Activation of the Wnt signaling cascade provides key signals during development and in disease. Wnt signals are transduced by seven-transmembrane Frizzled (Fz) and the single transmembrane low density lipoprotein receptor-related proteins 5 or 6. In the course of the analysis of genes regulated by bone morphogenetic protein-2 in mesenchymal cells we found a significant induction of murine Frizzled-1 (mFz1) gene expression. Unexpectedly overexpression of mFz1 dramatically repressed the induction of alkaline phosphatase mediated by either bone morphogenetic protein-2 or Wnt3a in these cells. Moreover mFz1 overexpression significantly repressed both β-catenin translocation into the nucleus and T cell factor signaling mediated by Wnt3a. Importantly microinjection of mFz1 transcript in Xenopus embryos inhibited the ability of Wnt1 to induce the expression of the Wnt/β-catenin target gene Siamois in animal cap assay and secondary axis formation in whole embryo. By using chimeric constructs in which N- and C-terminal segments of Fz3 were replaced by the corresponding parts of Fz3 we demonstrated that the antagonistic activity resides in the cysteine-rich domain of the N-terminal part. The antagonist activity of mFz1 could be prevented by overexpression of Gqα-Q305–359, which specifically uncouples Gq-coupled receptors, suggesting that Gqα signaling contributes to the inhibition of Wnt/β-catenin pathway by mFz1. This is the first time that a Frizzled receptor has been reported to antagonize Wnt/β-catenin.

The transforming growth factor (TGF)-β family plays a central role in regulating a broad range of cellular responses including cell growth and differentiation. Among members of this family, bone morphogenetic proteins (BMPs) have been shown to regulate growth and differentiation of chondroblast and osteoblast lineage cells and induce the commitment of mesenchymal cells into osteoblast/chondroblast phenotypes (1–3). In addition, when ectopically implanted, BMPs possess the capacity to induce bone and cartilage formation (4, 5). In a series of murine cell lines including C3H10T1/2, C2C12, and ST2, BMP-2 induces the expression of several osteoblast differentiation markers including alkaline phosphatase (ALP) and osteocalcin (6). In contrast, TGF-β1 has been shown to repress the expression of osteoblast markers, and it abolishes the increase in osteocalcin induced by calcitriol (7, 8). Moreover we have previously showed that TGF-β1 is able to antagonize BMP-2 activities in several mesenchymal cell lines (9).

Wnts are secreted proteins involved in a wide range of developmental processes such as embryonic axis specification and organogenesis (10). They have been shown to activate distinct pathways both in vertebrates and in invertebrates. The better described pathway is the Wnt/β-catenin pathway. In this pathway, the interaction between Wnt and Frizzled receptors leads to inactivation of glycogen synthase kinase-3β. Genetic epistasis experiments suggest that Dishevelled lies upstream and represses the activity of glycogen synthase kinase-3β. As a consequence, β-catenin is stabilized in the cytoplasm and then forms a complex with TCF/Lympoocyte enhancer factor to activate transcription of target genes. Frizzled proteins have been shown to function as Wnt receptors (11), and they constitute a large family of seven-transmembrane receptors with at least 10 members in mammals (12). All Frizzled receptors have a conserved extracellular cysteine-rich domain (CRD) followed by seven putative transmembrane segments. Their cytoplasmic regions differ in length and sequence. Functional analyses in Drosophila and Xenopus embryos indicate that Frizzled proteins have distinct functions in Wnt/β-catenin signaling (13).

More recently, members of the low density lipoprotein receptor-related protein (LRP) family have been shown to be co-receptors for Wnt ligands (14–18). The two closely related proteins LRP5 and LRP6 are single pass transmembrane receptors and associate with Frizzled receptors in a Wnt-dependent manner. There is an increasing body of evidence showing that Wnt/β-catenin signaling plays an essential role during osteoblast differentiation. Deletion of LRP6 gene in mice results in embryos with spina bifida, absence of tail, and malformed fore and hind limbs. The skeleton of the neonate also shows truncation of the axial skeleton and loss of distal dorsal limb structures (16). More importantly, mice deficient in LRP5 exhibit decreased osteoblast proliferation and osteopenia (19). Furthermore mutations resulting in a loss of function of LRP5 in humans lead to osteopenia (20), and, in contrast, mutation of G171V leads, in two separate families, to a marked increase in bone mass (21, 22). These results clearly suggest a genetic link between LRP5, and consequently Wnt, signaling and the regeneration of bone mass.

