Predominant role of PDGF receptor transactivation in Wnt3a-induced osteoblastic cell proliferation

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Abstract

This paper described the molecular mechanism by which Wnt3a stimulates the proliferation in osteoblasts.

Reference


DOI: 10.1002/jbmr.1748
Predominant Role of PDGF Receptor Transactivation in Wnt3a-Induced Osteoblastic Cell Proliferation

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ABSTRACT
Previous studies have shown that Wnt3a enhances the proliferation and inhibits the osteogenic differentiation of human mesenchymal stem cells (hMSCs). In this study, we investigated the signaling pathways involved in Wnt3a-induced osteoblastic cell proliferation. Experiments with DKK1, a natural antagonist of Lrp5/6, indicated that Wnt/β-catenin did not play a major role in Wnt3a-induced osteoblastic cell proliferation. The use of selective inhibitors of known mitogenic pathways implicates Src family kinases (SFKs) and a protein kinase C (PKC) in this cellular response. Time-dependent analysis of signaling molecules activated by Wnt3a in MC3T3-E1 cells revealed parallel activation of the canonical pathway and of several tyrosine kinases, including SFKs and PDGF receptors (PDGF-Rs). Functional analysis with specific inhibitors suggested a major role of PDGF-Rs in mediating Wnt3a-induced cell proliferation. Further investigation with an si-RNA approach confirmed a predominant role of this receptor in this cellular response. The use of soluble decoy PDGF-Rs that can sequester extracellular PDGFs excluding that part of the increased PDGF receptor phosphorylation by Wnt3a was the result of autocrine production of PDGFs. A selective SFK inhibitor blunted the enhanced PDGF-R phosphorylation and cell proliferation induced by Wnt3a. Studies of initial events involved in the regulation of this pathway suggest a role of dishevelled. In conclusion, data presented in this study indicate that cell proliferation induced by Wnt3a in osteoblastic cells is mediated by a dishevelled-dependent and β-catenin-independent pathway, which involves the transactivation of PDGF receptors. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: WNT3A; OSTEOBLASTS; DISHEVELLED; SRC KINASE; PLATELET-DERIVED GROWTH FACTOR RECEPTOR; PROLIFERATION; DIFFERENTIATION

Introduction
Wnts are secreted glycoproteins that can bind to Frizzled (Fzd) receptors and low-density lipoprotein coreceptors 5 and 6 (LRP5/6) and initiate signaling cascades with major roles in cell fate determination, proliferation, and survival. Pathways downstream of Wnt binding to Fzds include the well-described Wnt/β-catenin canonical pathway as well as β-catenin-independent pathways diversely classified as Wnt/calcium and Wnt PCP pathways, which are less understood. Dishevelled (Dvl) protein relays Wnt canonical and noncanonical signals from receptors to downstream effectors. In the canonical pathway, Dvl is recruited by the receptor frizzled and prevents the constitutive destruction of cytosolic β-catenin. In the noncanonical pathways, Dvl can signal through RhoA and Rac1 axis and other Wnt-related signals. These pathways have been reported to share components and crosstalk with other signaling networks, interactions that are cell type–dependent.

