Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells

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Genetic evidence indicates that Wnt signaling is critically involved in bone homeostasis. In this study, we investigated the functions of canonical Wnts on differentiation of adult multipotent human mesenchymal stem cells (hMSCs) in vitro and in vivo. We observe differential sensitivities of hMSCs to Wnt inhibition of osteogenesis versus adipogenesis, which favors osteoblastic commitment under binary in vitro differentiation conditions. Wnt inhibition of osteogenesis is associated with decreased expression of osteoblastic transcription factors and inhibition of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation, which are involved in osteogenic differentiation. An hMSC subpopulation exhibits high endogenous Wnt signaling, the inhibition of which enhances osteogenic and adipogenic differentiation in vitro. In an in vivo bone formation model, high levels of Wnt signaling inhibit de novo bone formation by hMSCs. However, hMSCs with exogenous expression of Wnt1 but not stabilized β-catenin markedly stimulate bone formation by naive hMSCs, arguing for an important role of a canonical Wnt gradient in hMSC osteogenesis in vivo.

Introduction

Growing evidence indicates that Wnt signaling plays a critical role in stem/progenitor self-renewal in adult tissues (Reya and Clevers, 2005), in which these cells serve as reservoirs for tissue renewal in response to trauma, disease, and aging. In the canonical pathway, Wnts signal through frizzled and LRP5/6 coreceptors, leading to inactivation of the axin–GSK3-β complex, which otherwise phosphorylates and directs degradation of β-catenin. Stabilized β-catenin translocates into the nucleus and forms a complex with T cell factor (TCF)/lymphoid enhancer-binding factor transcription factors to activate Wnt target genes (Reya and Clevers, 2005). Some Wnts lack this ability and stimulate noncanonical pathways through effectors including JNK, Rho GTPase, or Ca^{2+} (Veeman et al., 2003).

Adult mesenchymal stem cells (MSCs) isolated from bone marrow are multipotent and give rise to tissues, including bone, cartilage, muscle, and adipose (Pittenger et al., 1999). Recent studies have revealed critical transcription factors involved in the commitments of different MSC-derived lineages (for review see Harada and Rodan, 2003). For example, osteoblastic differentiation is controlled by Runx2, osterix (Osx), and Dlx5, whereas PPAR-γ is involved in adipocyte commitment (for review see Harada and Rodan, 2003). Genetic studies have also established that Wnt/β-catenin activity is essential for normal osteogenesis (Day et al., 2005; Hill et al., 2005). Enhancement of Wnt signaling either by Wnt overexpression (Bennett et al., 2007) or deficiency of Wnt antagonists (Morvan et al., 2006; ten Dijke et al., 2008) is associated with increased bone formation in mice and humans. Loss or gain of function mutations in LRP5 also cause osteoporosis or high bone mass phenotypes, respectively (Gong et al., 2001; Boyden et al., 2002; Little et al., 2002).

The in vitro effects of Wnt signaling on osteogenic differentiation of MSCs are controversial. Wnt/β-catenin signaling has been reported to stimulate differentiation of mouse MSCs toward the osteoblastic lineage (Gong et al., 2001; Gaur et al., 2005). However, both stimulatory (Gregory et al., 2005) and inhibitory (Boland et al., 2004; de Boer et al., 2004) effects of canonical Wnt signaling on osteogenic differentiation by human MSCs (hMSCs) have been observed. Because of their osteogenic potential, human adult bone marrow MSCs are one of the most promising stem cell populations

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Abbreviations used in this paper: CM, conditioned medium; dnTCF4, dominant-negative TCF4; ERK, extracellular receptor-dependent kinase; HA, hydroxyapatite; hMSC, human MSC; MSC, mesenchymal stem cell; Osx, osterix; PCNA, proliferating cell nuclear antigen; shRNA, short hairpin RNA; TCF, T cell factor; TCP, tri-Ca phosphate.

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for bone regeneration as well as repairing critical-size bone defects that fail to undergo spontaneous healing (Meijer et al., 2007). Thus, these experiments were undertaken in an effort to better define how Wnts influence hMSC commitment along osteoblastic and other lineages as well as the mechanisms involved.

Results and discussion

We first analyzed the in vitro biological effects of canonical Wnts on differentiation of adult hMSCs. Consistent with previous studies (Boland et al., 2004; de Boer et al., 2004), treatment of hMSCs with Wnt3a conditioned medium (CM) or lentiviral-mediated transduction of Wnt1 strongly inhibited osteogenic differentiation compared with that of respective control cultures, as reflected by reduced staining for the early osteoblastic marker AP and reduced mineralization as detected by Alizarin red staining (Fig. S1 A). Moreover, exogenous Wnt1 resulted in dramatic down-regulation of expression of several markers associated with osteoblastic differentiation (for review see Harada and Rodan, 2003), including AP, bone sialoprotein, and osteocalcin (Fig. S1 B).

In addition to its inhibition of osteogenic differentiation, Wnt stimulation was associated with increased cell proliferation (Fig. S1 C). Because terminal differentiation is commonly associated with exit from the cell cycle, we asked whether Wnt inhibition of differentiation might be caused by enhanced proliferation blocking exit from cell cycle. However, PDGF, which also increased hMSC proliferation and resulted in higher saturation density (Fig. S1 C), did not inhibit osteogenic differentiation, arguing that the Wnt effects could not be explained by stimulation of cell proliferation alone.

