MOLECULAR AND CELLULAR BIOLOGY, Aug. 2008, p. 4759–4771 Vol. 28, No. 15
0270-7306/08/$08.00+0 doi:10.1128/MCB.01849-07

Fibroblast Growth Factor Signaling Uses Multiple Mechanisms To Inhibit Wnt-Induced Transcription in Osteoblasts▽

Davide Ambrosetti,†* Greg Holmes,† Alka Mansukhani, and Claudio Basilico*

Department of Microbiology, New York University School of Medicine, New York, New York 10016

Received 10 October 2007/Returned for modification 15 December 2007/Accepted 17 May 2008

Fibroblast growth factor (FGF) and Wnt signals are both critical for proper bone development. We previously reported that the expression of activating FGF receptor mutations in osteoblasts downregulated the expression of many genes reported as targets of Wnt signaling, suggesting an antagonistic effect between Wnt signaling, which promotes osteoblast differentiation and function, and FGF signaling, which inhibits these processes. To analyze the effect of FGF on Wnt signaling in osteoblasts, we created reporter cell lines where a Wnt-responsive promoter drives luciferase expression and showed that Wnt3a-induced luciferase expression was specifically inhibited by FGF treatment. FGF specifically prevented the formation of a Wnt-induced transcriptional complex of TCF1 and -4 with β-catenin on DNA. FGF did not significantly affect the activation of β-catenin, although it reduced both the expression of TCF/LEF factors and their induction by Wnt. Microarray analysis using osteoblasts treated with Wnt3a and FGF alone or in combination showed that about 70% of the genes induced by Wnt3a were downregulated by combined FGF treatment. These included novel and previously identified Wnt target genes and genes involved in osteoblast differentiation. Furthermore, FGF alone could downregulate the expression of four Fzd Wnt receptor genes. Our results show that FGF antagonizes Wnt signaling by inhibiting Wnt-induced transcription and suggest that multiple mechanisms, including downregulation of TCFs and Wnt receptors, contribute to this effect.

The process of animal development requires the orchestrated interaction of several signaling pathways that are temporally and spatially controlled. During skeletal development, many signaling systems, such as those of fibroblast growth factor (FGF), bone morphogenic protein (BMP), Wnt, and insulin-like growth factor (IGF), play an important role (13, 18). Signals from each of these pathways vary depending on the specific cell type and stage of differentiation, and the cross talk between them generates cell-specific patterns of gene expression. The interplay between pathways can occur at different levels: they may converge on the same target gene or on protein components of the signaling cascade. Our studies have focused on the cross talk between the FGF and Wnt pathways in bone-forming cells and shed light on the negative effect of FGF on Wnt signaling in bone tissue (7, 24).

Activating mutations in FGF receptor 1 (FGFR1) and FGFR2 lead to skeletal disorders such as the craniosynostosis syndromes (27, 32), which are characterized by premature closure of the sutures between the skull bones and result from aberrant proliferation and differentiation of osteoblasts (22, 26). In cell culture, FGF signaling causes increased proliferation of immature osteoblasts and blocks the differentiation program (10, 25). This contrasts with the effect of other signaling pathways, notably those of BMPs, IGFs, and Wnt, in promoting osteoblast differentiation and function (18, 21, 40). By studying the effects of activating FGFR mutations on osteoblasts, we had found a striking downregulation of many genes that had been reported as targets of Wnt signaling (24). This suggested that the inhibitory effect of FGF signaling on osteoblast differentiation could have been due, at least in part, to its downregulation of Wnt signaling and that FGF and Wnt produced antagonistic signals to regulate osteoblast maturation. Furthermore, we have shown that the transcription factor Sox2 was strongly induced by FGF in osteoblasts and could antagonize Wnt signaling through its association with β-catenin (24).

Wnt signaling mediates a variety of cellular processes, determining cell fate during embryogenesis and tissue homeostasis in the adult animal (6, 29, 30). Wnt ligands bind a heterodimeric complex formed by the LRP5/6 coreceptor and a member of the frizzled (Fzd) receptor family. Activation of the receptor inhibits the destruction complex that includes anaphase-promoting complex, Axin, casein kinase Iα, and glycogen synthase kinase 3β (GSK3β). In the absence of Wnt signaling, β-catenin is constitutively phosphorylated by GSK3β on N-terminal residues and then targeted by ubiquitination. The inhibition of GSK3β activity by Wnt results in the accumulation of β-catenin in the cell cytoplasm followed by its nuclear translocation (15, 23, 29). Nuclear β-catenin functions as a transcriptional coactivator for the TCF/LEF family of transcription factors, which are otherwise bound to DNA in a repressive complex, regulating the expression of Wnt target genes (3, 9, 34). Among the Wnt/β-catenin target genes are many components of the Wnt pathway itself, such as the Axin2, Tcf1, and Dkk1 genes (Wnt target genes at http://www.stanford.edu/~rnusse/pathways/targets.html). Genetic studies have shown an important role for Wnt signaling in promoting os-

* Corresponding author. Mailing address for Claudio Basilico: Department of Microbiology, New York University School of Medicine, New York, NY 10016. Phone: (212) 263-5341. Fax: (212) 263-8714. E-mail: claudio.basilico@med.nyu.edu. Present address for Davide Ambrosetti: Department of Biology, University of Bologna, Bologna 40126, Italy. Phone: 39 051 209 4239. Fax: 39 051 209 4286. E-mail: davide.ambrosetti@unibo.it.

† These authors contributed equally to this work.

▽ Published ahead of print on 22 May 2008.

teoblast function and regulating bone mass in humans and mice. Activating mutations in the LRP5 Wnt coreceptor lead to high bone mass, while loss of LRP5 function causes osteoporosis-pseudoglioma syndrome, characterized by low bone density (14, 21). In mice, ablation of the Wnt10b gene causes osteoporosis (2). The essential role of the canonical Wnt pathway for osteoblasts is also shown by the severe osteopenia observed in the β-catenin null mouse model (12, 16).

In this report, we have examined the molecular basis of the antagonistic effect of FGF on Wnt target genes in osteoblasts. We have utilized reporter cells where luciferase expression is driven by a Wnt-responsive promoter containing consensus TCF/LEF binding sites and studied the kinetics of the inhibitory effect of FGF on the Wnt response and the effect of FGF on various components of the canonical Wnt signaling pathway. Additionally, we used microarray analysis to delineate a subset of Wnt-induced target genes that are negatively affected by FGF signaling. The results show that FGF severely affects the formation of Wnt-induced transcriptional complexes and Wnt/β-catenin-activated transcription and suggest that several molecular mechanisms contribute to this effect.

MATERIALS AND METHODS

Tissue culture and differentiation assay. Osteoblasts were grown in Dulbecco's modification of Eagle's medium (Gibco BRL) containing 10% fetal calf serum (growth medium). For the differentiation assay, confluent osteoblasts were grown for 5 days in growth medium plus 50 μg/ml ascorbic acid and 4 mM β-glycerophosphate, and the medium was changed every 2 days.

Antibodies and growth factors. Active β-catenin antibody (37) was from Upstate, Lake Placid, NY. β-Catenin monoclonal antibody was from BD Transduction Labs. TCF3/4 (6F123-3) and TCF4 (6H5-3) monoclonal antibodies were from Exalpha. Actin monoclonal antibody was from Sigma. Sox2 polyclonal antibody was from Chemicon. TFII-I antibody (4562) and TCF1 antibody (C4-C7) were from Cell Signaling Technology. Recombinant human Wnt3A was purchased from R & D Systems and recombinant human FGF1 was a gift from M. Mohammadi, NYU School of Medicine.

Plasmids. The plasmid pcDNA3.1(-CMV) was obtained by eliminating the BglII/BamHI DNA fragment, including the cytomegalovirus promoter region, from the pcDNA3.1(-) plasmid (Invitrogen). The TOP-luciferase construct contains the KpnI/NaeI cassette from the Super8xTOPFlash plasmid (39), including eight TCF/LEF binding sites (AGATCAAAGG), the TATA box from the herpesvirus thymidine kinase promoter, and the firefly luciferase gene cloned in the KpnI/Pme I sites of pcDNA3.1(-CMV). The FOP-luciferase construct is the same as the TOP-luciferase construct except for a double mutation in the TCF/LEF binding site (AGGCCAAAGG; mutation underlined).

Isolation of OB-TOP and OB-FOP cell clones. OB1 cells (250,000 cells per well) were transfected in six-well plates with 4 μg of TOP-luciferase or FOP-luciferase plasmid DNA by use of Lipofectamine 2000 (Invitrogen). Individual colonies were selected in growth medium containing 800 μg/ml neomycin.

Luciferase assay. OB-TOP cells (50,000 per well) were plated in 24-well plates. After overnight incubation, the medium was replaced with growth medium containing 2 μg/ml heparin. The growth factor(s) was added as indicated for each experiment in the figure legends. Treated cells were lysed in 50 μl of passive lysis buffer (PLB; Promega) and 20 μl was assayed for luciferase activity using the luciferase assay system (Promega).

DNA probes and DNA binding assays. To prepare the TOP and FOP DNA probes, TOP-Dir and TOP-Rev or FOP-Dir and FOP-Rev oligonucleotides were annealed. The sequences of the oligonucleotides are as follows: for TOP-Dir, TACCCCCTTTGATCTTACCC; for TOP-Rev, GGGTAAGATCAAAGGGGGTA; for FOP-Dir, GGGTAAGGCCAAAGGGGGTA; and for FOP-Rev, TACCCCCTTTGGCCTTACCC.