Previous studies in bone biology have demonstrated that Wnt/β-catenin signaling represents a major mechanism for the specification and development of mesenchymal precursors into osteoprogenitors and skeletal development. Conditional deletion of β-catenin, the central molecule of canonical Wnt signaling, in limb and head mesenchyme during early embryonic development has resulted in arrest of osteogenesis and lack of mature osteoblasts. In absence of β-catenin, osteochondro-progenitors differentiate into chondrocytes instead of osteoblasts. A key role of Wnt signaling in embryonic skeletal development has recently been confirmed in mesenchyme Lrp5 and Lrp6 double-deficient mutants. The phenotype of the double-deficient embryo closely resembles that of the β-catenin–deficient mutant. In vivo analysis of osteoblast-specific gain- and loss-of-function mutations of β-catenin has indicated that this Wnt signaling molecule does not affect postnatal bone formation but negatively influences osteoclast differentiation and bone resorption through deregulation of the osteoprotegerin gene. Genetic studies have also shown that LRPS plays...
an important role in the control of bone mass. Mutations in LRP5 lead to disorders associated with either low\(^{(12-14)}\) or high bone mass.\(^{(15-17)}\) LRP5 mutations affect bone density by altering osteoblast number and bone accrual.\(^{(12,14,15)}\) Controversial data have been published concerning the osteoblastic cell autonomous function of the Lrp5/β-catenin pathway in controlling bone metabolism.\(^{(18,19)}\) Despite this controversy, there is in vivo blastogenesis and chondrogenesis.\(^{(12,24-26)}\) In contrast, other mouse function of the Lrp5/β-catenin pathway has been published concerning the osteoblastic cell autonomous function of the Lrp5/β-catenin pathway in controlling bone metabolism.\(^{(18,19)}\) Despite this controversy, there is in vivo evidence for a negative regulation of bone formation by Wnt antagonists such as DKK1, Krm2, or Sfrp1.\(^{(20-23)}\) These observations supported the existence of an in vivo cell autonomous effect of Wnts in osteoblasts. The role of Wnt signaling in controlling preosteoblastic cell proliferation and differentiation is not clear. Studies using various in vitro systems have suggested that the Wnt/β-catenin pathway promotes osteoblastogenesis and chondrogenesis.\(^{(12,24-26)}\) In contrast, other studies have reported that Wnts either enhance the proliferation and/or suppress the differentiation of mesenchymal progenitor cells,\(^{(27-29)}\) preosteoblasts,\(^{(30)}\) or cementoblasts.\(^{(31)}\)

Other studies in Wnt signaling revealed a more complex transduction network than previously thought. For instance, in mesenchymal ST2 cells, a PKCd-dependent pathway that contributes to Wnt-induced bone formation has been described.\(^{(32)}\) This pathway is activated by Gaq heterotrimeric proteins in response to Wnt3a, probably through frizzled receptors. A Rac1-JNK noncanonical pathway has also been described in the same mesenchymal cell line and plays an important role in limb development. In these cells, Rac1 and JNK2 appear to be required for β-catenin nuclear localization and signaling.\(^{(33)}\) Finally, it has also been shown that Wnt proteins influence smooth muscle cell development via a tenasin/ PDGF-R paracrine signaling.\(^{(34)}\)

In this study, we investigated the molecular mechanism by which Wnt3a regulates cell proliferation in preosteoblastic MC3T3-E1, human mesenchymal stem cells, and mouse primary cultured osteoblasts. Data presented in this study confirm that Wnt3a enhances the proliferation of osteoblastic cells. We found that this effect is associated with a reversible Wnt3a-induced inhibition of their differentiated status. Effects of this Wnt protein on cell proliferation result in small part from the canonical β-catenin pathway and mainly from transactivation of PDGF receptors probably via activation of Dvl and Src kinase. These findings describe a previously unknown regulatory mechanism by which Wnt proteins influence osteoblast proliferation and differentiation.

### Materials and Methods

#### Reagents and antibodies

Fetal calf serum (FCS), glutamine, antibiotics, and trypsin/EDTA were obtained from Gibco (Life Technologies, Basel, Switzerland). Alpha-modified essential medium (a-MEM) was purchased from Amimed (Biocconcept, Allschwill, Switzerland). Go6983 and the Dv-PDGF domain inhibitor peptide Pen-N3 were from Merck-Millipore (Darmstadt, Germany). SU6656 was from Sigma-Aldrich (St. Louis, MO, USA). U0126 and SB202190 were from Tocris (Lucena Chem AG, Luzern, Switzerland). Anti-pPDGF-Ra/β, anti-PDGF-Ra, anti-PDGF-Rβ3, anti-p-Akt, anti-p-PLCg, anti-p-pan-PKC, anti-p-SFK, anti-CD1, anti-pan-actin, anti-dishevelled2 and anti-p-Lrp5/6 were from New England BioLabs (Cell Signaling Technology, Danvers, MA, USA). Anti-active β-catenin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DKK1 was a generous gift from Promega (San Luis Obispo, CA, USA). PDGFs, or FGF2 (Immunotools, GmbH, Friesoythe, Germany). Cell proliferation was determined after 48 hours (MC3T3-E1) or 96 hours (hMSCs and mPOBs) of incubation with agents by cell counting (Coulter Counter, Beckman, Brea, CA, USA).