The osteogenic inhibitory function of canonical Wnts seemed inconsistent with the fact that this pathway plays a positive role in bone homeostasis in vivo. It has been observed that aging leads to a decrease of bone-forming osteoblasts and an increase of marrow adipocytes (Verma et al., 2002). These findings are consistent with the possibility that alterations in hMSC lineage differentiation may have profound effects on bone formation. Because Wnt signaling can also inhibit adipogenic differentiation (Ross et al., 2000), we asked whether canonical Wnts might modify hMSC lineage commitment to favor osteoblastic differentiation. As shown in Fig. 1 (A and B), osteogenic differentiation was partially inhibited at 100 ng/ml Wnt3a, whereas concentrations of 25 ng/ml or lower failed to detectably inhibit either AP activity or mineralization. In contrast, Wnt3a was much more potent in inhibiting adipocyte differentiation, with ∼40% inhibition observed at 5 ng/ml and complete inhibition at observed at 100 ng/ml (Fig. 1 C). As might be expected for transcriptional signaling through Wnt/β-catenin, a dominant-negative TCF4 (dnTCF4) antagonized exogenous Wnt1-mediated inhibition of both osteogenic and adipogenic differentiation (Fig. S1 D).

To directly compare hMSC sensitivity to Wnt inhibition of commitment with the osteogenic versus adipogenic lineage under the same conditions, we cultured these cells in a binary differentiation medium that efficiently induced differentiation along both lineages. Wnt3a significantly blocked adipogenic differentiation at 5 ng/ml and completely abolished adipogenesis at 100 ng/ml (Fig. 1, D–G and I). In marked contrast, treatment with Wnt3a at both 5 and 25 ng/ml resulted in a significant relative increase in osteogenic differentiation, as manifested by increased AP activity and mineralization (Fig. 1, H and I). At 100 ng/ml, Wnt3a partially inhibited osteogenic differentiation as well (Fig. 1, H and I). These results indicate that under conditions permissive for binary lineage differentiation, differences in sensitivity to Wnt inhibition may alter the equilibrium and shift the commitment from adipocytes toward osteoblasts.

One mechanism underlying osteogenic inhibition by Wnt/β-catenin signaling has been attributed to direct interaction and inhibition of Runx2 transcriptional activity by the β-catenin–Lef1 complex (Kahler and Westendorf, 2003). However, it was also reported that Wnt/β-catenin signaling increases Runx2 level in mouse mesenchymal cells (Gaur et al., 2005). Thus, we examined the expression of osteoblastic transcription factors, including Runx2, Osx, and Dlx5, in hMSCs. As shown in Fig. 2 A, the expression levels of these genes were reduced in Wnt1-expressing hMSCs in basal medium as well as under osteogenic conditions compared with that in vector cells, which is consistent with high levels of Wnt signaling acting to down-regulate these osteoblastic transcription factors.

Extracellular receptor-dependent kinase (ERK) and p38 MAPK pathways have been shown to play critical roles in osteogenesis (Kratchmarova et al., 2005). As shown in Fig. 2 B, p38 and ERK as well as JNK were activated, as indicated by their increased phosphorylation in hMSCs undergoing osteogenic differentiation. Activation of p38 was detectable as early as 2 d, whereas increased phosphorylated JNK (p-JNK) was obvious by 6 d. Wnt1 expression largely inhibited activation of both kinases under osteogenic conditions, whereas there was no significant change in p-ERK levels in response to Wnt1 expression under the same conditions (Fig. 2 B).

The Ror2 receptor tyrosine kinase plays an important role in skeletal development as well as mediates Wnt5a signaling to the JNK pathway (Oishi et al., 2003). Other studies have shown that Wnt5a stimulates osteogenic differentiation (Baksh et al., 2007) and Ror2 short hairpin RNA (shRNA) inhibits hMSC osteogenic differentiation (Liu et al., 2007). When we examined Ror2 expression in response to osteogenic stimulation, we observed an increase, which was blocked by exogenous Wnt1 (Fig. 2 C). Moreover, when we inhibited Ror2 expression with shRNA under osteogenic conditions, neither p38 nor ERK activation was affected, whereas JNK activation was effectively blocked (Fig. 2 D), and osteogenic differentiation was partially inhibited as well (not depicted). Similarly, treatment of hMSCs under differentiation conditions with SP600125, a small molecule inhibitor of JNK, partially inhibited osteogenic differentiation (Fig. 2 E). These findings support the idea that canonical Wnt inhibition of Ror2 up-regulation and JNK activation contributes to its osteogenic inhibitory functions.

Canonical Wnt signaling has been implicated in stem/progenitor cell maintenance in several adult tissues (Reya and Clevers, 2005). Thus, we asked whether hMSCs demonstrated endogenous Wnt signaling activity. Using a lentiviral-based TCF luciferase reporter system, hMSCs showed an approximately sevenfold TCF reporter activity over that of the control reporter, and this activity could be completely inhibited by dnTCF4 expression (Fig. 3 A). We observed further that endogenous TCF activity

