Wnt Signaling Stimulates Osteoblastogenesis of Mesenchymal Precursors by Suppressing CCAAT/Enhancer-binding Protein α and Peroxisome Proliferator-activated Receptor γ*

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Mesenchymal precursor cells have the potential to differentiate into several cell types, including adipocytes and osteoblasts. Activation of Wnt/β-catenin signaling shifts mesenchymal cell fate toward osteoblastogenesis at the expense of adipogenesis; however, molecular mechanisms by which Wnt signaling alters mesenchymal cell fate have not been fully investigated. Our prior work indicates that multipotent precursors express adipogenic and osteoblastogenic transcription factors at physiological levels and that ectopic expression of Wnt10b in bipotential ST2 cells suppresses expression of CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) and increases expression of Runx2, Dlx5, and osterix. Here, we demonstrate that transient activation of Wnt/β-catenin signaling rapidly suppresses C/EBPα and PPARγ, followed by activation of osteoblastogenic transcription factors. Enforced expression of C/EBPα or PPARγ partially rescues lipid accumulation and decreases mineralization in ST2 cells expressing Wnt10b, suggesting that suppression of C/EBPα and PPARγ is required for Wnt/β-catenin to alter cell fate. Furthermore, knocking down expression of C/EBPα, PPARγ, or both greatly reduces adipogenic potential and causes spontaneous osteoblastogenesis in ST2 cells and mouse embryonic fibroblasts, suggesting that Wnt signaling alters the fate of mesenchymal precursor cells primarily by suppressing C/EBPα and PPARγ.

The Wnt family of secreted proteins regulates many aspects of cell biology, including mesenchymal cell fate. Since initial reports that mutations in a Wnt coreceptor are causally linked to alterations in human bone mass (1), positive effects of Wnt/β-catenin signaling on bone formation have been dissected through genetic manipulation of extracellular and intracellular components of the Wnt pathway (2–10). Our group previously reported that expression of Wnt10b in bone marrow results in an increase in trabecular bone density, whereas Wnt10b-deficient mice exhibit a low bone mass phenotype, indicating that Wnt10b is an endogenous regulator of bone formation (4). Furthermore, various Wnt signaling components, including Wnt10b, are induced in response to mechanical loading, further suggesting that Wnt signaling plays an important role in bone physiology (11).

One of the main mechanisms by which Wnt/β-catenin signaling increases bone mass is to increase the number of osteoblasts, which play a critical role in bone formation. Genetic and cell culture studies have indicated that Wnt signaling increases proliferation and decreases apoptosis of osteoblasts. For instance, targeted deletion of a Wnt coreceptor, low density lipoprotein-related protein 5, in mice resulted in low bone mass and reduced proliferation of osteoblasts (12), whereas transgenic expression of low density lipoprotein-related protein 5 with a gain of function mutation increased bone mass and decreased apoptosis of osteoblasts (3). Moreover, mice devoid of the Wnt inhibitor, secreted frizzled-related protein 1, exhibited high bone mass due to reduced apoptosis (2). In addition to effects on proliferation and apoptosis of osteoblasts, Wnt/β-catenin signaling also stimulates osteoblastogenesis (13).

Osteoblasts arise from mesenchymal precursor cells that have potential to differentiate into many different cell types, including myoblasts, chondrocytes, and adipocytes (13). Often, imbalance among the cell types is observed with bone loss. In particular, an increased ratio of adipocytes to osteoblasts is associated with age-related osteoporosis (14, 15). Thus, it has been suggested that targeting regulatory factors that alter mesenchymal cell fate to increase the number of osteoblasts has the potential to provide novel therapeutic approaches for osteoporosis and related bone diseases (14). Considerable evidence now indicates that Wnt signaling stimulates osteoblastogenesis (2, 6–8, 16) and represses differentiation to alternative cell fates, such as the adipocyte (4). However, our molecular understanding of how Wnt signaling shifts mesenchymal cell fate remains limited.

Although the transcriptional cascade for osteoblastogenesis is not fully defined, studies have provided insight into transcrip-
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transcription factors have been found to regulate adipogenesis. For example, a lipogenic basic helix-loop-helix transcription factor, SREBP1c, enhances adipogenesis by inducing expression of the PPARγ ligand (28). Zinc finger protein Kruppel transcription factors, KLF15 (29) and KLF5 (30), promote adipogenesis, whereas KLF2 (31, 32) and KLF7 (33) inhibit it. Other transcription factors, KLF15 (29) and KLF5 (30), promote adipogenesis, whereas KLF2 (31, 32) and KLF7 (33) inhibit it. Other transcription factors have been found to regulate adipogenesis. For example, a lipogenic basic helix-loop-helix transcription factor, SREBP1c, enhances adipogenesis by inducing expression of the PPARγ ligand (28). Zinc finger protein Kruppel transcription factors, KLF15 (29) and KLF5 (30), promote adipogenesis, whereas KLF2 (31, 32) and KLF7 (33) inhibit it. Other transcription factors, KLF15 (29) and KLF5 (30), promote adipogenesis, whereas KLF2 (31, 32) and KLF7 (33) inhibit it.