#### Alkaline phosphatase activity

Alkaline phosphatase activity (ALP) was determined as previously described.\(^{(36)}\) Essentially, cells were harvested in 0.1% Nonidet P-40 and disrupted by sonication. The homogenate was centrifuged at 15000 g for 5 minutes, and ALP was determined by the method of Lowry and colleagues.\(^{(37)}\)

#### Transfection of cells and transient reporter assay

To analyze the β-catenin-driven transcription, subconfluent cells were exposed to 45 μg/mL of the TCF reporter plasmid TOPflash (Upstate Biotechnology, Lake Placid, NY, USA) expressing the firefly luciferase and 9 μg/mL of the control plasmid pRLTK expressing the Renilla luciferase (Clontech, Takara Bio Europe, Saint Germain-en-Laye, France) with 225 μL of polyfect (Qiagen AG, Hombrechtikon, Switzerland) in 2-mL a-MEM medium containing 10% FCS for 18 hours. DKK1 and Wnt3a were then added for 24 hours before the determination of luciferase activity using the Promega (San Luis Obispo, CA, USA) dual-luciferase assay according to the manufacturer’s instructions.

#### Western blotting analysis

Cell layers treated with either vehicle or recombinant human Wnt3a were rapidly frozen in liquid nitrogen and stored at −80 °C until used for analysis. For cell lysate preparation, cells were incubated at +4 °C in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mM Na3VO4, 1% of the
protease inhibitor cocktail Set V (Calbiochem, Merck Ltd, Norringham, UK), 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS for 10 minutes. Lysates were then cleared by centrifugation at 6000g for 30 minutes. A 75-μL sample of lysate was diluted with an equal volume of 2x concentrated reducing sample buffer containing 125 mM Tris buffer (pH 6.8), 4% SDS, 20% glycerol, 0.05% bromophenol blue, and 200 μM dithiothreitol. The mixture was then heated at +70°C for 30 minutes and subjected to gel electrophoresis on 6% to 15% gels. After SDS-PAGE electrophoresis, proteins were transferred to Immobilon P membranes and immunoblotted with specific antibodies as previously described. Detection was performed using peroxidase-coupled secondary antibody, enhanced chemiluminescence reaction, and visualization by autoradiography (Amersham International, Little Chalfont, UK). Reprobed membranes were stripped according to the manufacturer's protocol.

RNA analysis

Total cellular RNA was extracted using peqGOLD TriFast (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and purified using an RNeasy Mini Kit (Qiagen). Single-stranded cDNA was synthesized from 2 μg of total RNA using a high-capacity cDNA archive kit (Applied Biosystems, Zug, Switzerland) according to the manufacturer's instructions. Two microliters of the cDNA sample was used in a 40-μL reaction containing a Red Taq PCR reaction buffer (Sigma-Aldrich), 1.5 mM MgCl₂, 0.2 mM dATP, dCTP, dGTP, dTTP, 0.4 μM of gene-specific primers, and 1 unit of Red Taq DNA Polymerase (Sigma-Aldrich). Primers were amplified using a program starting with 5 minutes of denaturation at 95°C, followed by 30 cycles with 45 seconds of denaturation at 95°C, 45 seconds of annealing at 58°C to 59°C, and 30 seconds of extension at 72°C and 5 minutes of final extension at 72°C. Mouse primer sequences were as follows: PDGF-Rβ, forward: 5’-CTTCCTCCCTATGGACTCCGAG-3’, reverse: 5’-CGACAGCGGTGTGTTGCTC-3’; Gapdh, forward: AGGTCTACCCAGAGCTGAACG, reverse: GTAGGCCATGAGGTCCACCAC.

Cell proliferation analysis with siRNA

Subconfluent MC3T3-E1 cells cultured in complete medium were transduced with 100 nM of either ON-TARGETplus mouse cyclophilin B Control siRNA or ON-TARGETplus SMART Pool mouse PDGF-Rβ-siRNA (Thermo Scientific Dharmacon, Fisher, Switzerland) with 60 μL of jetPRIME (Polyplus-Transfection SA, Illkirch, France) for 48 hours before exposure to either vehicle, 100 ng/mL of recombinant Wnt3a, or 10 ng/mL FGF2 for an additional 48 hours. Then, either mRNA or protein levels of PDGF-Rβ were determined by RNA and Western blot analysis as described above and proliferation by cell counting.