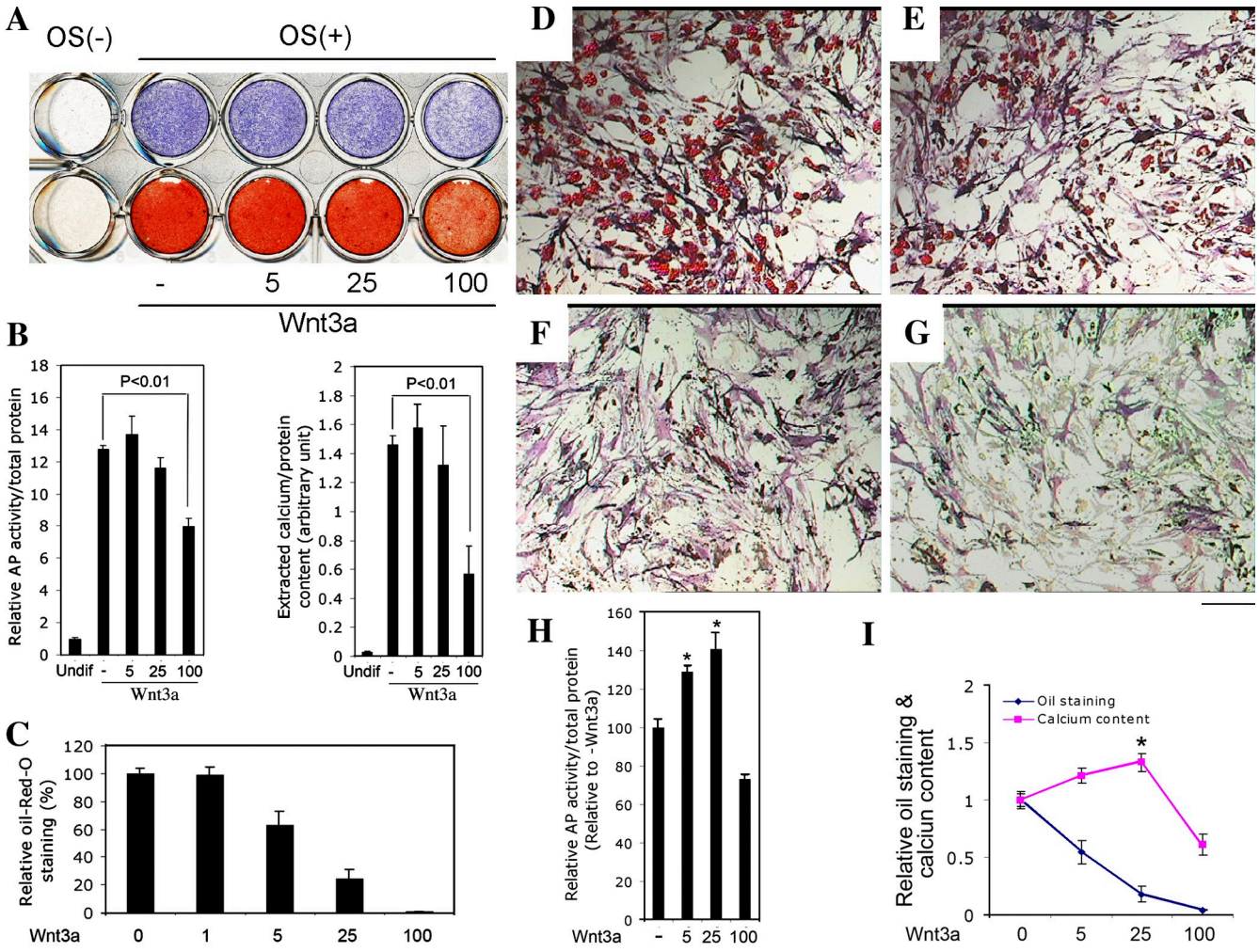

Figure 1. **Differential sensitivity of hMSCs to Wnt inhibition of osteogenic versus adipogenic differentiation.** (A) Scanned images of AP (top) or Alizarin red staining (bottom) in hMSCs after 3 wk in osteogenic medium (OS) in the presence of different concentrations (in nanograms/milliliter) of recombinant Wnt3a. (B) Relative AP activity and Ca deposition under conditions as in A. Undif, undifferentiated. (C) Quantification of adipocyte differentiation in response to Wnt3a. Bars represent relative extracted Oil red O staining after 3 wk in adipogenic medium. (D–G) AP and Oil red O staining in hMSCs after 3 wk in a mixed medium containing equal volumes of osteogenic and adipogenic media. Wnt3a was added at 0 (D), 5 (E), 25 (F), or 100 ng/ml (G). (H) Relative AP activity in hMSC cultures as in D–G. (I) Relative extracted Oil red O staining and Ca deposition in hMSC cultures as in D–G. (B, C, H, and I) Results represent mean values ± SD from two independent experiments performed in triplicate. *, $P < 0.05$. Bar, 20 μm.

in hMSCs was heterogeneous, as only ~5% of cells exhibited high activity using a GFP-based reporter system (Fig. 3 B). Moreover, this endogenous Wnt signaling was down-regulated during osteogenic differentiation as monitored by decreased levels of TCF reporter activity and expression of a known β-catenin target gene, *Axin2* (Fig. 3 C). In contrast, expression of the Wnt antagonist Dkk1 was markedly increased during osteogenic differentiation (Fig. 3 C), suggesting that Dkk1 may participate in down-regulating endogenous Wnt signaling during osteogenic differentiation.

To assess the biological effects of endogenous Wnt signaling, we compared the differentiation responses of dnTCF4 and vector-expressing hMSCs. In contrast to the osteogenic inhibition by exogenous Wnt1, dnTCF4 enhanced osteogenic differentiation (Fig. 3 D), as well as increased expression of genes associated with osteogenic differentiation (Fig. S3 A). dnTCF4 also enhanced adipogenic differentiation (Fig. S2 B) and increased PPAR-γ expression (Fig. S2 C), with evidence of spontaneous adipocyte commitment both in basal medium (Fig. S2 B) and under osteogenic conditions (Fig. S2 D), neither of which responses were observed in vector hMSCs (not depicted). All of these results argue that endogenous Wnt signaling plays an important role in maintaining hMSCs in a relatively undifferentiated state.