The control of osteoblast differentiation involves a complex combination of signals. However, the relationship and possible
mFz1 Inhibits Wnt Signaling

Cell Culture—C2C12 cells, kindly provided by Dr. G. Karsenty (Baylor College of Medicine, Houston, TX), and ST-2, C3H10T1/2, COS-7 and L-Wnt3a cells, obtained from ATCC, were maintained (5% CO2 at 37 °C) in Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum at appropriate concentration. DNA Extraction, Chip Hybridizations, and Quantitative RT-PCR—Total RNA samples were obtained from three C2C12 cell cultures (BMP-2-treated, 1 μg/ml; TGF-β-treated, 2.5 ng/ml; and solvent-treated control, 10 mM HCl) by use of the RNeasy kit provided by Quantum, harvesting from each culture at six time points (4, 6, 9, 1 day, 2, 3 days, and 4 days). Total RNA was also extracted from ST-2 cells and C3H10T1/2 cells under similar conditions. Total RNA samples from C2C12 cells were hybridized to the complete series of Affymetrix 35,000 mouse chips as described by Theilhaber et al. (23), and data assembly and analysis were carried as described by the authors (23). Quantitative RT-PCR was performed with TaqMan PCR reagent kits in the ABI PRISM 7700 sequence detection system (Applied Biosystems) as described by Spijnenburg et al. (6). The following (5′–3′) primer/probe set was designed using Primer-Express Version 1.0 software from Applied Biosystems: forward, 5′-GCCCTACATCGTGGCTTCTCTC-3′; reverse, 5′-CCCGTCCCTTCTGCCAAACTT-3′; probe, 5′-AGGAGCCGTTGCTGGGACAGA-3′.

Wnt3a-conditioned Medium Preparation—Wnt3a-conditioned medium (Wnt3a-CM) was prepared as described by Shibamoto et al. (24). Briefly, to collect the conditioned medium from cultures of Wnt3a-producing L cells, these cells were seeded at a density of 6 × 10^5 cells in a 125-cm^2 flask containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 24 h after seeding, medium was changed to Dulbecco’s modified Eagle’s medium with 2% fetal calf serum, and cells were cultured for 3 days. Then Wnt3a-CM was harvested, centrifuged at 1000 × g for 10 min, and filtered through a nitrocellulose membrane. The activity of Wnt3a-CM was assayed on normal L cells by examining increase in β-catenin as described by Willert et al. (25). Wnt3a-CM was added to cells at 20% final concentration in all subsequent experiments.

Measurement of Alkaline Phosphatase Activity—C2C12 cells were plated at 2 × 10^5/cm^2 and 24 h later were transfected with the indicated constructs (1 μg total) using FuGENE 6 (Roche Applied Science). At 16 h after transfection, cells were washed and cultured in media containing 2% fetal bovine serum. When indicated, cells were treated with BMP-2 (10 ng/ml) or 10 mM ethanol. For luciferase activity analysis, cells were transfected with the indicated constructs (1 μg total) using FuGENE 6 (Roche Applied Science). At 16 h after transfection, cells were washed and cultured in media containing 2% fetal bovine serum for an additional 24 h. Cell lysates were prepared, and luciferase assays were performed with the Dual Luciferase assay kit (Promega) according to manufacturer’s instructions. 10 μl of cell lysate were assayed first for firefly luciferase and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

Plasmids and Constructs—mFz1 and human Frizzled-1 (hFz1) were amplified by RT-PCR, and the nucleotide sequence was confirmed by DNA sequence analysis and subcloned with a Myc tag into pCDNA3.1 vector and into pTracer to express GFP protein (Invitrogen). Murine TCF expressing vector and the TCF-luciferase reporter construct, TOPflash (2) and FOPflash, were obtained from Upstate Biotechnology. Human β-catenin was RT-PCR amplified, confirmed by sequencing, and subcloned into pCDNA3.1. QuickChange was used to create a constitutive active mutant form of β-catenin (β-catenin*) with the S33Y missense mutation (27). Gea (305–359) expression vector was kindly provided Dr. W. J. Koch (Duke University, Durham, NC).