Statistical analysis

All experiments were carried out independently at least two times. Results are expressed as the mean ± SEM of triplicates. Comparative studies of means were performed using one-way analysis of variance followed by a post hoc test (projected least significant difference Fisher) with a significance value of p < 0.05.

Results

In vivo studies have shown that both Lrp5/6 and Fzd-dependent pathways influence osteoblast function and bone metabolism. However, the cellular and molecular mechanisms involved in the regulation of osteoblastic cells and bone mass by Wnt proteins remain unclear. In vitro studies have shown that Wnt3a promotes the proliferation and suppresses the differentiation of adult human mesenchymal stem cells. Whether this effect is also observed in more differentiated osteoblastic cells is unknown, and the molecular mechanism involved in this response has not been investigated. As shown in Fig. 1, Wnt3a stimulated the proliferation of MC3T3-E1 cells, hMSCs, and, to a lesser extent, of mouse primary cultured osteoblastic cells. As previously described in hMSCs, enhanced proliferation by Wnt3a was associated with a decreased
ALP activity in the three osteoblastic cell types, suggesting decreased cell differentiation (Fig. 1). Wnt3a also markedly reduced markers of cell differentiation in MC3T3-E1 cells, including ostein, BspI, alkaline phosphatase, collagen 1, and osteocalcin (Supplemental Fig. S1,[FIG S1] upper panel). Except for collagen 1 that was not affected by Wnt3a, similar effects were observed in mPOBs (Supplemental Fig. S1, lower panel).

Interestingly, the downregulation of osteoblastic markers induced by Wnt3a was a reversible process in both cell types. When this factor was removed in the culture medium after the initial treatment, we observed a substantial recovery of ostein (Supplemental Fig. S1) and of other osteoblast marker expression (data not shown). In hMSCs, the reversible downregulation of osteogenic markers by Wnt3a was not associated with enhanced expression of adipogenic markers assessed with FAB4 and PPARγ measurement (Supplemental Fig. S2).[FIG S2] To determine the contribution of the canonical Wnt signaling in Wnt3a-induced cell replication, we used DKK1, a selective antagonist of Lrp5/6. DKK1 marginally affected cell proliferation induced by Wnt3a but markedly reduced the β-catenin transcriptional activity assessed with the TOP flash assay in MC3T3-E1 cells (Fig. 2A, B) and hMSCs (Fig. 2C, D) and Lrp5/6 phosphorylation (Fig. 7A). This observation suggested a major role of a noncanonical pathway in mediating Wnt3a-induced osteoblastic cell proliferation and differentiation. To uncover the molecular mechanism involved in these effects, cells were preincubated with different selective signaling pathway inhibitors and cell proliferation induced by Wnt3a was determined. Data presented in Fig. 3 indicate that inhibitors of Src family kinase (SU6656 and CGP77675) and of protein kinase C (PKC; GO6983) markedly reduced the mitogenic effect of Wnt3a in MC3T3-E1 and hMSC cells. The selective MEK1/2 (U0126) and p38 (SB202190) inhibitors had no effect in MC3T3-E1 cells (Fig. 3A) but blunted this response in hMSCs (Fig. 3B). A selective JNK inhibitor (SP600125, 10 μM) had no effect on this cellular response in MC3T3-E1 cells (data not shown). This analysis suggested that Wnt3a-induced MC3T3-E1 and hMSC proliferation implicates Src family kinases (SFKs) and a PKC with differences in the involvement of the mitogen-activated protein (MAP) kinase pathways between the two cell types. Because SFKs, PKC, and MAP kinases are often activated by receptor tyrosine kinases (RTKs), we investigated whether, in addition to activation of the canonical Lrp5/β-catenin pathway, Wnt3a may also induce activation of some RTKs and their signaling pathways in MC3T3-E1 cells. As expected, Dvl, Lrp5/6 and β-catenin were transiently activated in response to Wnt3a (Fig. 4). Enhanced Dvl (band shift) and Lrp5/6 phosphorylation were detected after 1 hour with a maximal effect after 6 hours of incubation. This effect was maintained for at least 10 additional hours and then gradually decreased. Associated with this effect, activation of β-catenin was slightly delayed compared with Dvl and Lrp5/6 activation, but the duration of increased activity was similar (Fig. 4, upper panel). Among RTKs investigated, the phosphorylation of EGF-Rs and PDGF-Rs (Fig. 4, lower panel) but not FGF-Rs (data not shown) were found to be enhanced in Wnt3a-treated MC3T3-E1 cells. The amount of phosphorylated EGF-Rs was slightly increased in response to Wnt3a after a 12-hour incubation (Fig. 4, lower panel). This effect was maintained for 12 additional hours. The total amount of EGF-Rs was also slightly increased during this time period, suggesting that Wnt3a slightly and transiently increased the number of phosphorylated EGF-Rs. Compared with EGF-Rs, endogenous PDGF-Rs phosphorylation was very low, and Wnt3a markedly and transiently increased this phosphorylation in MC3T3-E1 cells (Fig. 4, lower panel). A slight increase was already detected after 6 hours of incubation with maximal stimulation between 12 hours and 16 hours with a gradual and rapid decrease after 20 hours. Of the two well-known PDGF-Rs, PDGF-Rα was rapidly and transiently downregulated in response to Wnt3a, whereas the level of PDGF-Rβ was not changed or slightly increased at later time points compared with vehicle. Initial increase in PDGF-R phosphorylation correlated with enhanced phosphorylation of SFKs by Wnt3a. Stimulation of SFKs, however, lasted much longer than phosphorylation of PDGF-Rs because it was still present after 24 hours and 42 hours of incubation (Fig. 4, lower panel). Associated
with phosphorylation of PDGF-Rs, the phosphorylation of well-known downstream signaling pathways of this type of receptor such as Akt, PLCg, and PKC were also enhanced, indicating that the change in PDGF-R phosphorylation corresponded to a change in activity. The duration of PLCg phosphorylation correlated with that of PDGF-Rs, whereas activation of Akt and PKC lasted much longer (Fig. 4, lower panel). Cyclin-D1, a cell cycle regulator in early to mid-G1 phase, was also enhanced by Wnt3a at time points similar with those of PDGF-Rs and PLCg activation, suggesting that these molecules are involved in Wnt3a-induced osteoblastic cell proliferation.