To investigate how canonical Wnt inhibitory effects on osteogenic differentiation in vitro might translate into a positive function in bone formation in vivo, we used an established ectopic bone formation model in immunodeficient mice (Krebsbach et al., 1997). When mixed with ceramic powder of hydroxyapatite (HA)/tri-Ca phosphate (TCP), vector-expressing hMSCs formed detectable small fragments of ectopic bone constituting ~1% of the total scaffold area (Fig. 4, A and G). Consistent with the inhibitory effects of high Wnt signaling levels on hMSC osteogenic differentiation in vitro, exogenous Wnt1-expressing hMSCs exhibited little if any detectable bone forming capacity under the same conditions (Fig. 4, B and G). Based on our

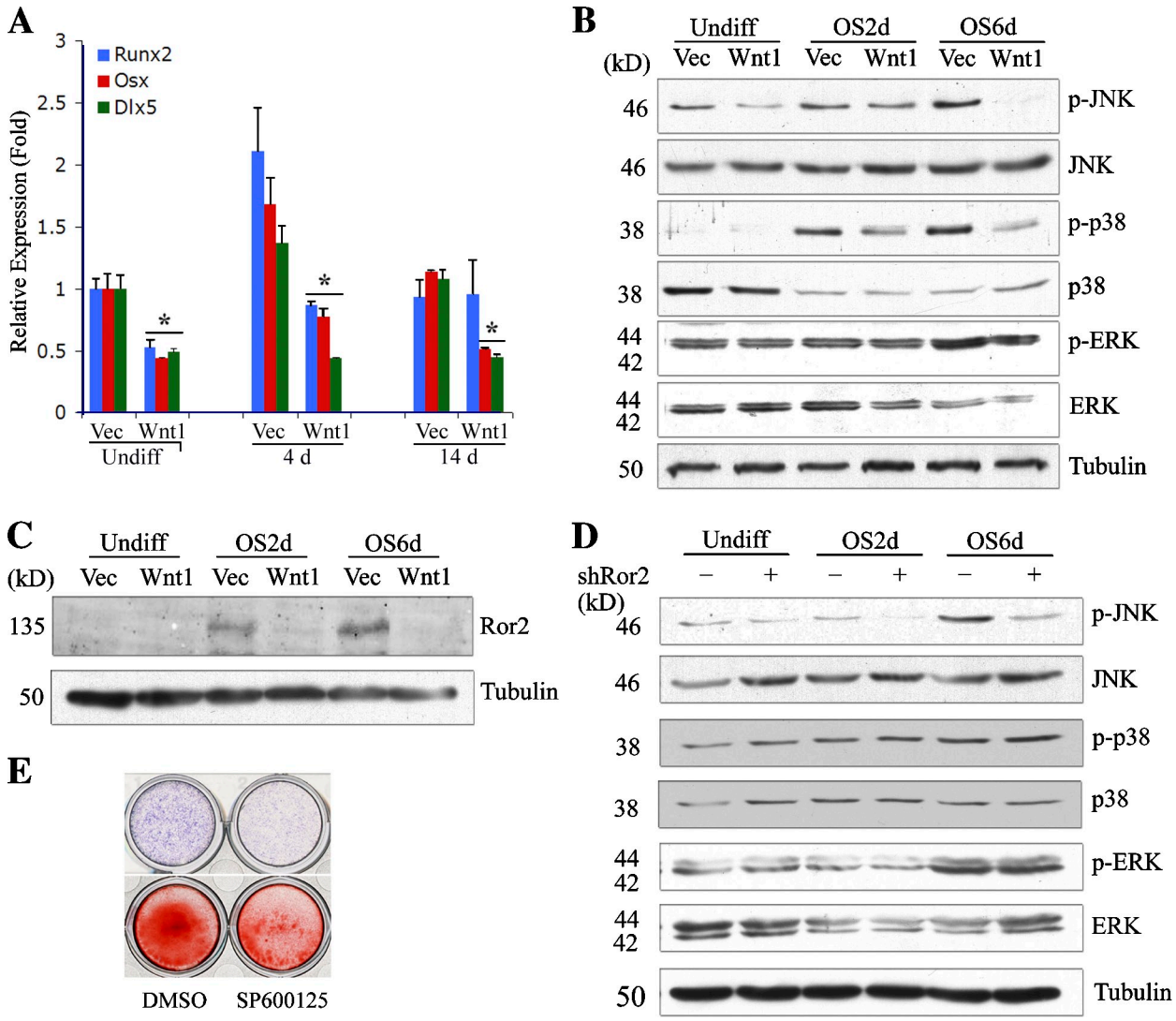

Figure 2. **Mechanisms involved in Wnt inhibition of osteogenic differentiation.** (A) Real-time PCR analysis of Runx2, Osx, and Dlx5 expression in vector (Vec)- or Wnt1-expressing hMSCs grown in basal (Undiff) or osteogenic medium for 4 and 14 d. Results represent mean values ± SD from two independent experiments performed in triplicate. *, $P < 0.05$. (B and C) Representative immunoblots for MAPK activation (B) and Ror2 expression (C) in vector- or Wnt1-expressing hMSCs grown in basal or osteogenic medium (OS) for 2 and 6 d. (D) Immunoblots of MAPK activation in control or Ror2 shRNA–expressing hMSCs grown as in B. (E) Scanned images of AP and Alizarin red staining in hMSCs after osteogenic induction in the presence or absence of 20 μM SP600125.

observations of heterogeneous endogenous Wnt activity in hMSCs, we sought to model higher levels of heterogeneous Wnt activity by mixing vector and Wnt1-expressing hMSCs at different ratios in the implants. These conditions led to a striking enhancement of bone formation with as much as 25% bone formation observed (Fig. 4, C–G).

To directly identify which cell population was responsible, we labeled hMSCs with LacZ by viral transduction. LacZ-expressing hMSCs alone formed only small amounts of bone (Fig. S3 A), which is similar to that observed with vector hMSCs (Fig. 4 A). When bone formation was significantly enhanced in implants with mixed LacZ- and Wnt1-expressing hMSCs (Fig. S3, B and C), the bone structures were predominately comprised of LacZ-staining cells. In contrast, Wnt1 immunostaining was observed in dense cell clusters surrounding bone structures (Fig. 5, A–F). These results strongly support the conclusion that Wnt1-expressing hMSCs act primarily to stimulate osteogenesis by Wnt1-negative hMSCs.