Xenopus Embryos and Microinjections—Xenopus eggs were obtained from females injected with 500 IU of human chorionic gonadotrophin (Sigma), and artificially fertilized with motile sperm. Eggs were dejellied with 2% cysteine hydrochloride (pH 7.8) and kept in 0.1× modified Barth solution. Synthetic capped mRNAs were made by in vitro transcription as described previously (29). Embryos were injected at the four-cell stage near the animal pole region (for ectodermal explants) or in the two ventrocentral blastomers (for secondary axis induction) at the 1/50 molar solution of each mRNA. Embryonic Barth solution containing 3% (w/v) sodium d-glucose, 20 mM NaCl, 0.1 M NaCl, and 20 μg/ml leupeptin, and then the protein content of cell lysates was determined with Bio-Rad DC protein assay. 50 μg of each cell lysates were separated by SDS-PAGE. Proteins were then electrophoretically transferred to nitrocellulose membranes and probed with the indicated primary antibody. Immune-reactive bands were visualized by ECL Western blotting technique (Amersham Biosciences) using horseradish peroxidase-labeled secondary antibodies. Anti-Myc antibody was from Santa Cruz Technology, and the anti-β-catenin antibody was obtained from Upstate Biotechnology.

Immunoblotting—Cells were transfected with expression vectors as described above, and 24 h post-transfection, cells were lysed (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.5% Nonidet P40, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin), and then the protein content of cell lysates was determined with Bio-Rad DC protein assay. 50 μg of each cell lysates were separated by SDS-PAGE. Proteins were then electrophoretically transferred to nitrocellulose membranes and probed with the indicated primary antibody. Immunoreactive bands were visualized by ECL Western blotting technique (Amersham Biosciences) using horseradish peroxidase-labeled secondary antibodies. Anti-Myc antibody was from Santa Cruz Technology, and the anti-β-catenin antibody was obtained from Upstate Biotechnology.

Luciferase Assay—COS-7 cells plated in 24-well plates at 2 × 10^5/cm^2 were transfected with the indicated constructs (1 μg total) using FuGENE 6 (Roche Applied Science). 20 ng of PRL-TK (Promega, Madison, WI), which encodes a Renilla luciferase gene downstream of a minimal herpes simplex virus thymidine kinase promoter, was systematically added to the transfection mixture to assess transfection efficiency. When required, controls were carried out by replacing constructs with empty vectors. 16 h after transfection, cells were washed and cultured in media containing 2% fetal bovine serum for an additional 24 h. Cell lysates were prepared, and luciferase assays were performed with the Dual Luciferase assay kit (Promega) according to the manufacturer’s instructions. 10 μl of cell lysate were assayed first for firefly luciferase and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

Confocal Microscopy Assay—COS-7 cells were plated at 40,000 cells/well in a 6-well plate; each well contained a sterile microcoverglass, which can be removed for observation. Cells were transfected with the
indicated plasmid (1 μg total) using FuGENE 6 (Roche Applied Science). Transfected cells were treated or not with Wnt3a-CM for 24 h, then fixed with 3.7% formaldehyde (Sigma) for 10 min, and washed two times with phosphate-buffered saline. Fixed cells were permeabilized by phosphate-buffered saline, 0.025% Triton X-100 (Sigma) for 5 min and blocked in phosphate-buffered saline, 3% bovine serum albumin for 15 min. After that cells were incubated overnight at 4 °C with the primary antibody, a rabbit polyclonal anti-β-catenin (Upstate Biotechnology), at 5 μg/ml in phosphate-buffered saline. After being washed two times cells were incubated for 1 h at room temperature with the secondary antibody, goat anti-rabbit conjugated to FITC (Santa Cruz Biotechnology) at a 1:100 dilution. The cells were washed three times, mounted on a glass slide, and viewed with a confocal laser scanning microscope (LSM510, Carl Zeiss).