Because PDGF-Rs were activated in response to Wnt3a, a selective PDGF-R inhibitor was used to further investigate their functional role. As shown in Fig. 5A, this inhibitor dose-dependently reduced the mitogenic effect of Wnt3a in MC3T3-E1 cells. Specifically, other inhibitors of EGF-Rs, FGF-Rs and VEGF-Rs had either no or much lower effects on this response (data not shown). The PDGF-R inhibitor also completely blunted the mitogenic effect of Wnt3a in hMSCs (Fig. 5B). In MC3T3-E1 cells, PDGF-BB was more potent than PDGF-AA in enhancing MC3T3-E1 cell proliferation (Fig. 5C), suggesting a predominant role of PDGF-Rb in mediating this response. To further investigate a role of PDGF-Rs in mediating Wnt3a-induced cell proliferation, we used an siRNA approach. Transfection of an si-PDGF-Rb in MC3T3-E1 cells reduced mRNA expression of this receptor by 65% to 70% (Fig. 5D) and its protein level by 80% (Fig. 5E). Downregulation of PDGF-Rb almost completely blunted the effect on Wnt3a on cell proliferation (Fig. 5F). This effect was selective because it did not blunt the proliferative effect of FGF2. Signaling pathway inhibitors were used to get insight into the molecular mechanism by which Wnt3a induces activation of PDGF-Rs. All inhibitors did not influence activation of Dvl induced by Wnt3a (band shift, Fig. 6A). CGP77675 was the only agent that completely blunted the phosphorylation of PDGF-Rs and associated signals induced by Wnt3a (Fig. 6A). This effect was observed without any change in β-catenin activation and with a 74% decrease in cyclin D1 expression induced by Wnt3a. SU6656 and GO6983, which also completely blunted cell proliferation...
induced by Wnt3a, had no effect on PDGF-R signaling but also reduced cyclin D1 expression (−42% and −64%, respectively). In contrast, the selective MEK1/2 inhibitor, U0126, which did not affect Wnt3a-induced cell proliferation, had no effect on signaling (Fig. 6A) and cyclin D1 expression (−16%). To assess whether the increased PDGF-R activity observed in response to Wnt3a was because of autocrine production of PDGFs, we used recombinant mouse PDGF-Rβ and PDGF-Rα Fc chimera to blunt the effect of extracellular PDGFs. As presented in Fig. 6B, C, the chimera completely blunted the mitogenic effect of PDGF-BB and PDGF-AA, respectively, but had no effect on the Wnt3a response, indicating that activation of PDGF-Rs by Wnt3a may not be because of the production of extracellular PDGFs. To investigate initial events involved in PDGF-R transactivation, we used a selective Dvl inhibitor (Dvl-PDZ domain inhibitor, peptide Pen-N3) and compared its effect with that of DKK1. Surprisingly, functional analysis indicated that this peptide inhibitor enhanced MC3T3-E1 cell proliferation in the absence of Wnt3a (Fig. 7A). Associated with this effect, activation of SFK(s), the phosphorylation of PDGF-R, and its downstream signaling protein Akt were enhanced to a similar extent compared with Wnt3a alone (Fig. 7B). In cells treated with the Dvl inhibitor, cell proliferation and the change in SFK(s), PDGF-R, and Akt phosphorylation induced by Wnt3a were either markedly reduced or not changed. As expected, this inhibitor reduced activation of both Dvl (reduction in the band shift) and of active β-catenin but had no effect on the phosphorylation of Lrp5/6 (Fig. 7B). In contrast, DKK1 markedly blunted the phosphorylation of Lrp5/6 induced by Wnt3a. It also reduced β-catenin and Dvl activity but had no effect on PDGF-R phosphorylation induced by Wnt3a (Fig. 7C).