Next, we examined the Wnt signaling activity in mixed implants by immunostaining for β-catenin because the presence of nonmembrane-bound β-catenin is a hallmark of canonical Wnt signaling activation. As shown in Fig. 5 G, cytoplasmic β-catenin was apparent within both LacZ-positive (hMSC-LacZ) and -negative (hMSC-Wnt1) cells surrounding bone-like structures but was generally undetectable in osteocytes embedded within the ectopic bone. These findings argue that only hMSCs with low Wnt signaling activity are capable of differentiating into bone-forming osteocytes.

Immunostaining for proliferating cell nuclear antigen (PCNA) revealed significant increases in both LacZ- and Wnt1-expressing hMSCs in mixed implants (Fig. 5, H–J) compared with hMSC-LacZ cells alone (Fig. 5 J and Fig. S3 D). These

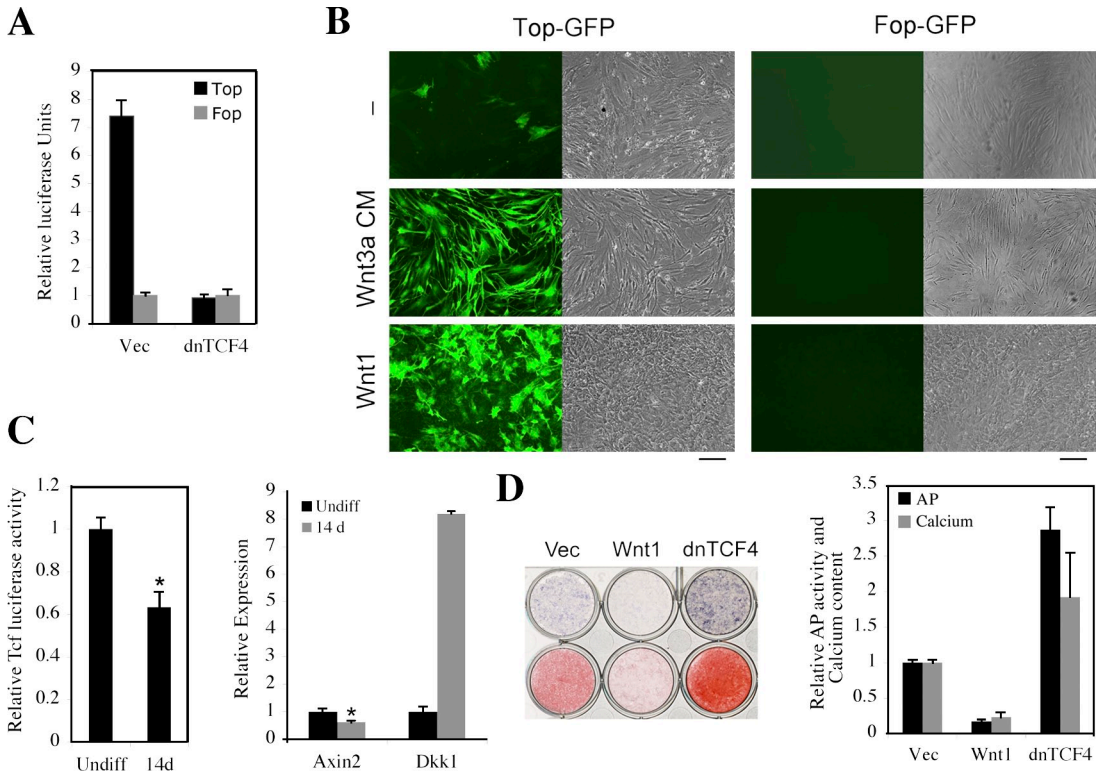

Figure 3. **Functions of endogenous Wnt signaling in hMSC osteogenic differentiation.** (A) TCF reporter analysis in hMSCs after infection with Top or Fop luciferase virus and Renilla luciferase virus together with either vector (Vec) or dnTCF4 virus. Luciferase activity was measured at 48 h and normalized to Renilla activity. (B) Parental or Wnt1-expressing hMSCs were transduced with Top- or Fop-GFP virus, and parental cells were treated at 24 h with or without Wnt3a CM. GFP was visualized by fluorescence microscopy at 72 h. (C) Top luciferase reporter activity (left) and *Axin2* and Dkk1 expression by real-time PCR (right) in hMSCs at 14 d in osteogenic medium. Fold changes were all relative to levels in undifferentiated (Undiff) cells. *, $P < 0.05$. (D) Scanned images of AP or Alizarin red staining (left) in hMSCs expressing vector or dnTCF4 after 3 wk in osteogenic medium. Quantification of relative AP activity and Ca content is shown (right). (A, C, and D) Results represent mean values ± SD from two independent experiments performed in triplicate. Bars, 10 µm.

results correlated with the increased numbers of both Wnt1- and LacZ-staining cells observed in mixed implants (Fig. 5 K). These results establish that canonical Wnt1 specifically stimulated in vivo proliferation of hMSCs in both autocrine and paracrine modes, with the latter increasing the population of naive hMSCs capable of undergoing osteogenesis under low or undetectable Wnt signaling.

To address whether or not the enhanced bone formation by Wnt1 was caused by other factors induced by activated β-catenin signaling, we expressed stabilized mutant β-catenin in hMSCs and examined the effects on bone formation in vivo. β-Catenin was specifically detected in hMSCs expressing stabilized β-catenin but not in mixed LacZ-expressing hMSCs in the same implants (Fig. S3 E) or implants with hMSC-LacZ cells alone (Fig. S3 F). Of note, expression of stabilized β-catenin significantly increased cell numbers in implants but had relatively little effect on the number of naive hMSC-LacZ cells under mixed conditions (Fig. S3 G). Consistent with the in vitro osteogenic inhibitory function of β-catenin reported previously (Jian et al., 2006), we observed little if any bone formation by hMSCs expressing mutant β-catenin alone and no increased bone formation when mixed with hMSC-LacZ cells (Fig. S3 H). These results establish that canonical Wnt ligands acting in a cell-nonautonomous manner are required for enhanced bone formation in this context.