To prepare nuclear and cytoplasmic fractions, adherent cells were washed twice with phosphate-buffered saline (PBS), scraped in 1 ml PBS, and spun down for 20 s in a microcentrifuge. The cell pellet was resuspended in 200 μl of hypotonic buffer (10 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 10 mM HEPES [pH 7.9], 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail) and incubated on ice for 10 min. Resuspended cells were lysed in a glass Dounce homogenizer (15 to 20 strokes), and nuclei were spun down for 5 min. Nucleus-containing pellets were washed with hypotonic buffer, resuspended in 50 μl of BC400N buffer (20 mM HEPES [pH 7.9], 400 mM NaCl, 2 mM EDTA, 20% glycerol, 0.02% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail), and incubated at 4°C for 30 min at constant rotation. After 5 min of centrifugation, nuclear fractions were recovered, aliquoted, and stored at −80°C. The DNA binding reaction mixture contained 10 fmoles of DNA probe, 4 μg of nuclear extract, 3 μg of poly(dG-dC) in 20 μl of binding buffer (40 mM HEPES, 40 mM KCl, 4% Ficoll, 0.05% NP-40). The reaction mixture was incubated on ice for 15 min and analyzed on a 4% polyacrylamide 0.25× Tris-borate-EDTA buffer gel. Supershift analysis was performed by adding 500 μg of the specific antibody to the DNA binding reaction mixture. Alternatively, scaled-down reaction mixtures with 1 μg of nuclear extract in a final volume of 10 μl were incubated on ice for 40 min before gel analysis at 4°C.

Microarray analysis. OB-TOP#1 cells were plated in 6-cm culture plates. After 10 h, the medium was replaced with 2.5 ml of growth medium plus 2 μg/ml heparin with or without 20 ng/ml FGF1. After 10 h of incubation, 2.5-ml portions of medium with heparin with or without 200 ng/ml recombinant human Wnt3a were added to the wells and further incubated for 6 or 12 h. Total RNA was extracted using Trizol reagent (Gibco BRL) from three independent plates for each treatment. RNA purification was carried out using the RNeasy kit (Qiagen). Biotinylated cRNA was prepared from 6.5 μg as detailed according to the one-cycle labeling kit protocol (Affymetrix). Fifteen micrograms of cRNA was hybridized to the mouse genome 430 2.0 array and scanned by the gene array scanner (Affymetrix) at the Columbia University Microarray Facility (New York, NY). Metrics files were generated from each chip by use of Microarray Suite 5.0 (Affymetrix). Expression data were further analyzed in the GeneSpring 7.0 program (Silicon Genetics) as previously described (8, 24). Normalization across all of the chips was performed by using the 50th percentile of all measurements as a positive control. Each measurement for each sample was divided by this value. The lowest 10th percentile was used as a test for correct for background subtraction ("per-chip normalization"). The median of each gene's expression value over all of the samples was used as a synthetic positive for each gene and divided into all measurements for that gene ("per-gene normalization"). The values for multiple samples of each time point were averaged and used for all further analyses. Genes induced at least 2.5-fold were identified by comparison of the expression level for each Wnt3a-treated sample (6 and 12 h) with that for the untreated (heparin-only) control sample; these data were combined using Venn diagrams.

Real-time RT-PCR. RNA was prepared from cultures in six-well plates with 1 ml of Trizol and purified after DNase treatment using Rneasy MinElute columns (Qiagen). cDNA was prepared with the SuperScript first-strand kit (Invitrogen). PCR primers were designed using the LightCycler probe design 2 program. The following primer sequences were used for real-time reverse transcription-PCR (RT-PCR): for *Axin2*, 5′GAAGAAATTCCATACAGGAGGAT and 3′GTCACTCGCCTTCTTGAAATAA; for *Edn1*, 5′TGTATCTATCAGCAGCTGGTG and 3′ATAGAGCTCAGCTTTCAACTT; for *Has2*, 5′TTAGTGATGTTTACATGGGCG and 3′ATACCCTTCCTGAGAAGTAAAGA; for *Rhou*, 5′TACCACTGGACTATTTATGTCACAA and 3′CAGTACAGTCAGACATTATGACTTC; for *Ctgf*, 5′GGTCAAATCCCTGTTGGTGAA and 3′GTCATAATCAAAGAAGCAGCAAG; for *Tgfb3*, 5′CACCAATTACTGCTTCCGCAA and 3′GCATAGTAACCCTTAGGTTCG; for *Timp3*, 5′AGAATTACTGGGACACAATCAC and 3′TCTTCTTCTTCCCACCCTTAG; for *Tcf1*, 5′CATGTACAAAGAGACTGTCTA and 3′GAATAAAATCCAGAGAGATCG; for *Tcf4*, 5′GTAACCTGTAGTGCCAACTCT and 3′CTGCATGTAATTCCTACACATGAT; for *Fzd1*, 5′GGCAGTGTGTCAAATGTAAATCT and 3′AATTTAGGTCCTATGTATGTATCGG; and for *Fzd2*, 5′GCACACACCACTTTAAGAATG and 3′CCCAATCCTACAAACAGCTAAC.

Real-time RT-PCR was performed with the LightCycler FastStart DNA master SYBR green 1 kit (Roche) on a LightCycler system (Roche). Expression levels relative to those for the untreated control were calculated using the level of actin as a reference.

RESULTS

FGF and Wnt signaling have opposite effects on cultures of differentiating osteoblasts. FGF signaling inhibits spontaneous osteoblast differentiation (25, 27), and Fig. 1A shows that FGF1 also inhibited Wnt3a-induced differentiation of osteoblasts, as measured by alkaline phosphatase activity. We have previously suggested that FGF downregulates the canonical

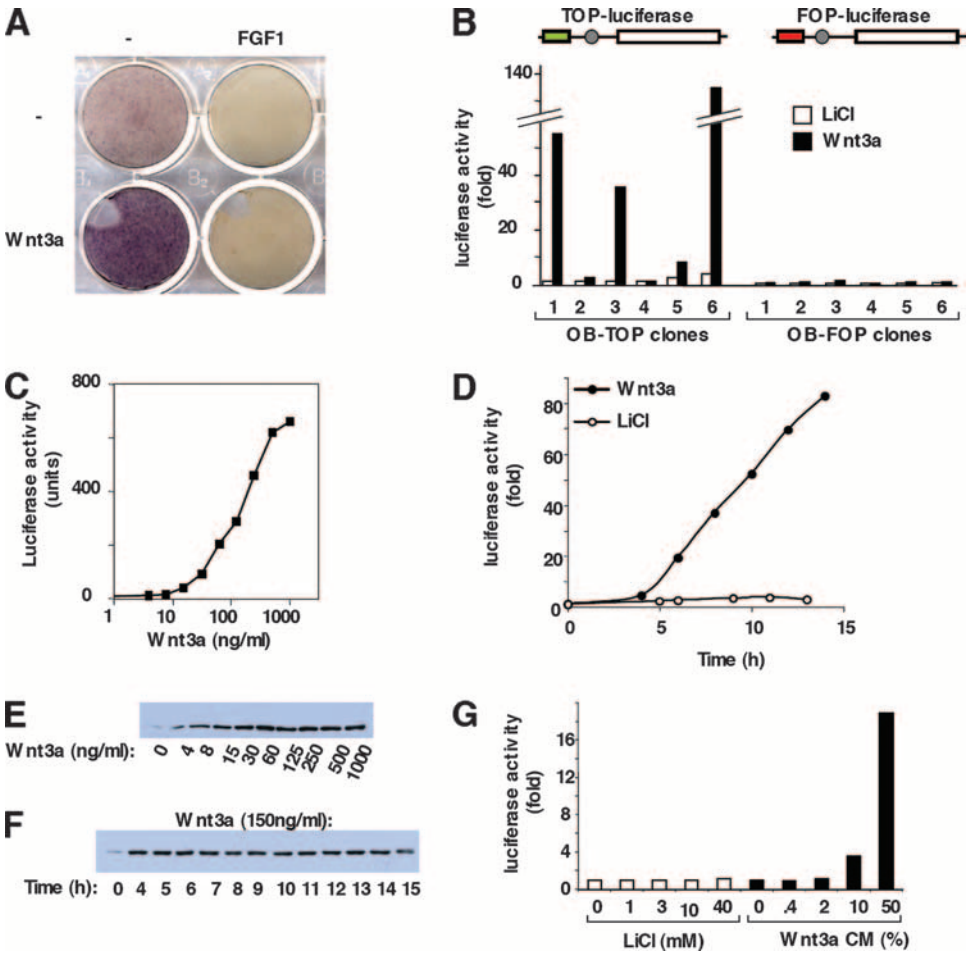

FIG. 1. Wnt3a induces luciferase in reporter osteoblast lines. (A) Alkaline phosphatase assay. Parental OB1 cells were plated and grown in differentiation medium for 5 days in the presence of PBS buffer as a control or 100 ng/ml Wnt3a or 10 ng/ml FGF1 or both. (B) Luciferase assay of OB cell reporter clones stably transfected with the TOP-luciferase or the FOP-luciferase cassette. Each cassette, schematically represented above the histograms, contains the luciferase reporter gene (white box) and the minimal herpesvirus thymidine kinase viral promoter (gray) cloned downstream of eight copies of wild-type (TOP; green box) or mutated (FOP; red box) TCF/LEF binding sites. The cell clones were treated for 18 h with 100 ng/ml Wnt3a or 20 mM LiCl. The resulting luciferase activity, expressed as induction compared to that for untreated cells, is the mean for duplicate samples. The experiment was repeated three times with similar results. (C) Wnt3a dose-response luciferase assay. OB-TOP#1 cells were treated for 20 h with increasing concentrations of Wnt3a. (D) Wnt3a and LiCl time course luciferase assay. OB-TOP#1 cells were treated with 150 ng/ml of Wnt3a or 20 mM LiCl for the indicated times. (E and F) Western blot analysis, with monoclonal antibodies raised against active β-catenin, of total lysates from OB-TOP cells treated with increasing concentrations of Wnt3a (E) or with 150 ng/ml Wnt3a (F) for the indicated times. (G) Dose-response luciferase assay of OB-TOP#1 cells to LiCl or Wnt3a conditioned medium (cm).

Wnt pathway (24). To define this antagonism and to analyze the molecular mechanisms underlying it, we generated a number of cellular clones independently derived from mouse osteoblastic OB1 cells containing either a stably integrated Wnt/β-catenin-responsive TOP-luciferase construct (OB-TOP) or a control construct mutated in the TCF/LEF consensus sequence (OB-FOP).