**Discussion**

Results presented in this study indicate that Wnt3a enhances the proliferation of preosteoblastic MC3T3-E1 cells and primary cultures of osteoblasts and confirm this effect in hMSCs. In MC3T3-E1 and mPOBs, enhanced proliferation by Wnt3a was

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**Fig. 5.** Role of PDGF-R in Wnt3a-induced cell proliferation. (A) MC3T3-E1 cells were preincubated with various concentrations of the selective PDGF-R inhibitor III for 15 minutes and then exposed to either vehicle (Veh) or Wnt3a (100 ng/mL) for 48 hours before the determination of cell proliferation by cell counting. (B) Human MSCs were preincubated with 1 μM of the selective PDGF-R inhibitor III and then exposed to either vehicle (Veh) or Wnt3a (100 ng/mL) for 6 days. Then, proliferation was determined by cell counting. (C) MC3T3-E1 cells were preincubated with either vehicle, PDGF-BB, or PDGF-AA at 25 ng/mL for 48 hours before determination of cell proliferation. (D, E) MC3T3-E1 were transfected with 100 nM of either cyclophilin (cyclo) siRNA or PDGF-Rβ siRNA for 48 hours before measurement of either PDGF-Rβ expression by PCR analysis (D), expression of the PDGF-Rβ protein by western blotting (E), or cell proliferation by cell counting in response to either Wnt3a (100 ng/mL) or FGF2 (10 ng/mL) (F). Cell proliferation and mRNA data are mean ± SEM of quadruplicates of a representative experiment. Western blot results are from one experiment. a, p < 0.01 compared with control; b, p < 0.01 compared with Wnt3a-treated cells in absence of either inhibitors or si-PDGF-Rβ.
associated with a decreased osteoblastic cell differentiation as previously reported, a process we found to be reversible. In hMSCs, although Wnt3a induced a downregulation of osteogenic markers, it did not promote adipogenic differentiation (Supplemental Fig. S2). In vitro, the cellular effects of Wnt3a has been reported to be cell type– and culture condition–dependent. In vivo, Wnt3a has been shown to accelerate the healing process of bone repair when delivered in liposomes at injury sites. This effect of Wnt3a was mainly the result of enhanced proliferation of skeletal progenitors and acceleration of their differentiation into osteoblasts. Thus, Wnt-induced mesenchymal cell proliferation is an important mechanism for enhanced bone formation and fracture repair. The cellular and molecular systems involved in Wnt3a-induced osteoblastic cell proliferation remain poorly understood and have been investigated in this study. Wnt3a has been assigned to the group of canonical Wnts that signal through the β-catenin pathway. However, information in endothelial cells has suggested that Wnt3a may regulate their proliferation via canonical and noncanonical Wnt signaling. To assess the contribution of the canonical pathway in Wnt3a-induced MC3T3-E1 and hMSCs proliferation, we used DKK1, a selective Lrp5/6 antagonist. Although DKK1 completely inhibited the canonical Wnt/β-catenin pathway, it only slightly reduced the proliferation of MC3T3-E1 cells induced by Wnt3a and had no effect in hMSCs, indicating that this proliferative response involves a noncanonical pathway. To characterize this noncanonical Wnt signaling, we first used selective inhibitors of SFKs, PKCs, MEK1/2, and p38 activities. Data obtained suggested the involvement of SFKs and PKCs in both cell types with a variable role of MAPKs between MC3T3-E1 and hMSCs. Whereas inhibitors of MAPKs had no effect in MC3T3-E1 cells, the MEK1,2 inhibitor completely blunted the effects of Wnt3a on cell proliferation in hMSCs. Because this inhibitor also significantly reduced basal hMSC proliferation, this pathway is perhaps essential for controlling their cell cycle and its effect may not be specific for Wnt3a. From the observation that SFKs and PKCs are probably involved in Wnt3a-induced cell proliferation, we studied which signaling molecules are activated by Wnt3a in MC3T3-E1 cells. Surprisingly, we found that two tyrosine kinase systems are stimulated by Wnt3a in MC3T3-E1 cells including SFKs and PDGF-Rs. Enhancement of these kinases is a delayed