Based on our present findings and the aforementioned previous studies, we propose a model for canonical Wnt modulation of osteogenesis by hMSCs in vivo (Fig. 5 L) in which a Wnt activity gradient results from the asymmetrical localization of Wnts and antagonists between hMSC stem/progenitor and osteoblasts/osteocyte compartments. For example, we observed Dkk1 up-regulation during osteogenesis, and previous studies have indicated increased expression of other Wnt antagonists, secreted frizzled-related proteins (Boland et al., 2004), and sclerostin by osteoblasts/osteocytes (Winkler et al., 2003). Such a gradient of Wnt activity likely plays important roles in stimulating self-renewal and expansion of hMSCs/progenitors at high Wnt activity levels while permitting osteoblastic differentiation where Wnt activity decreases. Differential sensitivity to Wnt inhibition of osteogenesis versus adipogenesis in vitro also provides a possible mechanism to favor osteoblastic commitment within a certain range of Wnt activity by restricting hMSCs from adopting adipocytic cell fate. Our findings strongly favor a direct role of canonical Wnt in bone formation but do not exclude a role for LRP5 on bone formation through regulation of the hormonal acting serotonin as recently reported (Yadav et al., 2008).

Despite their potential clinical use in regenerative medicine and tissue engineering, bone formation by hMSCs is poor, and, so far, repair of critical-size bone defects has not been achieved

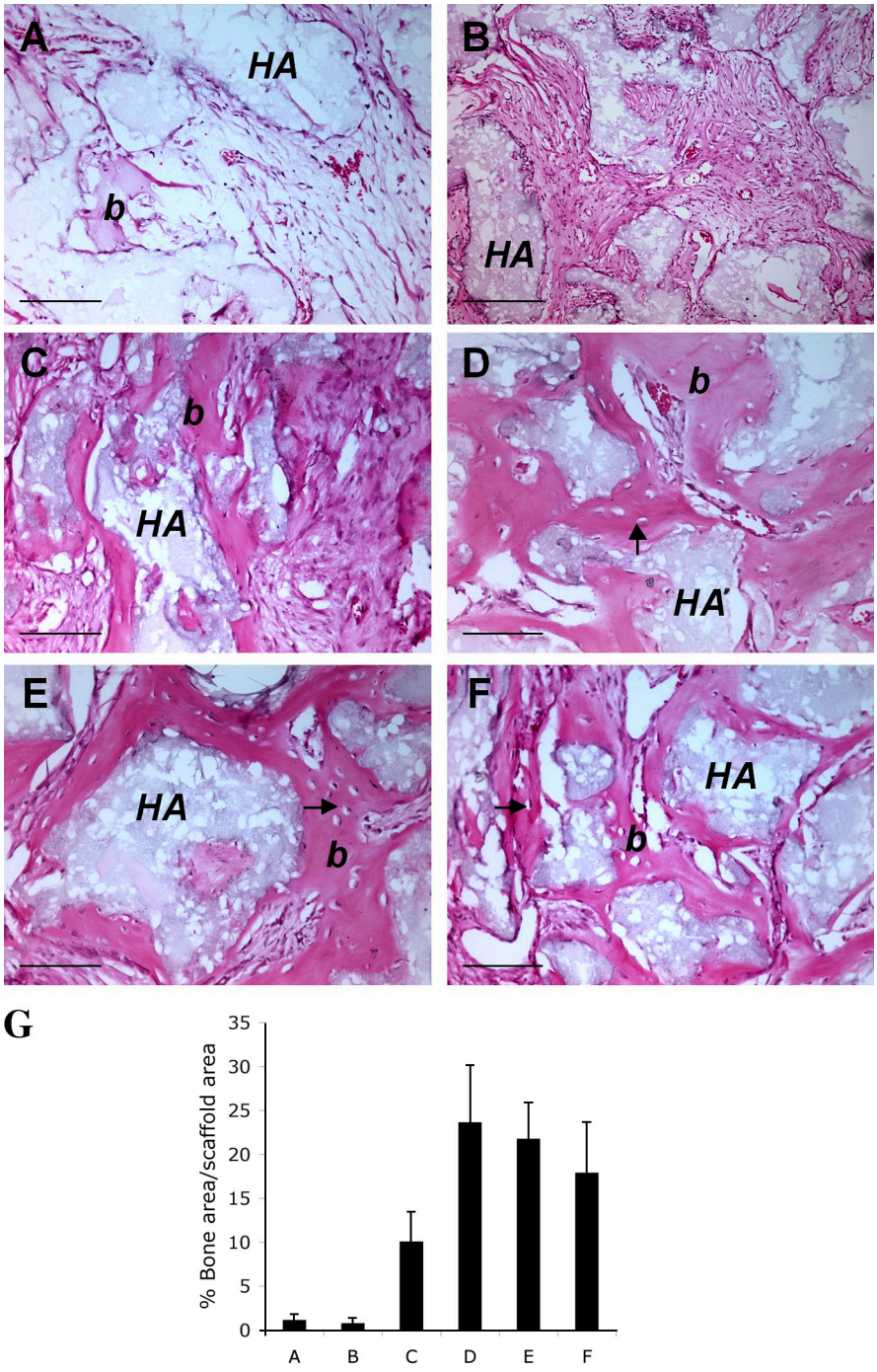

Figure 4. **Effects of canonical Wnt signaling on osteogenesis by hMSCs in an ectopic bone formation model.** (A–F) hMSC-vector cells alone (A), hMSC-Wnt1 cells alone (B), or hMSC-vector cells mixed with hMSC-Wnt1 cells (C–F) at ratio of 1:1 (C), 3:1 (D), 10:1 (E), or 20:1 (F). b, bone; HA, HA/TCP. Arrows designate osteocytes within bone structures in eosin-stained sections. (G) Quantitative analysis of bone formation by hMSCs in the recovered implants of A–F. Results represent mean values ± SD from experiments performed in triplicate. Bars, 100 µm.