Figure 1B shows that the addition of Wnt3a increased the luciferase activity from approximately 10- to 140-fold in four out of six independent OB-TOP clones. Under the same experimental conditions, none of the control OB-FOP clones showed induction of luciferase. This result suggests that the luciferase response of the OB-TOP clones is mediated by the binding of TCF factors to the reporter. Figures 1C and D show that the luciferase response of OB-TOP#1 cells to recombinant Wnt3a was dose and time dependent. Under these conditions, a lag of approximately 5 h is observed prior to significant induction. Similar results were obtained with conditioned medium from Wnt3a-producing L cells (Fig. 1G and data not shown).

As Wnt signaling stabilizes β-catenin, we carried out Western blotting as shown in Fig. 1E and F. Staining with an antibody against active β-catenin that recognizes an unphosphorylated epitope in the N terminus of the molecule showed increasing amounts of active β-catenin in response to Wnt. An increase in the expression of active β-catenin was also induced by Wnt3a in the time course experiment shown in Fig. 1F. Interestingly, LiC1, an inhibitor of GSK3β which has been widely used to mimic Wnt/β-catenin signaling, had only a very minor effect on luciferase expression in all the OB-TOP clones (Fig. 1B, D, and G), although it could induce the stabilization of β-catenin (not shown). Experiments using lower doses of

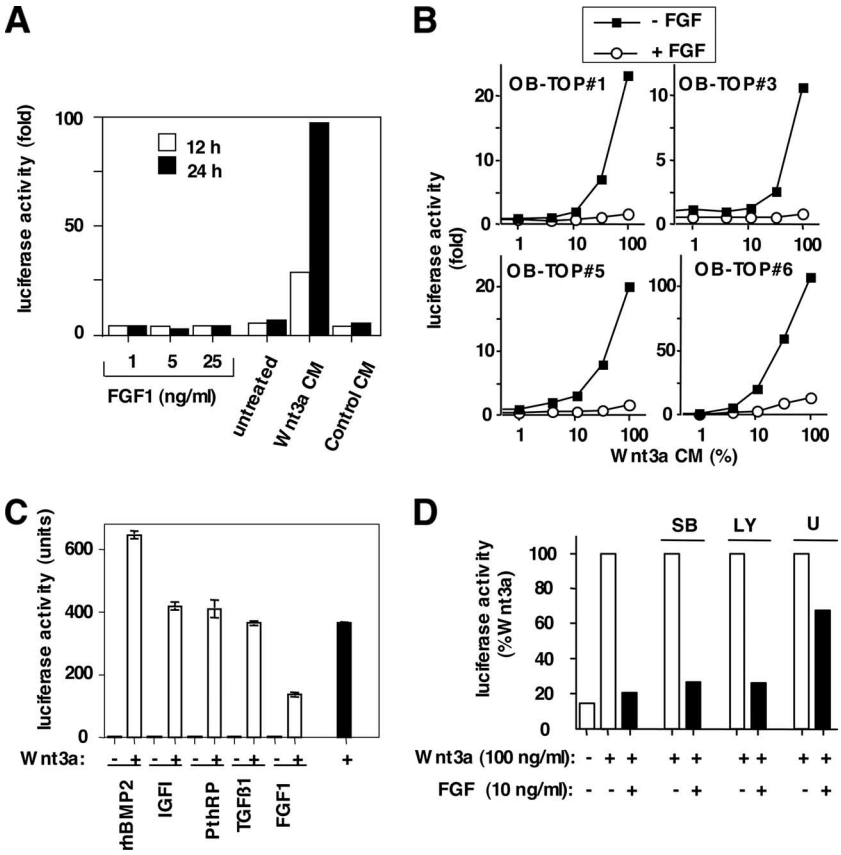

FIG. 2. Downregulation of Wnt/β-catenin signaling by FGF. (A) Luciferase assay of OB-TOP cells incubated with 1, 5, or 25 ng/ml of FGF1 and harvested after 12 or 24 h. One hundred percent Wnt3a conditioned medium (Wnt3a CM) and control CM were used as positive and negative controls, respectively. (B) Luciferase assay of four independent OB-TOP clones treated for 12 h with increasing amounts of Wnt3a CM in the absence or in the presence of 10 ng/ml of FGF1. (C) OB-TOP reporter cells were pretreated for 1 h with the indicated growth factor. Wnt3a at 100 ng/ml (+) or PBS buffer (−) was added and the cells were further incubated for 15 h before being harvested. Growth factor concentrations were 10 ng/ml FGF1, 50 ng/ml BMP2, 50 ng/ml IGF1, ng/ml TGFβ, and 100 ng/ml parathyroid hormone-related peptide (PTHrP). The resulting luciferase activity is from one representative experiment and is the mean of triplicates ± standard deviation. (D) Luciferase response to Wnt or to Wnt and FGF of OB reporter cells pretreated with dimethyl sulfoxide (−) or 20 uM of the indicated inhibitors: SB203580 (SB), LY294002 (LY), and U0126 (U). For each pretreatment, the luciferase activity obtained for Wnt3a-treated cells was arbitrarily set as 100%. The resulting luciferase activity is from one representative experiment and is the mean of duplicates.

LiCl (Fig. 1G), shorter times of exposure (Fig. 1D), or an alternative inhibitor of GSK3β (SB915286) (not shown) gave very similar results. Thus, these experiments suggest that stabilization of β-catenin is not sufficient to fully activate the reporter gene.

These results show that the Wnt-responsive reporter cell lines that we have generated respond specifically to Wnt treatment and can be used to study the relationship between Wnt and FGF signaling in osteoblasts.

FGF specifically inhibits Wnt signaling. To determine the effect of FGF signaling on the Wnt reporter clones, we treated OB-TOP cells with FGF1, Wnt3a, or both and measured luciferase activity. Figure 2A shows that an increasing concentration of FGF1, in the absence of Wnt, did not affect luciferase expression. Figure 2B shows that FGF1 inhibited almost completely the luciferase activity induced by Wnt3a. The inhibition was similar for each of the four independent OB-TOP clones tested. These results suggest that the FGF role is independent of the site of integration of the reporter construct in the genome and is specifically directed to the Wnt pathway. All subsequent experiments were carried out using OB-TOP#1 cells.

The specificity of the FGF function was tested by treating the cells with BMP2, IGF1, parathyroid hormone-related peptide, or transforming growth factor β (TGFβ), signaling molecules involved in osteoblast biology (13, 18). Figure 2C shows that only Wnt could activate the luciferase reporter. Furthermore, FGF1 was the only growth factor downregulating Wnt-induced transcription. As already reported (33), BMP further activated the Wnt3a response. Western blotting with specific antibodies was carried out to verify the activation of the corresponding pathways. As expected, BMP2 and TGFβ1 induced the phosphorylation of SMAD4/6, IGF1 induced the phosphorylation of AKT, and FGF1 induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation (data not shown).

FGF activates many different signaling pathways in osteoblasts, including the ERK1/2, p38, and phosphatidylinositol 3-kinase pathways (7). To investigate the contribution of these pathways to the inhibitory effect of FGF on Wnt signaling, we used the inhibitors SB203580, LY294002, and U0126, which

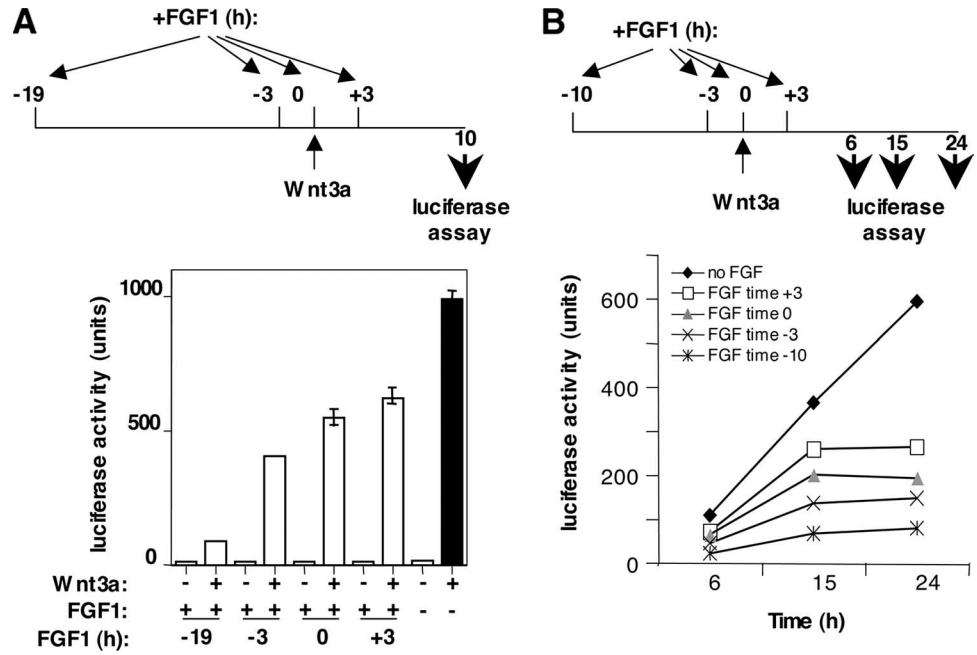

FIG. 3. Time course analysis of the inhibition of Wnt3a-induced luciferase activity by FGF. (A) (Top) Schematic representation of the experiment. The horizontal line represents time in h. The arrows indicate the time of treatment. (Bottom) OB-TOP#1 reporter cells were treated for 10 h with 100 ng/ml of Wnt3a alone (black bar) or together with FGF1 (white bars) at the indicated time. The resulting luciferase activity, expressed as luciferase units, is the mean of triplicates ± standard deviation. (B) (Top) Schematic representation of the time course experiment; (bottom) cells were treated for 6, 15, or 24 h with Wnt3a alone or together with FGF added at time +3, 0, −3, or −10.

block the activation of the p38, phosphatidylinositol 3-kinase, and ERK1/2 pathways, respectively (Fig. 2D). Under the experimental conditions used, each inhibitor showed the expected specific activity without affecting other signaling pathways (data not shown). The addition of SB203580 and LY294002 to the cells did not affect the downregulation of Wnt signaling by FGF. On the other hand, inhibition of the ERK1/2 signaling pathway by UO126 significantly decreased the negative effect of FGF on Wnt-induced luciferase activity. This suggests that FGF-mediated downregulation of Wnt signaling requires the activation of the ERK1/2 signaling pathway.

