE-2) activity in MSCs transfected with empty vector, LRP5 WT, or LRP5 TS and subsequently grown in (**A**) basal medium and (**B**) osteogenic medium in the presence or absence of Wnt3a or Wnt5a. OCN reporter luciferase activity (normalized to Renilla luciferase) was significantly enhanced in osteogenic conditions relative to basal conditions, and exposure to Wnt5a further increased reporter activity, relative to control (Ctrl) in osteogenic conditions. Day 4 results are shown. Bars represent the fold change in colony number normalized to control CM conditions. N = 3, performed in triplicate. Values are mean ± SD. $^*P < 0.05$. C: RT-PCR was used to show the relative changes of Runx2 expression in MSCs transfected with LRP5 TS under (**C**) basal and (**D**) osteogenic conditions, grown in the presence of control, Wnt3a, Wnt5a, and Wnt3a/5a-conditioned media. The results revealed that a higher detectable levels of Runx2 expression was observed in conditions where Wnt5a added to the growth media.

surface of MSCs in basal medium control conditions, while Fz3 expression was more prominent on MSCs cultured in osteogenic control conditions (Fig. 7C). Gene expression analysis confirmed the Fz receptor expression patterns obtained by flow cytometry (data not shown). Interestingly, overexpression of LRP5 resulted in elevated levels of Fz1 expression in basal conditions and even greater levels in osteogenic conditions, relative to EV conditions (Fig. 7A,B). Taken collectively, these flow cytometry results suggest that the expression repertoire of Frizzled receptors on MSCs was modulated as a function of their differentiation state, as well as the expression levels of Wnt mediators, such as LRP5 and TCF1.

DISCUSSION

In the present work, we have addressed the role of the LRP5 and Fz Wnt co-receptors and the Wnt downstream mediator, TCF1, in mediating canonical Wnt signaling in MSCs during the acquisition of an osteogenic phenotype in the presence of a prototypical canonical Wnt, Wnt3a, and prototypical non-canonical Wnt, Wnt5a. First, using a Wnt responsive reporter-promoter assay system (TOP-FLASH), we established that Wnt3a enhanced TOP-FLASH reporter activity in MSCs, while Wnt5a had no effect on TOP-FLASH reporter activity (Fig. 2A). Transfection with LRP5 WT resulted in an even greater elevated level of TOP-FLASH reporter activity in combination with Wnt3a, while LRP5 TS transfection inhibited this effect (Fig. 2A), suggesting synergy between Wnt3a and LRP5 with respect to Wnt/β-catenin signaling. Interestingly, Wnt5a itself did not activate TOP-FLASH transcriptional activity but exhibited the capacity to suppress canonical Wnt signaling in the presence of Wnt3a, suggesting a possible cross-talk between Wnt3a and Wnt5a



C

Relative expression levels of Frizzled receptors

Conditions	Frizzled Phenotype	
	Basal	Osteogenic
Ctrl (EV)	Fz4 > Fz1 = Fz7 = Fz3 > Fz6	Fz3 > Fz4 = Fz1 > Fz7 > Fz6
LRP5 WT	Fz1 > Fz4 = Fz7 = Fz3 > Fz6	Fz1 = Fz7 = Fz4 > Fz3 = Fz6
LRP5 TS	Fz7 = Fz1 = Fz4 = Fz3 > Fz6	Fz6 > Fz3 = Fz1 = Fz4 > Fz7
dnTCF1	Fz7 > Fz1 = Fz3 = Fz4 > Fz6	Fz3 > Fz4 = Fz1 = Fz7 > Fz6

> Represents a significant difference ($p<0.05$) within condition
= Represents no significant difference ($p<0.05$) within condition

Fig. 7. Relative expression levels of Fz receptors in MSCs as a function of LRP5 and TCF1 expression. Presence of Fz1, Fz3, Fz4, Fz6, and Fz7 was analyzed by flow cytometry on MSCs transfected with LRP5 WT, LRP5 TS, and dnTCF1 in (**A**) basal and (**B**) osteogenic conditions. The fold change in the percentage of cells positive for each Fz receptor was normalized to control (empty vector, EV) conditions (bars represent the mean fold change ± SD in three experiments, performed in triplicate. **C**: Tabulated presentation of the relative level of expression of each Fz receptor under various conditions. Statistical significance ($*P<0.05$) is as indicated. Analysis revealed the differential Fz receptor profile on MSCs cultured in basal and osteogenic conditions as well as a function of LRP5 expression.

in canonical Wnt signaling in human MSCs, a concept which has been reported by Mikels and Nusse [2006].