**RESULTS**

**Up-regulation of mFz1 Expression by BMP-2 in Mesenchymal Cell Lines**—The myoblast cell line C2C12 displays a certain degree of plasticity in terms of differentiation, and BMP-2 treatment is known to inhibit myotube formation and converts the differentiation pathway of C2C12 cells into that of the osteoblast lineage (31). To identify genes playing a role in the transdifferentiation of these pluripotent cells, we performed a
mFz1 Inhibits Wnt Signaling

Fig. 3. Nuclear β-catenin translocation in COS-7 cells is blocked by mFz1. A, COS-7 cells were transfected with a GFP expression plasmid and left unstimulated. B, COS-7 cells were transfected with a GFP expression plasmid, and then cells were stimulated with Wnt3a-conditioned medium for 24 h. C, COS-7 cells were transfected with a GFP and mFz1 expression plasmid, and then cells were stimulated with Wnt3a-conditioned medium for 24 h. i, endogenous β-catenin visualized by immunocytochemistry using an anti-β-catenin antibody. ii, GFP fluorescence. iii, i and ii merged. D, the percentage of cells that display both GFP fluorescence and β-catenin nuclear localization was determined in GFP-transfected cells (GFP CTRL), GFP-transfected cells treated with Wnt3a-CM (GFP Wnt3α), and mFz1-GFP-transfected cells treated with Wnt3a-CM (mFz1 GFP Wnt3α). Data are average ± S.D. of two independent experiments. E, COS-7 cells were transiently co-transfected with TCF-1 expression construct, TOPflash, and pTK Renilla. Where indicated empty vector, mFz1, or β-catenin* constructs were added to the co-transfection mixture. Luciferase activity was determined in cell lysates 24 h after transfection and normalized to Renilla signal. All experiments were performed in triplicate and repeated three times. Data ± S.D. from one representative experiment are presented.

Overexpression of mFz1 Represses Activity of BMP-2 and Wnt3a—BMP-2 is known to induce the osteoblast phenotype in distinct pluripotent mesenchymal cell lines, including C2C12, C3H110T1/2, and ST2. To investigate the significance of the up-regulation of mFz1 by BMP-2, we examined whether mFz1 overexpression could affect the responsiveness of these cells to BMP-2, mFz1, hFz1, and Xfz3 were cloned and expressed as Myc-tagged proteins and showed comparable expression levels when transfected in C2C12 cells (Fig. 2C) or in the other cells used (data not shown). As shown in Fig. 2A, mFz1 significantly inhibits the BMP-2-induced ALP activity in C2C12. Similar data were obtained using ST-2 and C3H110T1/2 cells (data not shown), suggesting that mFz1 antagonizes the effects of BMP-2. We have recently demonstrated that BMP-2 controls ALP expression by a Wnt autocrine loop (32). As shown in Fig. 2A, mFz1 overexpression also inhibited Wnt3a-mediated ALP activity. Moreover mFz1 was also capable of significantly decreasing Wnt3a-mediated TCF-1 activation in COS cells (Fig. 2B). In contrast, overexpression of human Frizzled-1, Xfz3 (Fig. 2, A and B), or Xfz4 (data not shown) did not decrease but rather enhanced either BMP-2 or Wnt3a activities in our assays. These observations demonstrate that mFz1 antagonizes the activity of BMP-2 and Wnt3a in different cell lines.