Maximal downregulation of the Wnt response requires preexposure to FGF. Although all initial experiments showed a very significant effect of FGF on the Wnt response when these factors were added to cells at the same time, we considered it of interest to study whether varying the time of FGF treatment would affect the reporter response. A schematic representation of the experiment is shown in Fig. 3. The histogram in Fig. 3A shows that approximately 90% downregulation is observed with 19 h of FGF pretreatment, while 40% downregulation is found for cells treated with FGF for 3 h following Wnt addition. In this experiment, all samples were assayed after 10 h of treatment with Wnt. We analyzed similarly treated samples in a Wnt treatment time course to establish if and how the FGF effect varies with time (Fig. 3B). The luciferase assay confirms that FGF downregulation increases with the length of Wnt treatment in all samples tested. Furthermore, the effect was detectable at the earliest time tested, i.e., 6 h after the beginning of Wnt treatment, even when FGF was added 3 h after Wnt. These results suggest a requirement for the FGF-induced accumulation or depletion of a critical molecule to obtain the strongest downregulation. On the other hand, FGF can also significantly inhibit the Wnt response when added 3 h after the Wnt treatment. Thus, it is possible that multiple molecular mechanisms are responsible for the downregulation of Wnt signaling by FGF.

FGF inhibits the assembly of a transcriptionally active Wnt-induced complex. Stabilization of β-catenin is the central molecular event of the canonical Wnt pathway (6, 29, 30). Figure 4A shows that FGF treatment had no significant effect on the stabilization of β-catenin induced by Wnt when FGF1 and Wnt3a were applied at the same time. The Western blot shows that FGF did not modify the localization of active β-catenin, which is present both in the cytoplasm and in the nuclei of Wnt-treated cells, suggesting that the nuclear translocation of activated β-catenin is not affected. The amounts of total β-catenin were also slightly increased in Wnt-treated cells, and FGF did not affect this increase. However, a small decrease in the amounts of active β-catenin was observed when cells were incubated with FGF for 10 h before Wnt treatment (Fig. 4B). Although this decrease could contribute to the inhibitory function of FGF, the experiments suggest that the main function of FGF on the Wnt canonical pathway is downstream of β-catenin activation.

Since FGF acts downstream of the nuclear translocation of β-catenin, its function may be directed upon the formation of the transcriptionally active complex with a TCF/LEF factor. Thus, we analyzed the formation of a TCF/DNA complex by electromobility shift assay (EMSA), using nuclear extracts of cells treated as described in the legend to Fig. 4 and a DNA probe containing one copy of the TCF/LEF binding site present in the reporter construct (Fig. 4C and D).

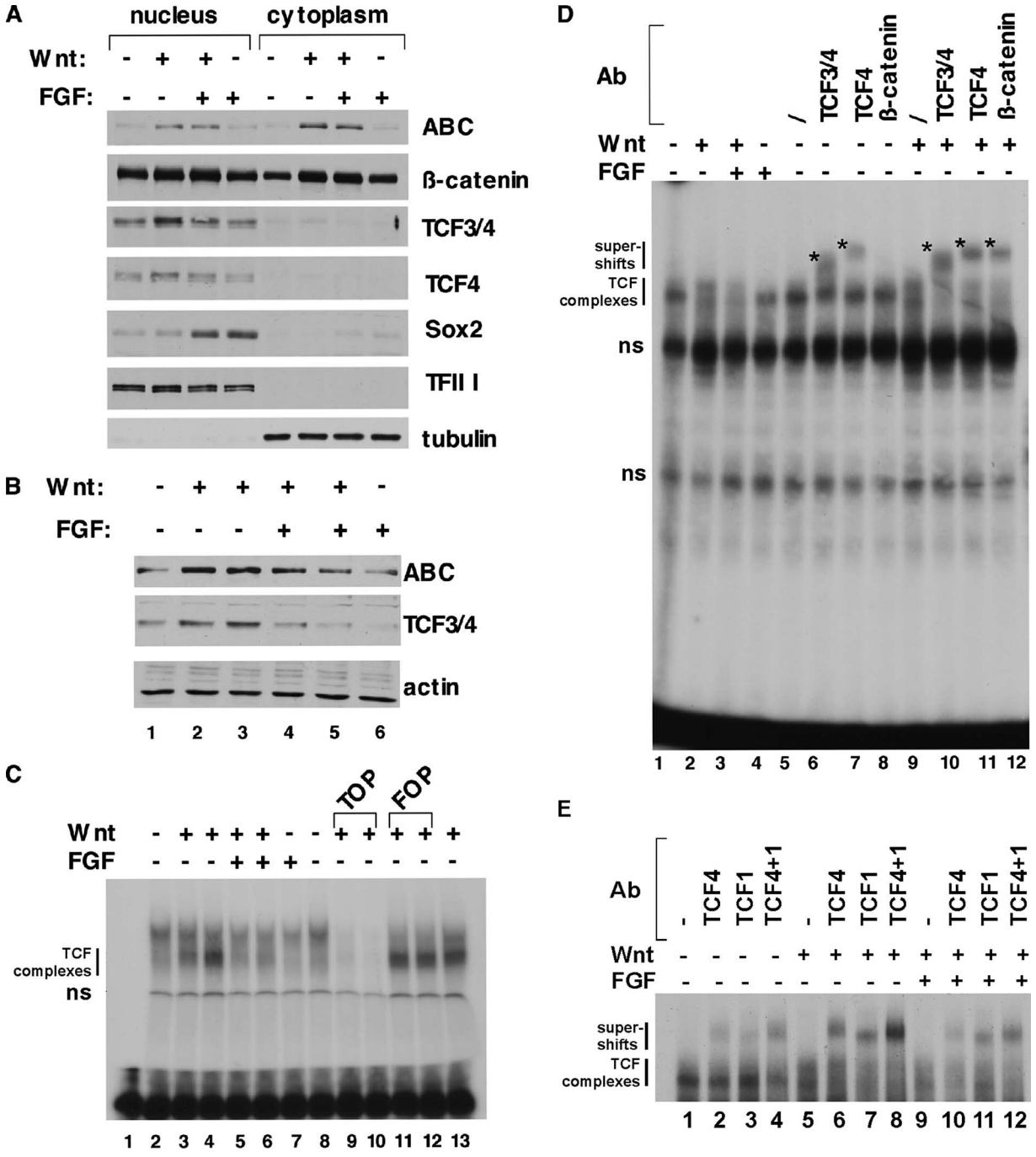

FIG. 4. FGF does not affect β-catenin stabilization by Wnt but blocks the formation of a β-catenin/TCF nuclear complex. (A) Western blot analysis of nuclear (lanes 1 to 4) and cytoplasmic (lanes 5 to 8) protein extracts from OB-TOP#1 cells untreated or treated with Wnt, with FGF, or with FGF and Wnt added together for 6 h. Antibodies raised against active β-catenin (ABC), total β-catenin, TCF3/4, TCF4, and Sox2 were used. Antibodies raised against tubulin (cytoplasmic) and TFII-I (nuclear) were used as controls to verify the purity and equal loading of the samples. (B) Western blot analysis of nuclear extracts from OB-TOP#1 cells that had been pretreated with FGF for 10 h before Wnt addition. The length of Wnt treatment was 5 h (lanes 2 and 4) or 10 h (lanes 3 and 5). Lane 6, FGF only, 20 h total. (C) EMSA comparing nuclear protein complex assemblies on the TCF/LEF consensus DNA probe. Nuclear extracts are from cells treated as described for panel B. ns, a nonspecific DNA/protein complex. Lanes 9 to 13, competition experiment with 10× and 100× molar excesses of unlabeled probes containing the wild-type (TOP) or mutated (FOP) TCF/LEF consensus. (D) EMSA of nuclear extract from OB-TOP#1 cells that are untreated or treated with Wnt, with FGF and Wnt together, or with FGF only (lanes 1 to 4) for 6 h. Supershift experiment: nuclear extracts from OB-TOP#1 cells untreated (lanes 5 to 8) or treated with Wnt3a for 6 h (lanes 9 to 12) were preincubated with 500 ng of the indicated antibodies (Ab) before EMSA. Asterisks indicate the positions of supershifted DNA complexes. (E) EMSA of nuclear extracts from the same experiment shown in panel D preincubated with 500 ng of the indicated antibodies before EMSA.

Figure 4C shows that 5 or 10 h of Wnt treatment induced a broad band of DNA/protein complex(es) that is absent from the untreated samples (compare lanes 2, 3, and 4). Lanes 9 to 12 show the results of a competition experiment on the lane 4 sample. The addition of cold wild-type probe competed the binding to the labeled probe (TOP), showing the specificity of the reaction (lanes 9 and 10). In agreement with this, the addition of a cold mutated probe (FOP) did not compete (lanes 11 and 12). Importantly, the Wnt-induced complex was not detected in the samples derived from cells receiving Wnt after 10 h of FGF pretreatment (lanes 5 and 6).