(Meijer et al., 2007). A recent study showed that preexposure of hMSCs to dibutyryl-cAMP, an activator of the PKA pathway, can enhance ectopic bone formation by these cells from 1.5% in the control group up to 6% (Siddappa et al., 2008). Our present findings indicate that canonical Wnts can have a major positive impact on hMSC-mediated bone formation in HA/TCP transplants, increasing bone formation up to 25% under our experimental conditions. The appropriate manipulation of Wnt ligand expression might lead to significant improvement in the efficiency of tissue engineering and enhance the therapeutic value of these stem cells for the restoration of bone defects.

Materials and methods

Cell culture, CM, growth factors, and inhibitors

Primary hMSC cultures were obtained from three independent donors (Lonza) and expanded in basal medium or induced with osteogenic or adipogenic medium (Lonza) according to the manufacturer's instructions. CM of control or Wnt3a was collected from L-cell transfectant as described previously (Boland et al., 2004). Recombinant Wnt3a (R&D Systems), PDGF-BB (PeproTech), and SP600125 (EMD) were commercially obtained.

Assays for in vitro osteogenic and adipogenic differentiation

Ca deposition was analyzed by staining with 2% Alizarin red (Sigma-Aldrich) or extracted with 0.5 N HCl and quantified with Liquicolor kit (Stan-Bio Laboratory). AP activity was detected histochemically with Leukocyte