Overexpression of mFz1 Blocks Nuclear β-Catenin Translocation in COS Cells—To investigate how mFz1 inhibits the activity of Wnt, we monitored nuclear β-catenin translocation induced by Wnt3a in the presence or absence of mFz1. COS cells were transfected with either a control vector expressing GFP or a construct that expresses both GFP and mFz1, and then cells were treated with Wnt3a-conditioned medium. Endogenous cellular β-catenin localization was determined by immunocytochemistry, and exogenous mFz1 expression was determined by GFP fluorescence. As shown in Fig. 3, in cells transfected with GFP expression vector β-catenin is located both in the cytoplasm and at the membrane but not in the nucleus (Fig. 3A). Wnt3a treatment resulted in nuclear localization of β-catenin (Fig. 3B). Importantly mFz1 transfection precluded the Wnt3a-induced β-catenin nuclear localization.
by immunoblotting using anti-Myc antibody. Furthermore, injection of Frizzled receptors such as Xfz3, Xfz4, and Xfz7 synergized with Wnt1 to induce Siamois expression. Embryos at the two-cell stage were injected with the indicated mRNAs near the animal pole region. Ectodermal explants were dissected at blastula stage and cultured to early gastrula stage for analysis of Wnt/β-catenin signaling. To examine this possibility, we generated various chimeric Frizzled receptors. Xfz3N/mFz1C is composed of the CRD and the seven transmembrane domains of Xfz3 fused to the C-terminal cytoplasmic region to activate the Wnt/β-catenin signaling pathway. To examine this possibility, we generated various chimeric Frizzled receptors. Xfz3N/mFz1C is composed of the CRD and the seven transmembrane domains of Xfz3 fused to the C-terminal cytoplasmic region to activate the Wnt/β-catenin signaling pathway. To examine this possibility, we generated various chimeric Frizzled receptors. Xfz3N/mFz1C is composed of the CRD and the seven transmembrane domains of Xfz3 fused to the C-terminal cytoplasmic region to activate the Wnt/β-catenin signaling pathway. To examine this possibility, we generated various chimeric Frizzled receptors. Xfz3N/mFz1C is composed of the CRD and the seven transmembrane domains of Xfz3 fused to the C-terminal cytoplasmic region to activate the Wnt/β-catenin signaling pathway.
Frizzled-1 (mFz1) Inhibits Wnt Signaling

Fig. 5. Analysis of chimeric receptors on Wnt/β-catenin signaling in Xenopus embryos. A, schematic representation of wild-type and truncated mFz1 (see text for details). B, RT-PCR analysis of Wnt/β-catenin target gene Siamois expression in ectodermal explants. Xfz3/mFz1C that is composed of the Xfz3 CRD and seven transmembrane domains fused with the C-terminal cytoplasmic tail of mFz1 induced Siamois expression similar to wild-type Xfz3. Mfz1N/Xfz3 is the converse of Xfz3/mFz1C and inhibited Siamois expression induced by Wnt1. Xfz3CRD/mFz1C that is composed of the Xfz3 CRD and the mFz1 seven-transmembrane domain (7TM) and the C-terminal cytoplasmic tail had no effect on Wnt1. ODC, ornithine decarboxylase; RT−, no reverse transcription reaction.

Fig. 6. Effect of truncated mFz1 on Wnt/β-catenin signaling in Xenopus explants. A, schematic representation of wild-type and truncated mFz1 alone or co-injected with Wnt1 as above. We have previously shown that Xfz3 is able to activate Wnt/β-catenin signaling in the absence of exogenous ligands (29). Like Xfz3, injection of Xfz3N/mFz1C mRNA (1 ng) induced Siamois expression (Fig. 5B). These results demonstrate that the C-terminal cytoplasmic tail of mFz1 is functional in transducing Wnt/β-catenin signaling. This is consistent with the fact that this region contains a conserved Lys-Thr-X-X-Trp motif necessary for Wnt/β-catenin signaling (29). In contrast, injection of mFz1N/Xfz3C mRNA (1 ng) failed to induce Siamois expression (Fig. 5B). When co-injected with Wnt1, this construct indeed inhibited Siamois expression similar to inhibition by the wild-type mFz1 (Fig. 5B). The chimeric receptor Xfz3CRD/mFz1C had no effect on Siamois expression either alone or co-injected with Wnt1 (Fig. 5B). Similar results were obtained when those chimeric receptors were tested for their capacity to interfere with Wnt3a signaling using the TOPFlash reporter assay in COS-7 cells (data not shown). Thus, mFz1 negatively regulates Wnt/β-catenin signaling independently of the C-terminal cytoplasmic domain.