In addition, two homeobox transcription factors, Msx2 and Dlx5, are also thought to regulate osteoblast differentiation. Mice with deletion of Msx2 or Dlx5 exhibit a marked delay of ossification in the bones of the skull (21, 22). Expression of Runx2 was reduced in Msx2-deficient mice (21), whereas Runx2 was intact in Dlx5-deficient mice (22), suggesting that Msx2 might lie upstream of Runx2, and Dlx5 might act downstream of or be independent of Runx2. However, interactions between these transcription factors are considerably more complex than a simple linear relationship causing differentiation. For example, one study showed that Dlx5 is required for BMP2 (bone morphogenic protein 2)-induced Runx2 expression (23), and the same group reported that BMP2 induced osterix through a Runx2-independent mechanism (24).

On the other hand, the genetic cascade of adipogenesis is well established based upon considerable analyses of in vitro and in vivo models. In response to adipogenic inducers, early transcription factors C/EBPβ, C/EBPδ, and PPARγ1 are rapidly induced/activated and initiate the adipogenic cascade (25–27). Together they increase expression of the two important transcription factors for adipogenesis, PPARγ2 and C/EBPα, which then induce downstream genes that are characteristic of mature adipocytes, including FABP4, Glut4, and adiponectin (25–27). In addition to this traditional cascade, several other transcription factors have been found to regulate adipogenesis. For example, a lipogenic basic helix-loop-helix transcription factor, SREBP1c, enhances adipogenesis by inducing expression of the PPARγ ligand (28). Zinc finger protein Kruppel transcription factors, KLF15 (29) and KLF5 (30), promote adipogenesis, whereas KLF2 (31, 32) and KLF7 (33) inhibit it. Other transcription factors with regulatory roles in adipogenesis, including STAT5 (34), EPAS1 (35), CREB (36), Krox20 (37), EBF1 (38), Bmal1 (39), and GATAs (40, 41). Herein, we report on our findings that Wnt signaling decreases expression of C/EBPα and PPARγ to regulate fate of mesenchymal precursors toward osteoblastogenesis and away from adipogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse embryonic fibroblasts (MEFs) and ST2 cells were cultured essentially as described previously (4, 42). Briefly, MEFs and ST2 cells were maintained in Dulbecco’s modified Eagle’s medium and in α-minimal essential medium, respectively, that contained 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. For adipogenesis, cells that had been confluent for a day were treated with 10% fetal bovine serum, 1 μM dexamethasone, 0.5 mM methylisobutylxanthine, 1 μg/ml insulin, and 1 or 5 μM troglitazone (day 0). Subsequently, cells were fed 1 μg/ml insulin in 10% fetal bovine serum media on day 2, and refed with 10% fetal bovine serum media every 2 days subsequently. To induce osteoblastogenesis, confluent MEFs were switched to maintenance media containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. ST2 cells were differentiated under the same conditions except that 25 μg/ml ascorbic acid was used. CHIR99021 (3 μM in Me2SO) was used to inhibit glycolgen synthase kinase 3. Accumulation of neutral lipids in adipocytes was evaluated with Oil Red-O staining (43). Degree of mineralization in osteoblasts was determined with von Kossa or Alizarin Red staining. In von Kossa staining, silver substitutes for calcium in salts with phosphate and carbonate. Thus, detection of silver ions is an indirect measure of calcium content (44). Alizarin Red S is an anthraquinone derivative that complexes with calcium, resulting in an orange-red-colored product (45, 46).