The Wnt effects on reporter activity translated to effects on MSC proliferation. Specifically, Wnt3a-activated canonical Wnt signaling resulted in a significant increase in cell numbers relative to EV control conditions, which was further enhanced in LRP5 WT-transfected MSCs, suggesting that the canonical Wnt ligand, Wnt3a and LRP5 act synergistically to enhance Wnt responsive activity leading to enhanced cell proliferation, as similarly demonstrated in 293T human embryonic kidney cells [Liu et al., 2005]. We confirmed using high-throughout Wnt gene array analysis that the likely cause for the increase in MSC proliferation is the upregulation of cyclin D1 and c-myc, which are known Wnt target genes. Furthermore, the finding that cell proliferation was significantly impaired when MSCs were co-transfected with LRP5 TS and dnTCF1 indicate that a Wnt-LRP5 co-receptor association is necessary to mediate the canonical Wnt signaling events that lead to the upregulation in cell proliferation. Our findings thus corroborate with similar observations reported in other cell systems [Ueda et al., 2002], suggesting a common involvement of LRP5-dependent canonical signaling in the regulation of cell proliferation.

Results from the reporter-promoter studies suggest that Wnt5a does not signal through β-catenin/TCF/LEF transcription factor complexes in human MSCs to enhance cell proliferation, as similarly reported in other cell types [Kuhl et al., 2000], and instead acts as a non-canonical Wnt in undifferentiated MSCs. Expo-

sure to Wnt5a did not alter the basal level of gene expression of cyclin D1 and c-myc, but resulted in elevated gene expression of DKK3 (Fig. 4C), which has been shown to be a potent antagonist of canonical Wnt signaling [Katoh and Katoh, 2006]. Interestingly, the effects of Wnt3a/Wnt5a co-treatment showed that Wnt5a was able to suppress the Wnt3a-mediated canonical Wnt signaling pathway, with concomitant reduction in both canonical Wnt transcriptional activity and cell proliferation. Cyclin D1 was significantly downregulated when MSCs were co-treated with Wnt3a and Wnt5a, compared to Wnt3a alone (Fig. 4A). Taken together, we hypothesize that Wnt5a antagonizes canonical Wnt signaling, likely via the upregulation of DKK3, resulting in the downregulation of target genes, for example, cyclin D1.

Topol et al. [2003] reported that Wnt5a also has the capacity to antagonize the canonical Wnt pathway by promoting the degradation of β-catenin, and thus downregulation of target transcription factors. Given these findings, we propose that Wnt5a may act to maintain MSCs in a quiescent, non-cycling state via the suppression of canonical Wnt signaling that otherwise would lead to cell proliferation cues. This concept has been proposed for human and mouse embryonic stem cell systems, where the activation of the canonical Wnt pathway is sufficient to maintain the self-renewal of both of these stem cell types and is downregulated upon differentiation [Sato et al., 2004]. Studies are underway to determine the mechanism of DKK3 action on downstream components involved in the suppression of canonical Wnt signaling in the presence of a non-canonical Wnt stimulus, such as Wnt5a and its effects of MSCs differentiation.

To gain insights into the involvement of Wnt signaling during osteogenesis, the various transfected cells were also evaluated for their osteogenic potential, in the presence of Wnt3a and Wnt5a. Our studies revealed that Wnt5a treatment alone enhanced ALP activity in MSCs, whereas Wnt3a alone resulted in very few ALP-positive cells (Fig. 5A–C). However, in the presence of Wnt3a/Wnt5a medium, the presence of Wnt3a was able to suppress the ability of Wnt5a to enhance osteogenesis (Fig. 5D). The ability of Wnt3a to suppress osteogenesis may be due in part by maintaining MSCs in an undifferentiated state via activation of canonical Wnt signaling (as hypothesized above), enhanced by the overexpression of LRP5, leading to the upregulation of WISP-1, as seen in undifferentiated MSCs exposed to Wnt3a (Fig. 5D). However, when MSCs were transfected with LRP5 TS, dnTCF1, or both, these cells were able to respond to osteogenic stimulation, with restored ALP expression, even in the presence of Wnt3a (Fig. 5J,N,R).