Inhibition of Wnt/β-Catenin Signaling by Different Truncated Forms of mFz1—To further examine the mechanism by which mFz1 negatively regulates the Wnt/β-catenin pathway, we generated different truncated forms of mFz1 (see Fig. 6A). The expression of the truncated forms of mFz1 was confirmed by immunoblotting in both Xenopus embryos and COS cells (data not shown). mFz1ΔC, which retains only seven residues...
Frizzled proteins are G protein-coupled receptors that appear to act through heterotrimeric guanine nucleotide-binding proteins (G proteins) (35). Recently Gq signaling has been shown to inhibit Wnt/β-catenin pathway (36). We have therefore investigated whether the effect of Gq-(305–359), the C-terminal peptide of the α-subunit Goq, specifically uncouples Gq-coupled receptors (28), in the inhibitory activity is mediated by mFz1. As shown in Fig. 8A, overexpression of Gq-(305–359) almost totally restores Wnt3a activity in the presence of mFz1 in COS-7 cells. Gq stimulates phospholipase-Cβ that subsequently causes calcium release from internal stores. We have assessed the effect of phospholipase-Cβ inhibitor U-73122 and the cell-permeable calcium chelator 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetate acetoxymethyl ester (BAPTA/AM). Both U-73122 and BAPTA/AM were able to relieve the inhibition of Wnt3a activity (Fig. 8B). To determine that the effects of Gq-(305–359), U-73122, or BAPTA/AM were not due to differences in the expression of β-catenin or mFz1, we looked at the expression of β-catenin and mFz1 both in cells overexpressing Gq-(305–359) and in cells treated with U-73122 or BAPTA/AM, and no differences compared with control cells were found (Fig. 8C). These data strongly suggest that Gq signaling contributes to the inhibition of Wnt/β-catenin pathway mediated by mFz1.

**DISCUSSION**

The Wnt family of secreted signaling molecules binds to two co-receptors: the Frizzled-type receptor and LRPS/6. The Frizzled gene family encodes proteins that have a seven-transmembrane-spanning motif characteristic of receptors that couple to G proteins (G protein-coupled receptors). To date there are at least nine members identified in mammals (10). In a similar way to Wnt proteins, vertebrate Frizzled homologues have been shown to activate distinct signaling pathways. The downstream targets of Frizzled actions include the canonical Wnt...
pathway, which acts through β-catenin, and the Ca\(^{2+}\) pathway, in which Frizzled appears to act through G proteins (for a review, see Ref. 37). In this study we demonstrated that in contrast with other Frizzled family members, mFz1 overexpression leads to the repression of Wnt/β-catenin canonical signaling. Actually overexpression of mFz1 significantly repressed TCF signaling mediated by either Wnt5a or a constitutive active mutant form of β-catenin and precludes Wnt5a-induced β-catenin nuclear localization in COS-7 cells. Moreover microinjection of mFz1 transcript in Xenopus embryo inhibits the ability of Wnt1 to induce the expression of the Wnt/β-catenin target gene Stamos in animal cap assay and secondary axis formation in whole embryo.

Previous reports have suggested that some Wnts display inhibitory effects on other Wnts. Mainly Wnt5 was shown to activate nemo-like kinase, which inhibits TCF transcriptional activity (39). Very recently, genetic evidence has demonstrated that mFz1 could be acting as a receptor for Wnt5a or other potential “antagonistic Wnts.” Further investigations are required to elucidate this possibility.