The experiment shown in Fig. 4D and E was performed using nuclear extracts from cells treated simultaneously with Wnt3a and FGF1 and analyzed 6 h after addition of the growth factors. Figure 4D also includes supershift experiments in which antibodies against TCF factors or β-catenin were mixed with the nuclear extracts to identify the protein components of the complexes. In this assay, in which the EMSA products were run further to improve separation, untreated cells (lanes 1 and 5) show more clearly a basal DNA/protein complex that appears to contain TCF3/4 but not β-catenin (lanes 6 to 8), since the antibodies against β-catenin did not generate a supershift (lane 8). Nuclear extracts from FGF-treated cells showed no significant change from the controls (lane 4). Lanes 2, 3, and 9 to 12 of Fig. 4D contain the Wnt-treated samples. Wnt treatment caused the appearance of two new bands, one migrating more slowly and the other more quickly than that present in the controls, which are not detected in extracts from cells which had also received FGF. Interestingly, the basal DNA/protein complex was no longer detectable in the Wnt- or Wnt/FGF-treated samples, indicating that Wnt signaling remodels and/or alters the composition of this complex. Indeed, the addition of each of the three antibodies to the Wnt-treated samples induced a supershift band, showing that in addition to TCF3/4, β-catenin was also present in the Wnt-induced complex.

To better identify the factors that bind to our Wnt target DNA probe, we also conducted a supershift experiment using antibodies against TCF1, one of the major TCF factors involved in Wnt signaling (Fig. 4E). The results show that both TCF1 and TCF4 were present in the basal DNA/protein complex (lanes 1 to 4), and their association with the probe was increased by Wnt treatment (lanes 5 to 8). FGF strongly reduced the amounts of TCF1 and TCF4 associated with the probe (lanes 9 to 12).

The nuclear and cytoplasmic protein extracts from cells treated with Wnt, with Wnt/FGF, or with FGF only were analyzed by Western blotting using antibodies against TCF3/4, TCF4, or active β-catenin (Fig. 4A and B). An antibody recognizing both TCF3 and -4 clearly showed that at least one of these factors is induced by Wnt, and this induction did not occur in cells which had been also treated with FGF. The suppression of TCF3/4 induction by FGF was more pronounced in cells pretreated with FGF for 10 h (Fig. 4B). The immunoblot with specific anti-TCF4 antibodies suggests that the main TCF factor involved here is TCF4. TCF1 was also induced by Wnt3a at the RNA and protein levels and downregulated by FGF (Table 1 and data not shown). We also verified that as previously reported (24) Sox2 was strongly induced by FGF. Sox2 induction was unaffected by Wnt.

Several conclusions can be made from these experiments. (i) In addition to the activation and nuclear translocation of β-catenin, Wnt stabilizes/induces TCF factor(s). The formation of a complex containing both proteins correlates with increased transcriptional activity. (ii) FGF prevents the formation of a Wnt-induced transcription complex on DNA, while not significantly affecting the activation of β-catenin. (iii) FGF signaling inhibits the induction of TCF protein by Wnt.

Many genes that are targeted by Wnt in osteoblasts are downregulated by FGF. To determine whether the inhibition of Wnt-mediated luciferase response produced by FGF in our OB-TOP clones was reflected by a similar response of endogenous osteoblast genes, a microarray analysis of gene expression using Affymetrix 430 2.0 mouse gene chips was performed on cells that had been pretreated with FGF for 10 h and then with recombinant Wnt3a for 6 or 12 h (Fig. 5 and Table 1). Luciferase activity was monitored during the experiment and showed the expected reduction of about 90% in cells treated with FGF and Wnt compared to what was seen for treatment with Wnt alone at 12 h (not shown). Of about 34,000 genes and expressed sequence tags (ESTs) represented in the microarray, 19,000 remained after eliminating those scored as absent from all samples. Considering genes which both were flagged as present by the GeneSpring program and had a raw expression score over 50 in the Wnt3a-treated chips, 157 unique genes or ESTs were upregulated at least 2.5-fold compared to control levels at either 6 or 12 h. Table 1 shows that at 6 h after Wnt3a addition, 67 genes or ESTs were significantly upregulated by Wnt3a, of which the induction of 53 was reduced to some degree by pretreatment with FGF, 34 of them by greater than 2.5-fold. We divided these latter genes into the following two groups (Table 1): (i) genes whose upregulation by Wnt is blocked by FGF, on which FGF alone has no significant effect; and (ii) genes for which FGF alone also causes downregulation of expression. It should be noted that although these two broadly defined groups are quite distinct, the adjudication of a specific gene to the first or second category may not be definitive, as the inhibition of expression caused by FGF alone cannot be assessed very exactly for genes which have extremely low basal levels of expression (e.g., the Axin2 gene).

Figure 5 shows a graphic representation of the results at 6 and 12 h for some genes typical of the two main groups of Wnt-responsive, FGF-inhibited genes outlined above. In addition, a few genes that are known to play a role in Wnt signaling but were not significantly upregulated by Wnt treatment were found to be regulated by FGF alone or by treatment with both FGF and Wnt (Table 2). The most notable in this group are genes of the Fzd Wnt receptor family (Fzd1, -2, -7, and -8 genes) that are downregulated by FGF. Downregulation of Wnt receptor expression could therefore contribute to the inhibition of Wnt signaling by FGF. It is also of interest that several Wnt target genes also play a role in Wnt signaling with effects that are either stimulatory (e.g., *TcP1*) or inhibitory (e.g., *Axin2*). FGF appears to inhibit the Wnt-induced expression of these genes irrespective of whether they promote or impair Wnt signaling.

For selected Wnt target and Fzd genes, the data were validated in an independent experiment by real-time RT-PCR (Fig. 5B). FGF cotreatment clearly reduced Wnt induction of mRNA levels in each case. Although marked as "absent" in

TABLE 1. FGF regulation of Wnt target genes in osteoblasts

Wnt target gene characteristic[a]	Gene symbol	Accession no.	Fold induction seen for treatment with[b]:			Description
			Wnt3a	Wnt3a + FGF1	FGF1	
FGF inhibited; not significantly regulated by FGF alone	*A930038C07Rik*	BE632850	28.73	2.33	1.50	Fibronectin type III-like fold
	Has2	NM_008216	11.77	3.53	0.63	Hyaluronan synthase 2
	Rhou	AF378088	8.76	1.81	0.86	*ras* homolog gene family, member U
	Tcf7 (*Tcf1*)	AI323642	5.74	1.64	0.66	Transcription factor 7; T cell specific
	Sstr2	NM_009217	5.38	1.69	1.37	Somatostatin receptor 2
	Fndc1	AK003938	4.66	1.13	0.79	Fibronectin type III domain containing 1
	Ahr	BE989096	4.54	1.68	1.35	Aryl-hydrocarbon receptor
	Ctgf	NM_010217	3.77	1.16	0.63	Connective tissue growth factor
	Mab21l1	AF228913	3.76	0.56	0.50	*mab*-21-like 1
	Gdnf	NM_010275	3.58	0.70	0.80	Glial cell line-derived neurotrophic factor
	1500005K14Rik	BF101721	3.44	0.78	0.44	RIKEN cDNA 1500005K14 gene
	Slc7a2	AV244175	3.35	1.13	0.48	Solute carrier family 7, member 2
	Ifi1	NM_008326	3.16	0.67	0.55	Interferon-inducible protein 1
	Tmem23	BE629162	2.84	1.12	0.69	Transmembrane protein 23
	Cyr61	BB533736	2.74	0.72	0.48	Cysteine-rich protein 61
	Zfpn1a2	BB291816	2.69	0.89	0.47	Zinc finger protein, subfamily 1A, 2 (Helios)
FGF inhibited; downregulated by FGF alone	*Edn1*	D43775	16.83	0.17	0.19	Endothelin 1
	Axin2	BB398993	10.22	3.16	0.19	Axin2
	Tgfb3	BC014690	9.92	0.53	0.23	Transforming growth factor, beta 3
	Timp3	BI111620	6.39	0.47	0.15	Tissue inhibitor of metalloproteinase 3
	EST	BI076661	5.15	0.48	0.07	Clone 9030002L20
	Gadd45g	AK007410	4.80	0.44	0.20	Growth arrest- and DNA-damage-inducible 45 gamma
	Serpinb1a	AF426024	4.71	0.04	0.02	Serine proteinase inhibitor, clade B, member 1a
	Tmepai	AV291712	4.01	1.39	0.30	Transmembrane, prostate androgen-induced RNA
	Thbs1	AI385532	3.59	0.34	0.17	Thrombospondin 1
	2310039E09Rik	NM_026509	3.39	0.11	0.10	RIKEN cDNA 2310039E09 gene
	Irs1	BB345784	3.36	0.81	0.37	Insulin receptor substrate 1
	EST	BM229155	3.19	0.90	0.26	Transcribed sequences
	FHOS2	BG066491	2.99	0.41	0.33	Formin-family protein FHOS2
	Prickle1	BC022643	2.87	0.96	0.33	Prickle-like 1
	Lrrc17	BB503935	2.86	0.35	0.17	Leucine-rich repeat containing 17
	Acpl2	BB183525	2.85	0.87	0.29	Acid phosphatase-like 2
	Tgfb2	BF144658	2.80	0.17	0.07	Transforming growth factor B2
	4930579P08Rik	AK016333	2.58	0.14	0.14	Clone 4930579P08
Variably affected by FGF-Wnt combination	*Dusp4*	AK012530	7.52	14.11	6.75	Dual-specificity phosphatase 4
	Sema4f	BB271145	6.75	3.29	0.41	Semaphorin 4f
	2610035D17Rik	BB760848	4.84	2.18	1.07	RIKEN cDNA 2610035D17 gene
	Il33	NM_133775	4.36	8.88	5.69	Interleukin 33
	Irx3	NM_008393	4.11	3.39	0.98	Iroquois-related homeobox 3
	EST	BB042558	3.64	2.26	2.24	EST
	Arl4c	BI964400	3.63	2.97	1.77	ADP ribosylation factor-like 4c
	Grem1	BC015293	3.62	3.22	3.05	Cysteine knot superfamily 1, BMP antagonist 1
	Flvcr1	BC010797	3.49	3.63	1.78	Feline leukemia virus subgroup C cellular receptor 1
	Ier3	NM_133662	3.37	9.10	7.28	Immediate early response 3
	Nudt6	BB043522	3.26	12.56	4.41	Nudix (nucleoside diphosphate linked moiety X)-type motif 6
	Sema3c	NM_013657	3.25	5.34	2.63	Semaphorin 3C
	Cxcl1	NM_008176	3.19	1.31	1.17	Chemokine (C-X-C motif) ligand 1
	Dyrk3	BC006704	3.19	3.79	3.10	Dual-specificity tyrosine-(Y) phosphorylation-regulated kinase 3
	Prdc	NM_011825	3.04	3.87	3.40	Protein related to DAN and cerberus
	Fmnl2	AK017338	2.99	2.62	1.85	Formin-like 2
	4931408A02Rik	AK016443	2.94	1.95	1.16	RIKEN cDNA 4931408A02 gene
	Tmem16a	AU040576	2.93	11.14	6.06	Transmembrane protein 16a
	Ptar1	AK008237	2.91	2.10	2.09	Protein prenyltransferase alpha subunit repeat containing 1
	Gas2l3	BB770972	2.79	1.46	1.45	Growth arrest-specific 2-like 3
	Tcf7l2 (*Tcf4*)	BB175494	2.78	1.19	0.84	Transcription factor 7-like 2; T cell specific; HMG-box