Retroviral Infection and Constructs—Genes were stably introduced into MEFs and ST2 cells by retroviral infection as described previously (43). pLHCX-Wnt10b was as described by Bennett et al. (4). pMSCV and pMSCV-PPARγ were provided by Dr. David Rosen (Harvard Medical School). pMSCV-C/EBPα was subcloned at BglII (5’’) and Xho (3’’) restriction sites from pBabe-C/EBPα at BamHI (5’) and SalI (3’’). Cell Lysates and Immunoblotting—Cells were washed once with phosphate-buffered saline and scraped in a lysis buffer containing 1% SDS and 60 mM Tris-HCl, pH 6.8. Lysates were boiled for 5 min, vortexed, and then boiled for an additional 5 min prior to storing at −20 °C. The samples were then analyzed by SDS-PAGE. Briefly, equal lysate volumes were loaded and separated by electrophoresis in 10 or 4–20% gradient polyacrylamide gels (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad). An equal amount of protein loading between lanes was confirmed by Ponceau staining. Membranes were immunoblotted with the appropriate primary antibodies, and secondary antibody (1:4000 dilution; IgG-peroxidase) was visualized with Super Signal (Pierce) enhanced chemiluminescence. Polyclonal FABP4 antibody (1:5000 dilution) was from Dr. David Bernlohr (University of Minnesota). PPARγ, C/EBPα (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and laminin (1:1000 dilution; Cell Signal Transduction) antibodies were obtained commercially.

Quantitative RT-PCR—One μg of total RNA was transcribed to cDNA using the TaqMan system (Applied Biosystems). Quantitative PCR was performed according to the manufacturer’s protocol. SYBR green I was used to monitor amplification of DNA on the I-Cycler thermal cycler and IQ real time PCR detection system (Bio-Rad). After amplification, melting curve analysis was performed as described in the manufacturer’s protocol. Gene expression was normalized to cyclophilin or 18 S RNA. All primers were evaluated as described previously (4). The corresponding primer sequences are 18 S (forward, 5’-cga tgc tct tag_ctg agt gt-3’; reverse, 5’-ggt cca aga att tca cct ct-3’), 4 The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome; MEF, mouse embryo fibroblast; shRNA, short hairpin RNA; RT, reverse transcription.
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RESULTS

Wnt10b Inhibits Adipogenesis and Promotes Osteoblastogenesis in Pluripotent Cell Culture Models—As reported previously (4), enforced expression of Wnt10b in bone marrow-derived ST2 cells stimulates osteoblastogenesis (Fig. 1A). Here we used MEFs to test whether activation of Wnt/β-catenin signaling regulates cell fate in another type of multipotent precursor cells. Although MEFs have been extensively used as a model for adipogenesis (42, 48–50), they are also able to differentiate into myoblast-like (51) or osteoblast-like cells (52), depending upon the inducing stimulus. For instance, a bona fide bone anabolic protein, BMP4 stimulates mineralization (Fig. 1B) and increases expression of osteoblast genes, including type I collagen (data not shown). To activate Wnt signaling in MEFs, we ectopically expressed Wnt10b and exposed them to induction media for osteoblast or adipocyte differentiation (Fig. 1, B and D). Control MEFs rarely undergo mineralization in osteogenic medium, which provides ascorbic acid and β-glycerophosphate to promote mineralization of extracellular matrix (Fig. 1, A and B). However, prolonged treatment with BMP4 stimulates mineralization of extracellular matrix, as assessed by Alizarin Red staining. Importantly, Wnt10b-expressing MEFs spontaneously differentiate into osteoblast-like cells, and BMP4 further enhances mineralization (Fig. 1B). Moreover, Wnt10b increases total deposition of calcium in cell matrix by ~4- and ~8-fold compared with control ST2 cells and MEFs, respectively (Fig. 1C). In response to adipogenic stimuli, control ST2 cells and MEFs infected with empty retrovirus vector differen-

cyclophilin (forward, 5′-tcc aaa gac aga aga aaa ctt tcg; reverse, 5′-tct ttc tgc tgg tct ctc cat tcc-3′), C/EBPα (forward, 5′-tga aca aga caa gca aca ag-3′; reverse, 5′-tca ctt gtc acc tcc agc ac-3′), PPARγ (forward, 5′-gga aag aca aca gac aaa tca c-3′; reverse, 5′-tac gga tcc aag caa ctc gca c-3′).

Calcium Assay—Cells were washed twice with phosphate-buffered saline and decalcified with 0.6 N HCl and rotation of cell culture plates overnight at room temperature. Calcium content of the supernatant was estimated relative to a standard provided within the LiquiColor kit (Stanbio Laboratories) (47). The reaction between calcium and ortho-cresolphthalein complexone produces a purple complex at 580 nm, and color intensity is directly proportional to the concentration of calcium in the sample. After decalcification, cells were solubilized with 0.1 N NaOH, 0.1% SDS, and the amount of protein was assayed (Bio-Rad) according to the manufacturer’s protocol.