Vertebrate Frizzled homologues have been shown to activate distinct signaling pathways. Some Frizzled receptors induce the expression of target genes of β-catenin when overexpressed in Xenopus ectodermal cells, whereas others stimulate calcium release and protein kinase C activation (13, 42, 43). Nevertheless, the lack of activity of a Frizzled receptor in Wnt/β-catenin signaling in a particular context may be due to the absence of an appropriate ligand. For example, Xfz3 activates Wnt/β-catenin in the absence of exogenous ligands, while Xfz4 and Xfz7 interact with Xwnt5A to activate this pathway (29). Two Frizzleds have been previously found to reduce the activity of Wnt: Drosophila Dfz3 (44) and Caenorhabditis elegans MOM-5 (45, 46). These two Frizzleds do not display the Lys-Thr-X-XX-Trp motif conserved among all Frizzled receptors reported to be required for activation of the Wnt/β-catenin pathway and for membrane relocalization and phosphorylation of Dishevelled (28). Dfz3 and mom-5 may therefore represent two naturally occurring “defective” Wnt receptors that reduce Wnt/β-catenin signaling. In contrast to Dfz3 and mom-5, mFz1 contains the conserved Lys-Thr-X-XX-Trp motif in its C-terminal cytoplasmic tail. In fact, the rat and human Frizzled-1 counterparts have been reported to positively affect Wnt/β-catenin signaling in different models (13, 43, 47). Although the structure of mFz1 is not distinguishable from that displayed by the closest homologues, human or rat Frizzled-1, subtle differences, especially in the extracellular region, exist. Interestingly the data obtained with chimeric receptors show that the antagonistic activity of mFz1 resides in the cysteine-rich domain of the N-terminal part of mFz1, while the intracellular portion of the receptor is capable of activating Wnt/β-catenin. Recently it was demonstrated that the CRD of certain Frizzled receptors (Frizzled-3 and Frizzled-8) exhibit the dimerization potential, which may be a feature of Wnt/β-catenin signaling (48). Therefore, it would be interesting to evaluate the capacity for dimerization of mFz1.

It is important to note that mFz1 not only blocks β-catenin nuclear translocation induced by Wnt, but it also dramatically reduces the transcriptional activity of a constitutive active mutant form of β-catenin. Previous studies have implicated calcium signaling in the regulation of Wnt/β-catenin pathway, and Li et al. (36) have recently shown that the Gq pathway inhibits the Wnt/β-catenin signaling at the level of β-catenin. Importantly the Gq pathway promotes β-catenin nuclear export. Interestingly we have shown that the antagonist activity of mFz1 could be prevented by overexpression of Goq (305–359), which specifically uncouples Gq-coupled receptors (28). Moreover the phospholipase Cβ inhibitor U-73122 and the cell-permeable calcium chelator BAPTA/AM were able to relieve the mFz1 inhibition of Wnt3a. These data strongly suggest that Gq signaling contributes to the Wnt/β-catenin inhibitory activity displayed by mFz1.

BMP-2 is a potent activator of osteoblast differentiation, and it induces the expression of a number of osteoblast differentiation markers in distinct pluripotent mesenchymal cell lines. We have shown here that mFz1 expression is up-regulated by BMP-2 and that mFz1 overexpression represses BMP-2-induced osteoblast differentiation. The regulation of mFz1 by BMP-2 is specific because another member of the TGF-β family, TGF-β1, had little effect on its expression. Although the present data did not allow us to conclude whether mFz1 represents a direct target gene of BMP-2, it is likely that the increase in mFz1 expression level may be a result of BMP-2-induced osteoblast differentiation. Indeed the time course analysis showed that the up-regulation of mFz1 was observed 24 h after BMP-2 treatment and was maintained until 4 days of culture. Our functional analyses suggest that this up-regulation may serve as a fine tuning of BMP-2 activity. Transfection experiments in different mesenchymal cell lines clearly show that mFz1 inhibits BMP-2-induced ALP expression. These data strongly suggest that the activity of mFz1 on BMP-2 is not direct but mediated by inhibition of Wnt/β-catenin signaling. Consistent with this hypothesis is the fact that mFz1 also inhibits the induction of ALP by Wnt3a. Moreover we have recently reported that dominant negative forms of the Wnt co-receptor LR5 are capable of inhibiting the BMP-2-mediated ALP induction in mesenchymal cells (20). There are several lines of evidence showing that Wnt/β-catenin signaling plays a crucial role in bone biology. In particular, loss of function of the Wnt co-receptor LR5 affects bone development both in mouse and human (20–22). One possibility is that other Frizzled receptors cooperate with LR5 to activate Wnt/β-catenin signaling during bone development, whereas mFz1 represents a feedback mechanism to modulate this signal. Preliminary experiments show that overexpression of LR5 does not overcome mFz1 inhibition, indicating that mFz1 is not interfering with LR5 signaling to inhibit the β-catenin pathway.

In summary our data show that mFz1 is capable of antagonizing Wnt/β-catenin. The induction of this receptor by BMP-2 in mesenchymal cells may represent a feedback mechanism to modulate the activity of BMP-2.

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5733

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