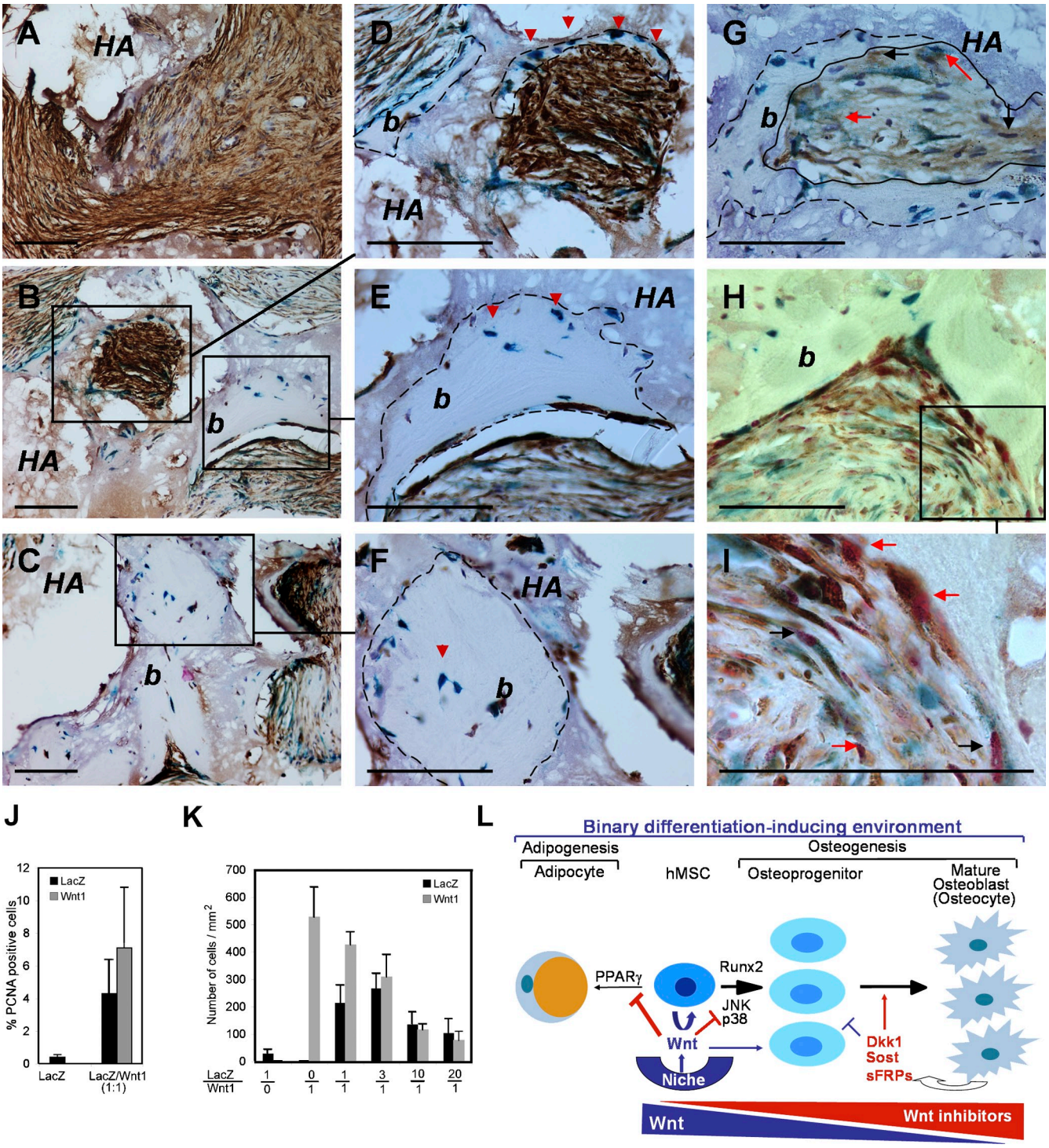

Figure 5. **Canonical Wnt1 stimulates bone formation by hMSCs in a paracrine mode in vivo.** (A–C) hMSC-Wnt1 alone (A) or hMSC-LacZ mixed with hMSC-Wnt1 (B and C) at ratio of 1:1 (B) or 3:1 (C). Sections in A–C were stained for Wnt1 protein (brown) and LacZ (blue), with higher magnification images shown in D–F as indicated. Dashed lines outline bone. Red arrowheads indicate LacZ-positive osteocytes. (G) β-Catenin staining (brown) in section with 1:1 mixture as in B. Black arrows indicate Wnt1 cells, and red arrows indicate LacZ cells with positive β-catenin staining. (H) A section with bone structures surrounded by mixed hMSCs as in B was stained for Wnt1 (brown), LacZ (blue), and PCNA (red), with a higher magnification image shown in I as indicated. Red and black arrows indicate positive PCNA staining in Wnt1- and LacZ-expressing hMSCs, respectively. All sections were counterstained with hematoxylin (dark blue). (J) Quantification of PCNA staining in implants with hMSC-LacZ alone or 1:1 mixed hMSCs as in B. (K) Quantification of cell numbers in implants of LacZ- and Wnt1-expressing hMSCs. (J and K) Numbers shown are mean values ± SD of experiments performed in triplicate. (L) Model for the effects of a canonical Wnt gradient on bone formation by hMSCs in vivo. sFRPs, secreted frizzled-related proteins; Sost, sclerostin. Bars, 100 μm.

Alkaline Phosphatase kit (Sigma-Aldrich) or quantified with a chemiluminescent substrate (Roche) and normalized to total proteins. Adipocytes were stained with Oil red O (Sigma-Aldrich) as previously described (Ross et al., 2000). For quantification, the incorporated dye was extracted with isopropanol and measured at 500 nm for optical density.

Constructs, virus production, and transduction of hMSCs

Mouse Wnt1, mutant β-catenin (S33Y), dnTCF4, and LacZ cDNA were all cloned into lentiviral vectors. shRNA targeting Ror2 (Liu et al., 2007) or a control shRNA was also expressed by lentiviral vector. Viruses were produced in HEK293T cells by transfection with lentiviral and packaging plasmids. hMSCs were selected for stable expression after viral transduction.

Western blotting and luciferase reporter assay

Western blotting was performed as previously described (Liu et al., 2005). Antibodies for p-JNK, JNK, p-p38, p38, p-ERK, and ERK were all purchased from Cell Signaling Technology, and α-Ror2 was purchased from R&D Systems. A lentiviral-based TCF reporter system containing 14 repeats of TCF binding sites (Top) was used to assay Wnt signaling, with control Fop reporter containing mutated TCF binding sites. Cells were coinfected with Renilla luciferase virus and assayed using the Dual Luciferase Assay System (Promega).

Quantitative real-time PCR

cDNA was synthesized from total RNA extracted with TRIZOL (Invitrogen). Real-time PCR was conducted with ∼50 ng cDNA using FastStart SYBR Green Master mix (Roche). Two independent experiments were performed for each reaction in triplicate. All data were normalized to the TATA box–binding protein.

In vivo transplantation and histological analysis of bone formation

Detailed procedures followed protocols described previously (Krebsbach et al., 1997). For each transplant, 1×10^6 cells in total were loaded onto 40 mg HA/TCP powder (Zimmer) and transplanted subcutaneously into immunodeficient 8–15-wk-old female mice. Transplants were recovered at 12 wk, fixed, decalcified, and embedded into paraffin blocks. Consecutive sections were prepared from three different levels and subjected to hematoxylin and eosin staining.

LacZ detection and immunostaining of implant sections

For LacZ staining, implants were fixed in 4% paraformaldehyde, 0.8% glutaraldehyde, 0.02% NP-40, and 1 mM $MgCl_2$ in PBS for 1h, stained overnight at 37°C with 400 μg/ml X-gal, 0.1 mM $MgCl_2$, 20 mM potassium ferrocyanide, and 20 mM potassium ferricyanide in PBS, and subjected to decalcification and histological processing. Wnt1 or PCNA was immunostained with α-Wnt1 (Invitrogen) or α-PCNA (Santa Cruz Biotechnology, Inc.) followed by secondary antibodies (Invitrogen) according to the manufacturers' protocol.

Microscopy imaging

Images of hMSCs (Fig. 1, D–G and Figs. S1, C and D, and S2, B and D) were acquired with a laser capture system (PixCell II; Arcturus Engineering, Inc.) with a 10× objective for Fig. 1 (D–G) and a 20× objective for other images (except Fig. S1 C, middle, with a 40× objective) using Arc200 software (Arcturus Engineering, Inc.). Top- and Fop-GFP experiments (Fig. 3 B) were analyzed with a fluorescence microscope (Eclipse TE200; Nikon) using a 20× objective. Images were acquired with a camera (7.1 Monochrome W/IR; Diagnostic Instruments, Inc.) using Spot advanced software (Diagnostics Instruments, Inc.). Sections (Figs. 4 and 5 and Fig. S3) were analyzed with a microscope (Eclipse E200; Nikon) with 20× (Figs. 4, A–F, and 5, A–C, and Fig. S3, A–C), 40× (Fig. 5, D–H, and Fig. S3, D–F), and 100× objectives (Fig. 5 I). Images were acquired with a 7.1 Monochrome W/IR camera using Spot advanced software.

Online supplemental material

Fig. S1 shows the effects of canonical Wnts on hMSC differentiation and proliferation. Fig. S2 shows the effects of inhibition of endogenous Wnt/β-catenin signaling on hMSC differentiation in vitro. Fig. S3 shows that paracrine Wnt signaling stimulates ectopic bone formation by hMSCs in vivo. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200810137/DC1.

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