Short Hairpin RNAs—Knock-down of endogenous genes was accomplished with short hairpin RNA (shRNA) and pSUPERIOR-retro and pSUPERIOR.retro.neo + GFP retroviral vectors (Oligoengine). Target sequences were selected with software available on the Dharmacon, Invitrogen, and/or Ambion Web sites. Oligonucleotides synthesized by Invitrogen were annealed and subcloned into retroviral vectors at HindIII and BglII sites. The target sequences were 5′-ggtttgatccgtggaaaaggtt-3′ for PPARγ and 5′-gcagactgcagggacagaaaca-3′ for C/EBPα. shRNA constructs were introduced into cells using retroviral transduction as detailed above. Efficiency and specificity of suppression by shRNAs were evaluated with analyses of protein and/or RNA levels as indicated.

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FIGURE 1. Wnt10b stimulates osteoblastogenesis and inhibits adipogenesis in multipotent cells. ST2 cells and MEFs were infected with control (NH23) or Wnt10b retroviruses. At confluence, ST2 cells were switched to osteogenic media, and MEFs were switched to osteogenic media in the presence or the absence of 20 ng/ml BMP4 from day 0 to 6, after which cells were fed with osteogenic media. At day 14, cellular mineralization was assessed with von Kossa (A; ST2) or Alizarin Red (B; MEF) staining. Mineralized calcium was also quantified (C). A representative experiment is presented as mean ± S.D. for ST2 cells (n = 3) and MEFs (n = 5). Significant differences between control and Wnt10b cells are depicted with an asterisk (Student’s t test, p < 0.05). D, 2 days after confluence, control and Wnt10b-ST2 cells and MEFs were induced with adipogenic media, and cells were stained with Oil Red-O to assess lipid accumulation on days 5 and 8 of differentiation. These results are representative of at least three independent experiments.
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tiate into adipocytes and accumulated lipid droplets in cytoplasm as visualized by Oil Red-O staining (Fig. 1D), whereas Wnt10b-expressing cells showed a dramatic reduction in adipogenesis and accumulation of lipid droplets (Fig. 1D). Taken together, these data provide further evidence that activation of Wnt signaling inhibits adipogenesis and promotes osteoblastogenesis in pluripotent precursor cells.

Wnt Signaling Represses Expression of C/EBPα and PPARγ—To investigate mechanisms whereby Wnt/β-catenin signaling regulates cell fate, we measured mRNA expression of transcriptional components regulating adipogenesis and osteoblastogenesis in ST2 cells infected with control or Wnt10b retroviruses (4). At confluence and in the absence of inducing agents, some osteoblastogenic transcription factors, including Runx2, osterix, and Dlx5, were up-regulated in cells that expressed Wnt10b (4), whereas adipogenic transcription factors C/EBPα and PPARγ and their target gene FABP4 were down-regulated, suggesting that Wnt10b tips the molecular balance governing commitment between two cell lineages.

To establish whether primary effects of Wnt signaling are on adipogenic or osteoblastogenic transcription factors, we examined temporal changes in gene expression following transient activation of Wnt signaling with the GSK3 inhibitor, CHIR99021, which stimulates nuclear localization of β-catenin (53). Confluent ST2s were switched to osteogenic media 12 h prior to treatment with CHIR99021 to adjust cells to cellular conditions more favorable to osteoblastogenesis. Cell lysates were collected at various time points, and mRNA expression was analyzed by quantitative RT-PCR. Although CHIR99021 repressed mRNA expression of C/EBPα within 4 h and PPARγ by 12 h (Fig. 2A), expression of the early adipogenic transcription factor, C/EBPβ, was not altered, consistent with observations in cells constitutively expressing Wnt10b (4). CHIR99021 increases expression of alkaline phosphatase, an early marker for osteoblastogenesis, until 72 h, but expression then decreases substantially by day 7 (Fig. 2A). In contrast, expression of osteoblastogenic transcription factors was not substantially changed over this time frame (data not shown). In addition, the gene expression profile was similar when the same experiment was performed in the absence of ascorbic acid and β-glycerophosphate, although suppression of PPARγ tended to occur earlier and stronger under these conditions (data not shown).