Continued on following page

TABLE 1—*Continued*

Wnt target gene characteristic[a]	Gene symbol	Accession no.	Fold induction seen for treatment with[b]:			Description
			Wnt3a	Wnt3a + FGF1	FGF1	
	Sema5a	AV375653	2.76	4.57	4.29	Semaphorin 5a
	EST	AV352204	2.73	1.23	0.86	Transcribed sequences
	Inhba	NM_008380	2.67	1.67	1.28	Inhibin beta-A
	Runx1	D13802	2.64	3.87	3.54	Runt-related transcription factor 1
	Ptgs2	M94967	2.62	5.60	3.60	Prostaglandin-endoperoxide synthase 2
	EST	BB433596	2.62	1.70	0.83	Transcribed sequences
	Sox4	BG083485	2.60	2.00	0.82	SRY-box containing gene 4
	Zbtb38	BE456566	2.59	2.07	1.23	Zinc finger and BTB domain containing 38
	Tnfrsf21	BG972377	2.58	1.57	1.25	Tumor necrosis factor receptor superfamily, member 21
	C79267	BG066466	2.57	4.06	3.53	Expressed sequence C79267
	Adam12	NM_007400	2.56	1.63	0.83	Disintegrin and metalloproteinase domain 12
	2010002N04Rik	BI963682	2.55	6.13	3.98	RIKEN cDNA 2010002N04 gene

[a] Genes induced by Wnt3a are listed in order of decreasing change. Those genes significantly downregulated in combination with FGF1 are grouped according to the effect of FGF1 alone on their expression levels. The third group consists of those genes variably affected in combination with FGF1. Note that several of these genes are downregulated by FGF but by less than 2.5 fold.

[b] Changes in gene expression in OB-TOP#1 cells treated with Wnt3a, Wnt3a plus FGF1, and FGF1 at 6 h relative to levels in untreated cells.

control conditions in the microarray assay, the Axin2 and Edn1 genes were detectable by RT-PCR and were not significantly downregulated by FGF alone in this experiment.

Sox2 contributes to the inhibition of Wnt signaling by FGF. We previously reported (24) that Sox2 was specifically and strongly induced by FGF in osteoblasts and could contribute to the inhibition of Wnt signaling by virtue of its association with β-catenin. To determine the contribution of Sox2 expression to the overall inhibitory effect of FGF on Wnt-induced transcription, we analyzed the ability of Wnt3a to activate the expression of a number of osteoblastic Wnt target genes in cells overexpressing Sox2 in the absence of FGF treatment. We compared the Sox16 clone of OB1 cells previously described (24) with two other OB1 "sister" clones created in the same experiment that exhibit basal levels of Sox2 expression. Figure 6 shows that the induction of four of the six genes tested was significantly reduced in the Sox2-overexpressing clone relative to what was seen for the controls. Although it is not immediately apparent why the induction of some genes would be affected more than that of others, these results support the hypothesis that Sox2 induction contributes to the inhibition of Wnt-induced transcription by FGF signaling.

DISCUSSION

Numerous reports have established that cross talk between FGF and Wnt signaling takes place in many tissues and organisms during embryonic development. Depending on the cell type and receptor context, the results of this cross talk can vary from a synergistic effect in determining cell fate and differentiation or promoting specific gene expression to an antagonistic effect (4, 7, 19, 28, 36). Our previous results indicated that FGF signaling could inhibit osteoblast differentiation through downregulation of Wnt signaling and that this effect was likely to contribute to the osteoblast/bone pathologies caused by excessive or unregulated FGF signaling (24). In this study, we have investigated the biochemical mechanisms by which FGF interferes with Wnt signaling by use of a series of reporter osteoblast lines where canonical Wnt signaling induces the expression of luciferase. In addition, we have conducted an extensive microarray analysis to identify endogenous osteoblast genes induced by Wnt and regulated by FGF.

FGF inhibits Wnt-induced transcription. The results obtained with our Wnt reporter osteoblast lines indicate that the major mechanism by which FGF antagonizes Wnt signaling is by preventing Wnt-induced transcription. Consistent with this conclusion, a striking inhibition of the formation of a Wnt-induced transcriptional protein complex on a consensus TCF/LEF binding DNA probe was observed for osteoblasts treated with FGF. The accepted view of the canonical Wnt pathway is that in the absence of Wnt ligand, TCF/LEF factors are bound to DNA in a repressive complex together with other corepressor proteins, such as Groucho (Gro)/Tle. β-Catenin activation by Wnt results in its binding to TCF, displacement of Gro/Tle, and the recruitment of other factors to form a transcriptionally active complex (3, 31). Consistent with this model, we detected in EMSAs a basal DNA/protein complex that contains TCF in untreated cells and a complex that contains both TCF(s) and β-catenin following Wnt treatment. FGF does not appear to prevent the activation of β-catenin but greatly decreases the formation of the Wnt-induced complex, while having only a slight effect on the basal complex. Interestingly, Wnt induces TCF4 expression at the protein and RNA levels and also elevates the RNA and protein (not shown) levels of TCF1. Together with TCF3, which is actually downregulated both by Wnt and by FGF, these are the TCF/LEF factors significantly expressed in OB1 cells. FGF inhibits the induction of both TCF1 and -4 by Wnt and also downregulates their expression in the absence of Wnt.

Our data suggest that FGF inhibits the formation of a Wnt-induced transcriptional complex by blocking the induction of TCF factors, thus dampening the amplification of the Wnt response. It is however unclear whether the lack of induction of TCF expression caused by FGF is sufficient to explain the almost complete block to the formation of a Wnt-induced

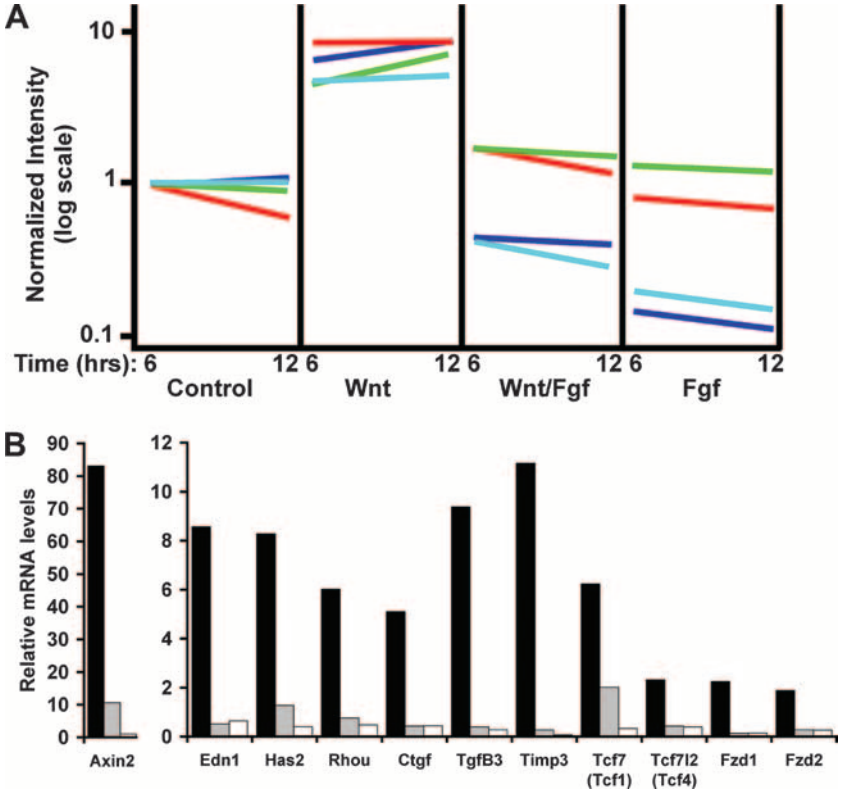

FIG. 5. Regulation of mRNA levels by Wnt3a and FGF1. (A) The graph shows representative expression patterns of selected genes at the 6-h and 12-h time points assayed by microarray analysis and classified by the criteria used to compile Table 1. The log values of the normalized intensity values are shown rather than absolute changes. Four Wnt3a-upregulated genes are shown, including two unchanged (*Ahr*, green; *Rhou*, red) and two downregulated (*Gadd45g*, light blue; *Timp3*, dark blue) by FGF1 alone. (B) The effects of Wnt and FGF treatment on various Wnt target and Fzd receptor genes were validated in an independent experiment by RT-PCR. Relative mRNA levels are expressed compared to the levels in untreated cultures. Bars indicate Wnt only (black), Wnt plus FGF (gray), and FGF only (white). OB-TOP#1 cells were pretreated with FGF for 10 h and with Wnt3a for an additional 10 h.

transcriptional complex. FGF-treated cells still contained significant levels of TCF1, -3, and -4; moreover, we did not detect a significant FGF effect on the formation of the basal TCF/DNA complex, and β-catenin activation was not affected. It is possible that similar to what has been shown for β-catenin (41), newly synthesized TCFs are required for the assembly of a transcriptionally active TCF/β-catenin/DNA complex or that a threshold level of TCF proteins is required.