Our observations that canonical Wnt effects altered activities in MSCs via misexpression of LRP5 are contrary to those reports which show that loss-of-function mutations in the *LRP5* gene leads to a low bone mass phenotype [Gong et al., 2001], while activating mutations in the *LRP5* gene leads to increase bone mass [Little et al., 2002]. In the low bone mass and high bone phenotypes reported by Gong et al. [2001] and Little et al. [2002], respectively, osteoblasts were shown to be the cells responsible for these phenotypes, while in our studies, undifferentiated MSCs, undergoing osteogenesis, were responsible for the observed outcomes. Thus, the difference in these observations may be explained by the state of differentiation of the cell type, that is , stem versus differentiated cell. Our results, however, are supported, in part, by observations reported by Kahler et al. [2005] who demonstrated that Lef1-overexpressing MSCs produced ALP later and at lower levels than control cells. Thus, these findings suggest the role of canonical Wnt signaling transcription factors (i.e., TCF1 and Lef1) in delaying osteogenic maturation in MSCs. Taken together, our results indicate that enhancement of canonical Wnt signaling is required to maintain MSCs in an undifferentiated state, and its abrogation may render the cells amenable to osteogenesis, as shown in this study.

Our findings suggest that canonical Wnt signaling, for example, mediated by Wnt3a, maintain MSCs in a proliferative state while Wnt5a, which does not signal through the canonical Wnt pathway, stimulates osteogenic differentiation. This is supported by enhanced 6xOSE-2 OCN promoter-reporter activity in Wnt5a-transfected MSCs cultured under osteogenic conditions. Interestingly, there was elevated reporter activity observed even in Wnt5a-transfected MSCs that were grown in the absence of dexamethasone, suggesting that Wnt5a may promote osteogenesis via direct regulation of osteogenic genes. These findings are supported by the role that glucocorticoids

(such as dexamethasone) play in suppressing canonical Wnt signaling in osteoblasts [Ohnaka et al., 2004, 2005]. In these studies, dexamethasone was shown to suppress TOP-FLASH transcriptional activity in a dose-dependent manner. Taken together, our findings suggest that osteoinductive growth conditions interfere with canonical Wnt signaling in MSCs, and that the suppression of canonical Wnt signaling, perhaps by reagents such as dexamethasone in osteogenic medium, may be restored if Wnt mediators, for example, LRP5, are abundantly available and presumably exceed a certain threshold level.

Over/misexpression of LRP5 and TCF1 also influenced the expression profile of Fz receptors on MSCs both in undifferentiated and osteogenic conditions. Fz1 and Fz4 were highly expressed in undifferentiated MSCs, and Fz1 expression was further increased in the LRP5 WT-transfected group, relative to Ctrl (EV) conditions (Fig. 7C), while Fz3 was more highly expressed on MSCs under osteogenic conditions (Fig. 7C). Fz3 (in mouse) has been implicated as receptors involved with non-canonical signaling, and have been shown to be negative regulators of canonical Wnt signal [Sheldahl et al., 1999; Golan et al., 2004], while Fz1 (in rat) has been shown to mediate signaling through the Wnt/β-catenin pathway [Yang-Snyder et al., 1996]. These findings suggest that the commitment of MSCs to a differentiation pathway, such as osteogenesis, is coupled with a switch from canonical to non-canonical signaling, and is characterized by changes in the expression profile of Frizzled receptors and most likely the functional involvement of specific Fzs in Wnt signaling via canonical and other signaling pathways (e.g., non-canonical and planar cell polarity pathways). Furthermore, our flow cytometry data also provide indication of a potential "Frizzled signature" for canonical