process with a kinetic similar to β-catenin activation. These findings indicate that Wnt3a-induced osteoblastic cell proliferation implicates a change in phosphorylation/activation of PDGF-Rs, an observation that is consistent with previous observations reported on PDGF/PDGF-R effects in bone-forming cells. Indeed, PDGF-Ra is required for the growth and differentiation of cranial and cardiac neural crest cells and normal craniofacial bone development. A role of PDGF-Rβ in bone development has not yet been reported. In general, PDGF-BB, which can activate both PDGF-Ra and PDGF-Rβ, is more potent than PDGF-AA, which only activates PDGF-Ra for enhancing the proliferation and migration of osteoprogenitors and osteoblastic cells. Associated with this effect, PDGF-BB also preferentially inhibits osteoblastic cell differentiation. PDGF-Rβ is also a potent regulator of mesenchymal stromal cell function. Genetic depletion of PDGF-Rβ in these cells decreased mitogenic and migratory responses and enhanced osteogenic differentiation. The use of a selective PDGF-R inhibitor further supported the hypothesis that PDGF-R activation is involved in Wnt3a-induced osteoblastic cell proliferation. This inhibitor dose-dependently reduced Wnt3a-induced cell proliferation in MC3T3-E1 cells, and a low concentration of this molecule completely blunted this response in hMSCs. This effect was specific because selective inhibitors of EGF-Rs, FGF-Rs, and VEGF-Rs had nearly no effect on this response (data not shown). To investigate which type of PDGF-Rs is involved in Wnt3a-induced cell proliferation, we initially assessed the effects of PDGF-AA and -BB on MC3T3-E1 cell proliferation. Clearly, PDGF-BB was more potent than PDGF-AA, confirming that signaling by PDGF-Rβ plays a predominant role in controlling osteoblastic cell growth. Downregulation of PDGF-Rβ using an siRNA approach indicated that this receptor is a major contributor in Wnt3a-induced MC3T3-E1 cell proliferation. Whether activation of PDGF-Rs by Wnt3a in osteoblastic cells involves an autocrine production of PDGFs was assessed using either recombinant PDGF-Rβ or PDGF-Ra Fc chimera that can sequester extracellular PDGFs. Whereas each chimera blunted the effect of their preferential PDGF ligand, none of them blunted the effect of Wnt3a on cell proliferation, strongly suggesting that activation of PDGF-Rs by Wnt3a in MC3T3-E1 cells involves a transactivation mechanism. Analysis of pathway inhibitors on Wnt3a-induced signaling indicated that transactivation of PDGF-Rs induced by
this Wnt protein probably involves an SFK because PDGF-R phosphorylation and activation of downstream signaling molecules were completely blunted by CGP77657. Surprisingly, however, the other SFK inhibitor, SU6656, had no effect on PDGF-R transactivation but also completely blunted Wnt3a-induced cell proliferation. This observation suggests that this inhibitor probably acts on a different pathway to blunt osteoblastic cell proliferation. Further studies are required to clarify this issue. The PKC inhibitor, GO6983, also did not influence the transactivation of PDGF-Rs by Wnt3a, suggesting that it probably acts downstream of PLCγ activation by PDGF-Rs. To our knowledge, this is the first report on transactivation of PDGF-Rs by a Wnt protein, whereas transactivation of EGFRs has already been reported in mammary epithelial cells, NIH3T3 cells, and breast cancer cells. The molecular mechanism by which Wnt proteins transactivate some receptor tyrosine kinases remains poorly understood. A study has shown that Wnt3a can stimulate Src docking to dishevelled-2 (Dvl2) and activates its tyrosine kinase activity. It is interesting to note that Dvl2 is a key phosphoprotein in Wnt signaling for activation of both canonical as well as noncanonical pathways and that SFKs have been shown to be able to transactivate tyrosine kinase receptors. Based on this information, we investigated whether Dvl is involved in PDGF-R transactivation induced by Wnt3a in MC3T3-E1 cells. Data provided in this study indicate that this protein is likely a proximal event in this cellular response because its activation by Wnt3a precedes SFK activation by several hours (Fig. 4) and was not inhibited by the SFK inhibitor CGP77675 (Fig. 6) that completely prevented the transactivation of PDGF-R. Unexpectedly, we found that a Dvl-PDZ-domain peptide inhibitor mimicked the effects of Wnt3a on PDGF-R transactivation and cell proliferation (Fig. 7A, B). These data suggest that Dvl is involved in activation of the β-catenin-independent pathway that induces the transactivation of PDGF-R and cell proliferation in response to Wnt3a. We speculate that the Dvl-PDZ-domain peptide inhibitor competes with a Dvl endogenous substrate involved in controlling the process of PDGF-R transactivation. Further studies are required to clarify this issue. In summary, Wnt3a binding to its receptor Lrp5/6-Fzd triggers activation of Dvl, which stimulates β-catenin-dependent and β-catenin-independent pathways in osteoprogenitors/osteoblasts. The β-catenin-independent pathway plays a prominent role in cell proliferation induced by Wnt3a via Src family kinases and phosphorylation/activation of PDGF-Rs (Supplemental Fig. S3, Fig S3).