Alternatively, other factors may contribute to the inhibition of Wnt signaling in FGF-treated cells by interfering with the association of β-catenin with TCF/LEF factors. The most likely candidate for this function is Sox2, which we have previously

TABLE 2. Regulation of genes affecting the Wnt signaling pathway by Wnt3a and/or FGF1[a]

Gene symbol	Accession no.	Fold induction seen for treatment with:			Description
		Wnt3a	Wnt3a + FGF1	FGF1	
Fzd1	BB259670	1.15	0.22	0.21	Frizzled homolog 1
Fzd2	BB371406	1.66	0.25	0.23	Frizzled homolog 2
Fzd7	NM_008057	1.34	0.28	0.30	Frizzled homolog 7
Fzd8	AV345166	1.01	0.30	0.25	Frizzled homolog 8
Sfrp	NM_009144	0.93	0.18	0.16	Secreted frizzled-related sequence protein 2
Dkk1	NM_010051	2.59	0.85	1.15	Dickkopf homolog 1
Dkk3	AK004853	0.73	0.13	0.15	Dickkopf homolog 3
Tcf3	NM_009332	0.54	0.24	0.26	Transcription factor 3
Mitf	NM_008601	1.02	3.22	3.98	Microphthalmia-associated transcription factor
Sox2	U31967	0.95	9.28	9.88	Sox2

[a] The genes shown affect the Wnt signaling pathway and were regulated to various degrees by Wnt or FGF treatment. Change values listed are from the 6-h point of the microarray assay. Other such genes (e.g., the Axin2, Tcf1, and Tcf4 genes) are included in Table 1. The Dkk1 gene is excluded from Table 1 because its expression level was below the cutoff used for Wnt target genes.

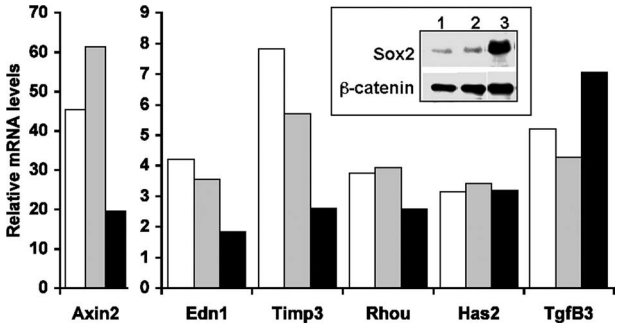

FIG. 6. Wnt induction of several target genes is attenuated in Sox2-expressing cells. Real-time RT-PCR analysis of six Wnt target genes in OB1 clones treated with Wnt3a for 16 h. Relative mRNA levels are expressed compared to the level for untreated cells for each clone. Lanes: 1, control OB1 cells transfected with vector alone (open bars); 2, OB1 clone expressing basal levels of Sox2 (gray bars); 3, OB1#16 expressing high levels of Sox2 (black bars). (Inset) Levels of Sox2 protein. β-Catenin was used as a loading control.

shown to be strongly induced by FGF in osteoblasts and which can associate with β-catenin to inhibit its transcriptional activation of a Wnt reporter plasmid (24). We also show in this report that in the absence of FGF, Sox2 overexpression can dampen the induction of several osteoblast genes by Wnt3a. Although Sox factors are DNA binding transcriptional regulators, Sox3 and Sox17 in *Xenopus laevis* and Sox9 in chondrocytes have also been shown to interfere with Wnt signaling through their association with β-catenin (1, 42). A different mechanism by which Sox proteins interfere with Wnt signaling has been recently proposed for SoxN in *Drosophila melanogaster*, which would involve binding to sequences adjacent to or overlapping with TCF binding sites and potentiation of TCF repression and/or its interaction with the Gro corepressor (5). However, EMSA supershift experiments with two different Sox2-specific antibodies did not detect binding of Sox2 to the DNA probe in extracts from FGF-treated cells (data not shown), and we previously showed that the ability of Sox2 to inhibit β-catenin-stimulated transcription does not require its DNA binding domain (24). It is also possible that other mechanisms not yet identified may affect the association of β-catenin with TCFs in FGF-treated cells. For example, the Mitf gene, which is induced by FGF (Table 2), has been shown to interact with β-catenin and redirect its activity (35).

An additional mechanism by which FGF inhibits Wnt signaling is suggested by the microarray analysis, which showed that all members of the Fzd Wnt receptor family expressed in our osteoblast lines are significantly downregulated (three- to fourfold) by FGF. This is likely to be one of the reasons why the strongest downregulation of the Wnt response is obtained by treating cells with FGF 10 h before the addition of Wnt3a. Downregulation of Wnt receptors could therefore contribute to the inhibitory effect of FGF on Wnt signaling but is unlikely to be the major factor, since β-catenin activation is not substantially prevented by FGF.

FGF signaling affects osteoblast Wnt target genes. Microarray analysis of gene expression conducted on osteoblasts that were exposed to Wnt3a for 6 or 12 h identified a number of genes whose RNA levels were significantly (>2.5-fold) increased. The induction of about 70% of these genes was reduced to some extent by pretreatment with FGF. For the remaining 30% of the Wnt-induced genes, Wnt induction was not affected by FGF. These genes could be targets of noncanonical Wnt signaling (20, 38). Alternatively, they could be induced by FGF through the action of other transcription factors that target independent enhancers or promoter elements so that the inhibition of Wnt induction would be masked by the positive effect of FGF on their expression. Indeed, many of these genes (e.g., *Grem1* and *Sema5a*) are induced by FGF alone (Table 1). While these data clearly show that the FGF inhibition of the Wnt response is not restricted to the TOP-luciferase reporter gene of our OB-TOP cells, a number of other interesting observations were made.

Many of the Wnt-induced genes that are downregulated by the combined Wnt/FGF treatment are not affected by FGF alone, suggesting a specific effect of FGF on Wnt-induced transcription. However, a substantial proportion of Wnt-induced genes is also significantly downregulated by FGF alone. The following two hypotheses could explain this latter observation: (i) in addition to inhibiting Wnt-induced transcription, FGF signaling could also inhibit the expression of these genes at the transcriptional or posttranscriptional level independent of Wnt; and (ii) it is possible that a degree of cell-autonomous Wnt signaling is taking place in our osteoblast cultures and that these genes are exquisitely sensitive to it.

While several genes previously identified in many cell types as Wnt targets (http://www.stanford.edu/~rnusse/pathways/targets.html) were also found to be induced by Wnt3a in our experiments (e.g., the Axin2, Endothelin-1, Tcf1, and Ahr genes) and other Wnt target genes such as the Wisp1 and -2 genes were weakly induced (<2.5-fold), a number of genes previously identified as Wnt targets in other cell types (e.g., the Myc, Engrailed, and Msx2 genes) did not score in our experiments. This is likely to reflect a cell-type- or context-specific response and highlights the notion that the response to exogenous signals is cell type specific.

Although the main objective of this work was not to identify the role of Wnt target genes in osteoblast maturation, since Wnt signaling promotes osteoblast commitment and function, we expected to find many genes involved in osteoblast differentiation among the Wnt target genes regulated by FGF. Although some of the genes we found upregulated by Wnt and downregulated by FGF are known to be involved in osteoblast differentiation and function (e.g., the insulin receptor substrate 1, tissue inhibitor of metalloproteinase 3, TGFβ, connective tissue growth factor, and Endothelin-1 genes), we did not observe any significant effect of Wnt on the expression of other genes that had been previously implicated in the Wnt response of osteoblasts, such as the Runx2, Osterix, and Msx2 genes (2, 11, 17). It is possible that the differentiation pathway is not fully functional in the immortalized osteoblast lines used in these experiments. Alternatively, as the OB1 cell line is already a committed osteoblast, fate markers such as Runx2 and Osterix may not be further responsive to Wnt.

In summary, we show in this report that FGF antagonizes canonical Wnt signaling in osteoblasts, thus resulting in the inhibition of Wnt-induced osteoblast maturation. This effect is ultimately manifested in the inhibition of Wnt-induced transcription, but the mechanisms through which FGF inhibits Wnt signaling appear to be multiple and complex (Fig. 7). FGF treatment of osteoblasts downregulates the expression of the

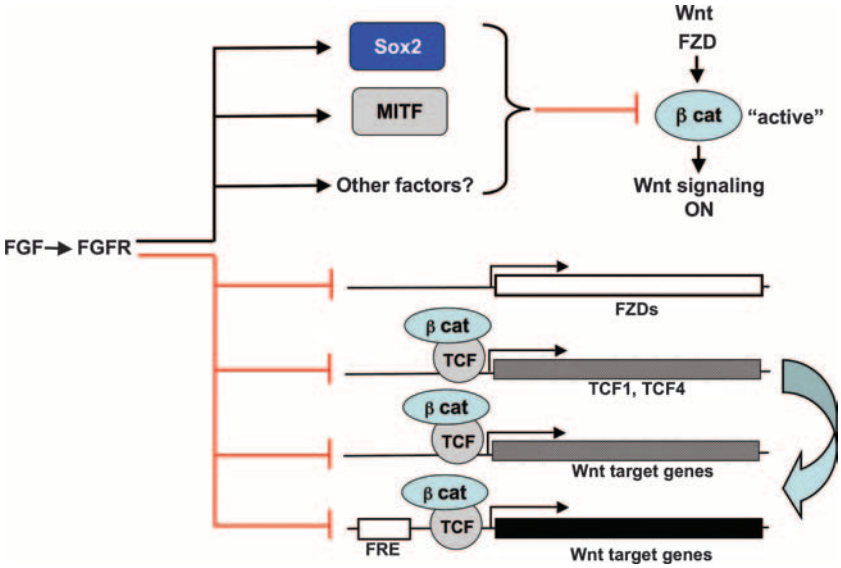

FIG. 7. Schematic representation of the multiple mechanisms by which FGF inhibits canonical Wnt signaling in osteoblasts. FGF signals (i) upregulate factors such as Sox2 and Mitf that can interfere with Wnt signaling by binding β-catenin (β cat); (ii) lower the transcript levels of Fzd receptors Fzd1, -2, -7, and -8; and (iii) decrease the formation of Wnt-induced transcriptional complexes containing TCFs and β-catenin on TCF/LEF binding sites by inhibiting TCF1 and -4 expression, thus preventing the transcription of Wnt target genes. Some Wnt target genes may also be directly downregulated by FGF signaling through FGF response elements (FRE) in independent regulatory regions or by destabilizing transcripts.