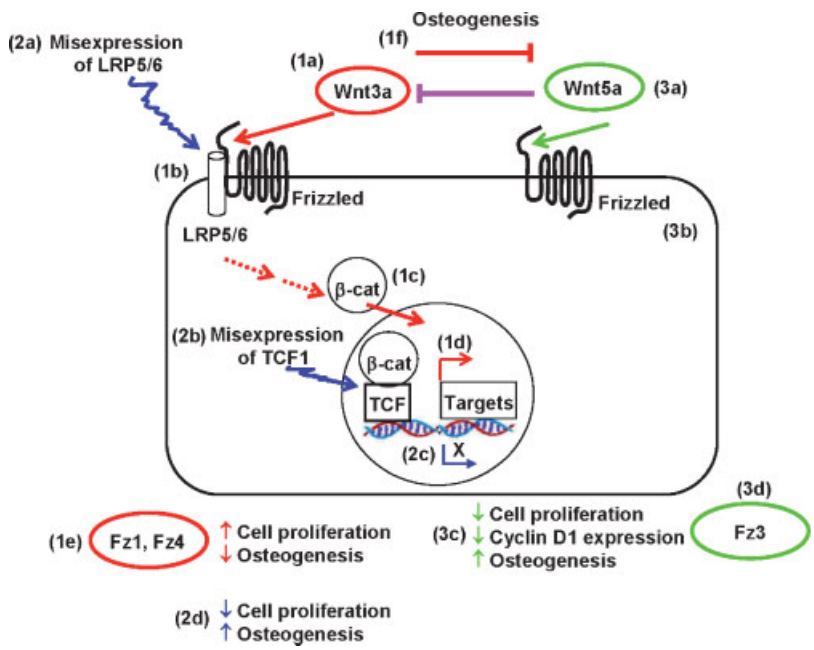


Fig. 8. Schematic of functional roles of key Wnt mediators in MSC cell fate determination. **1a**: Undifferentiated MSCs are responsive to Wnt3a. Wnt3a binds to LRP5 and (**1b**) Fz receptor, (**1c**) resulting in increased intracellular β-catenin. **1d**: β-Catenin translocates to the nucleus, associates with transcription factors, such as TCF, and upregulates expression of target genes, resulting in the enhancement of cell proliferation (**1e**). 1e: Fz1 and Fz4 are highly expressed on undifferentiated MSCs, and appear to be involved in canonical Wnt signaling transduction. **1f**: Under osteogenic conditions, Wnt3a suppresses the ability of MSC to differentiate into osteogenic cells. **2a**: Inhibition of LRP5 (e.g., expression of LRP5 TS) and (**2b**) misexpression of TCF1 result in (**2c**) reduction of TCF/LEF transcriptional activity, (**2d**) leading to the reduction of cell proliferation. 2d: However, under osteogenic conditions, inhibition of LRP5 expression and TCF1 result in the enhancement of osteogenesis. **3a**: Wnt5a binds Fz receptors but its signaling is not mediated through β-catenin in undifferentiated MSCs, but acts to suppress the ability of Wnt3a to enhance TCF/LEF transcriptional activity, perhaps by competition for Fz receptor binding (**3b**). The presence of Wnt5a results in the reduction of cell proliferation and cyclin D1 expression; however, under osteogenic conditions, (**3c**) Wnt5a enhances osteogenesis in MSCs. Wnt5a signaling is mediated via Fz receptors, and (**3d**) Fz3 is highly expressed in MSCs undergoing osteogenesis.

Wnt signaling in human MSCs. Our ongoing work is focused on isolating enriched populations of MSCs expressing specific Fz receptors to determine the "stemness" potential of these pools of cells.

In summary, our results lead us to propose a working model that summarizes how key Wnt mediators, such as LRP5 and TCF, are critical in transducing Wnt/β-catenin signaling in MSCs in undifferentiated conditions (Fig. 8). However, the presence of prototypical non-canonical Wnts, such as Wnt5a, has the potential to suppress a canonical Wnt-mediated signal in undifferentiated MSCs. In the absence of other competitive Wnts, such as Wnt5a, MSCs are maintained in a self-renewing and undifferentiated state. However, under differentiation conditions, Wnt5a promotes or enhances the cells toward a differentiation pathway. Interestingly, a canonical Wnt, such as Wnt3a, demonstrates the capacity to inhibit the ability of Wnt5a to enhance osteogenesis, but the ability of the cell population to undergo osteogenesis is recovered upon removal of the Wnt3a-mediated stimulus (data not shown). The potential cross-talk between the canonical and non-canonical Wnt signaling pathways provides important insights into understanding the regulation of MSCs both in vitro and vivo.

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