Data presented in this study also supports the idea that signaling pathways activated by a Wnt protein is dependent on its cellular context and that a Wnt protein can activate both β-catenin-dependent and –independent pathways in the same cell type. With this report and previous studies describing transactivation of receptor tyrosine kinases by Wnt protein, it seems that the network of signaling pathways activated by Wnt proteins is becoming more complex, especially when a Wnt protein activates several tyrosine kinase receptors in a particular cell type.

In conclusion, data presented in this study describe a noncanonical Wnt pathway controlling osteoblastic cell proliferation. In MC3T3-E1, hMSCs, and primary osteoblasts, we found that Wnt3a enhances cell proliferation by transactivating PDGF receptors. Associated with this effect, Wnt3a induced a reversible inhibition of osteoblast differentiation. These observations suggest that Wnt proteins may enhance the pool of osteoprogenitors/osteoblastic cells through activation of PDGF-Rs to increase bone formation.

Disclosures
All authors state that they have no conflicts of interest.

Acknowledgments
We thank P Apostolides for his expert technical assistance. This study was supported by the Swiss National Science Foundation (310030-127638). CGP77675 was generously provided by Dr. D Fabbro at Novartis Switzerland.

Authors’ roles: Study design: JC and CT. Study conduct: JC, EB, and CT. Data collection: EB and CT. Data analysis: EB and CT. Data interpretation: JC, EB, and CT. Drafting manuscript: JC and CT. Revising manuscript content: JC, EB, and CT. Approving final version of the manuscript: JC, EB, and CT. JC takes the responsibility for the integrity of the work.

References
7. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell. 2005;8(5):739–50.


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