four Fzd Wnt receptors expressed in our cell lines. TCF/LEF factors are the major transcriptional mediators of the canonical Wnt pathway: FGF both blocks their induction by β-catenin and further downregulates their basal levels of expression. In some cases, FGF may also directly downregulate Wnt target genes independently of Wnt. Furthermore, our previous studies show that the induction of Sox2 expression by FGF could lead to its association with β-catenin, thus further hampering Wnt-induced transcription. The contribution of each of these factors to the antagonistic effect of FGF on Wnt signaling is likely to vary depending on the relative strength of the two signals, the stage of osteoblast commitment and differentiation, and the length of exposure to each signal. While further work will be necessary to answer these questions, our findings highlight the complex cross talk between signaling systems that control cell lineage and differentiation.

ACKNOWLEDGMENTS

We thank R. Moon for the gift of the TOP-FLASH plasmids and L. Dailey and M. L. Melli for critical reading of the manuscript.

This investigation was supported by PHS grant AR 051358 from the NIAMS.

REFERENCES

1. **Akiyama, H., J. P. Lyons, Y. Mori-Akiyama, X. Yang, R. Zhang, Z. Zhang, J. M. Deng, M. M. Taketo, T. Nakamura, R. R. Behringer, P. D. McCrea, and B. de Crombrugghe.** 2004. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev. **18:**1072–1087.
2. **Bennett, C. N., K. A. Longo, W. S. Wright, L. J. Suva, T. F. Lane, K. D. Hankenson, and O. A. MacDougald.** 2005. Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc. Natl. Acad. Sci. USA **102:**3324–3329.
3. **Billin, A. N., H. Thirlwell, and D. E. Ayer.** 2000. Beta-catenin-histone deacetylase interactions regulate the transition of LEF1 from a transcriptional repressor to an activator. Mol. Cell. Biol. **20:**6882–6890.
4. **Canning, C. A., L. Lee, C. Irving, I. Mason, and C. M. Jones.** 2007. Sustained interactive Wnt and FGF signaling is required to maintain isthmic identity. Dev. Biol. **305:**276–286.
5. **Chao, A. T., W. M. Jones, and A. Bejsovec.** 2007. The HMG-box transcription factor SoxNeuro acts with Tcf to control Wg/Wnt signaling activity. Development **134:**989–997.
6. **Clevers, H.** 2006. Wnt/beta-catenin signaling in development and disease. Cell **127:**469–480.
7. **Dailey, L., D. Ambrosetti, A. Mansukhani, and C. Basilico.** 2005. Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev. **16:**233–247.
8. **Dailey, L., E. Laplantine, R. Priore, and C. Basilico.** 2003. A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. J. Cell Biol. **161:**1053–1066.
9. **Daniels, D. L., and W. I. Weis.** 2005. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat. Struct. Mol. Biol. **12:**364–371.
10. **Debiais, F., M. Hott, A. M. Graulet, and P. J. Marie.** 1998. The effects of fibroblast growth factor-2 on human neonatal calvaria osteoblastic cells are differentiation stage specific. J. Bone Miner. Res. **13:**645–654.
11. **Gaur, T., C. J. Lengner, H. Hovhannisyan, R. A. Bhat, P. V. Bodine, B. S. Komm, A. Javed, A. J. van Wijnen, J. L. Stein, G. S. Stein, and J. B. Lian.** 2005. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J. Biol. Chem. **280:**33132–33140.
12. **Glass, D. A., II, P. Bialek, J. D. Ahn, M. Starbuck, M. S. Patel, H. Clevers, M. M. Taketo, F. Long, A. P. McMahon, R. A. Lang, and G. Karsenty.** 2005. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev. Cell **8:**751–764.
13. **Harada, S., and G. A. Rodan.** 2003. Control of osteoblast function and regulation of bone mass. Nature **423:**349–355.
14. **Hartmann, C.** 2006. A Wnt canon orchestrating osteoblastogenesis. Trends Cell Biol. **16:**151–158.
15. **Henderson, B. R., and F. Fagotto.** 2002. The ins and outs of APC and beta-catenin nuclear transport. EMBO Rep. **3:**834–839.
16. **Holmen, S. L., C. R. Zylstra, A. Mukherjee, R. E. Sigler, M. C. Faugere, M. L. Bouxsein, L. Deng, T. L. Clemens, and B. O. Williams.** 2005. Essential role of beta-catenin in postnatal bone acquisition. J. Biol. Chem. **280:**21162–21168.
17. **Hussein, S. M., E. K. Duff, and C. Sirard.** 2003. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. J. Biol. Chem. **278:**48805–48814.
18. **Karsenty, G., and E. F. Wagner.** 2002. Reaching a genetic and molecular understanding of skeletal development. Dev. Cell **2:**389–406.
19. **Kim, Y., A. Kobayashi, R. Sekido, L. DiNapoli, J. Brennan, M. C. Chaboissier, F. Poulat, R. R. Behringer, R. Lovell-Badge, and B. Capel.** 2006. Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. PLoS Biol. **4:**e187.
20. **Kohn, A. D., and R. T. Moon.** 2005. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium **38:**439–446.

21. **Krishnan, V., H. U. Bryant, and O. A. Macdougald.** 2006. Regulation of bone mass by Wnt signaling. J. Clin. Investig. **116:**1202–1209.
22. **Lomri, A., J. Lemonnier, M. Hott, N. de Parseval, E. Lajeunie, A. Munnich, D. Renier, and P. J. Marie.** 1998. Increased calvaria cell differentiation and bone matrix formation induced by fibroblast growth factor receptor 2 mutations in Apert syndrome. J. Clin. Investig. **101:**1310–1317.
23. **Malbon, C. C.** 2004. Frizzleds: new members of the superfamily of G-protein-coupled receptors. Front. Biosci. **9:**1048–1058.
24. **Mansukhani, A., D. Ambrosetti, G. Holmes, L. Cornivelli, and C. Basilico.** 2005. Sox2 induction by FGF and FGFR2 activating mutations inhibits Wnt signaling and osteoblast differentiation. J. Cell Biol. **168:**1065–1076.
25. **Mansukhani, A., P. Bellosta, M. Sahni, and C. Basilico.** 2000. Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. J. Cell Biol. **149:**1297–1308.
26. **Marie, P. J.** 2003. Fibroblast growth factor signaling controlling osteoblast differentiation. Gene **316:**23–32.
27. **Marie, P. J., J. D. Coffin, and M. M. Hurley.** 2005. FGF and FGFR signaling in chondrodysplasias and craniosynostosis. J. Cell. Biochem. **96:**888–896.
28. **Mikels, A. J., and R. Nusse.** 2006. Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS Biol. **4:**e115.
29. **Moon, R. T., B. Bowerman, M. Boutros, and N. Perrimon.** 2002. The promise and perils of Wnt signaling through beta-catenin. Science **296:**1644–1646.
30. **Nusse, R.** 2005. Wnt signaling in disease and in development. Cell Res. **15:**28–32.
31. **Nusse, R.** 1999. WNT targets. Repression and activation. Trends Genet. **15:**1–3.
32. **Ornitz, D. M., and P. J. Marie.** 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev. **16:**1446–1465.
33. **Rawadi, G., B. Vayssiere, F. Dunn, R. Baron, and S. Roman-Roman.** 2003. BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J. Bone Miner. Res. **18:**1842–1853.
34. **Roose, J., M. Molenaar, J. Peterson, J. Hurenkamp, H. Brantjes, P. Moerer, M. van de Wetering, O. Destree, and H. Clevers.** 1998. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature **395:**608–612.
35. **Schepsky, A., K. Bruser, G. J. Gunnarsson, J. Goodall, J. H. Hallsson, C. R. Goding, E. Steingrimsson, and A. Hecht.** 2006. The microphthalmia-associated transcription factor Mitf interacts with beta-catenin to determine target gene expression. Mol. Cell. Biol. **26:**8914–8927.
36. **Takemoto, T., M. Uchikawa, Y. Kamachi, and H. Kondoh.** 2006. Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. Development **133:**297–306.
37. **van Noort, M., J. Meeldijk, R. van der Zee, O. Destree, and H. Clevers.** 2002. Wnt signaling controls the phosphorylation status of beta-catenin. J. Biol. Chem. **277:**17901–17905.
38. **Veeman, M. T., J. D. Axelrod, and R. T. Moon.** 2003. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev. Cell **5:**367–377.
39. **Veeman, M. T., D. C. Slusarski, A. Kaykas, S. H. Louie, and R. T. Moon.** 2003. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. **13:**680–685.
40. **Westendorf, J. J., R. A. Kahler, and T. M. Schroeder.** 2004. Wnt signaling in osteoblasts and bone diseases. Gene **341:**19–39.
41. **Willert, J., M. Epping, J. R. Pollack, P. O. Brown, and R. Nusse.** 2002. A transcriptional response to Wnt protein in human embryonic carcinoma cells. BMC Dev. Biol **2:**8.
42. **Zorn, A. M., G. D. Barish, B. O. Williams, P. Lavender, M. W. Klymkowsky, and H. E. Varmus.** 1999. Regulation of Wnt signaling by Sox proteins: XSox17 alpha/beta and XSox3 physically interact with beta-catenin. Mol. Cell **4:**487–498.