To assess influence of Wnt/β-catenin signaling on gene expression at the protein level, confluent ST2 cells were treated with vehicle (Me2SO), purified recombinant Wnt3a or GSK3 inhibitor CHIR99021 (Fig. 2B). Similar to regulation of mRNAs, both Wnt3a and CHIR99021 repressed protein expression of C/EBPα and PPARγ, the major isoform expressed in confluent undifferentiated ST2 cells, within 4–8 h, although suppression was more profound with CHIR99021. Expression of C/EBPα and PPARγ proteins increased slightly during this time frame, but this observation was not consistently found in other experiments; however, the internal control, laminin, remained constant at all time points (Fig. 2B). Although the rapid decline of PPARγ protein appears to precede that observed for PPARγ mRNA (Fig. 2A), the protein experiment was performed without ascorbic acid and β-glycerophosphate. Under these conditions, mRNA for PPARγ showed similar kinetics of suppression (data not shown), and expression of Runx2 protein was not changed during this time frame (data not shown). Taken together, these results suggest that activation of Wnt signaling rapidly suppresses expression of adipogenic transcription fac-

![FIGURE 2. Activation of Wnt/β-catenin signaling rapidly represses expression of C/EBPα and PPARγ. A, confluent ST2 cells were switched to osteogenic medium. Twelve hours later, cells were treated with vehicle (Me2SO; DMSO) or 3 μM CHIR99021, and RNA was isolated at the indicated times. Cyclophilin, C/EBPα, PPARγ, C/EBPβ, and alkaline phosphatase mRNAs were evaluated by quantitative RT-PCR. Expression of mRNAs is presented relative to cyclophilin mRNA. A representative experiment is presented (n = 3), and significant changes are depicted with an asterisk. Similar results were observed in four independent experiments. B, confluent ST2 cells were treated with vehicle (Me2SO), 20 ng/ml purified mouse Wnt3a recombinant, or 3 μM CHIR99021 for the indicated time points. Expression of C/EBPα, PPARγ, and laminin proteins was evaluated by immunoblotting. These results were observed in three independent experiments. C, confluent plates of ST2 cells were incubated with cycloheximide (CHX; 5 μg/ml) or vehicle for 30 min prior to treatment with 85 ng/ml Wnt3a or vehicle for 6 h (n = 3). Cells were lysed, RNA was purified, and expression of PPARγ (mean ± S.D.) was evaluated by quantitative RT-PCR.](image-url)
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ST2 cells were infected with retroviruses that express C/EBPα (A) or PPARγ2 (B). Confluent ST2 cells were treated with vehicle (−), purified mouse Wnt3a recombinant (W), or 3 μM CHIR99021 (G) for the indicated times. Expression of C/EBPα, PPARγ1/2, and laminin proteins was evaluated by immunoblotting. These results are representative of two independent experiments.

PPARγ and C/EBPα Are Required for Wnt/β-Catenin Signaling to Stimulate Osteoblastogenesis—In a prior study, we observed that PPARγ is required for Wnt to regulate cell fate between adipogenesis and osteoblastogenesis in bipotential ST2 cells (4). Rapid repression of both C/EBPα and PPARγ by activation of Wnt/β-catenin signaling prompted us to determine whether suppression of C/EBPα is also required for Wnt to shift cell fate toward osteoblastogenesis. To address this question, we ectopically expressed C/EBPα or PPARγ2 in control or Wnt10b ST2 cells and confirmed expression of these transcription factors with immunoblot analyses (Fig. 4A). As expected, introduction of either C/EBPα or PPARγ2 was sufficient to partially rescue accumulation of lipid in Wnt10b cells (Fig. 4B), although PPARγ2 appeared to be slightly more potent, as assessed by Oil Red-O staining. Although control ST2 cells did not undergo osteoblastogenesis in the osteogenic media (Fig. 4C), Wnt10b cells showed robust mineralization of their extracellular matrix. The addition of either C/EBPα or PPARγ2 substantially decreased the ability of Wnt10b to induce osteoblastogenesis as assessed by von Kossa staining (Fig. 4C). These findings suggest that suppression of C/EBPα and PPARγ by Wnt/β-catenin signaling is required for the switch of mesenchymal cell fate from adipogenesis to osteoblastogenesis.

PPARγ1 Is Sufficient to Stimulate Osteoblastogenesis—We next asked whether suppression of adipogenic transcription factors is sufficient to stimulate the program of osteoblastogenesis (Fig. 5). To stably repress expression of C/EBPα and PPARγ, shRNA constructs were generated in a retroviral vector that constitutively generates shRNA transcripts from the H1 RNA polymerase III promoter. Several shRNA constructs targeting C/EBPα and PPARγ were tested, and the most efficient ones were chosen for analyses of cellular effects. As expected, cells expressing two independent shRNAs to C/EBPα selectively lost expression of endogenous C/EBPα, whereas cells expressing two independent short hairpins targeting PPARγ had diminished expression of PPARγ1 (Fig. 5A). Cells with shRNAs for both factors expressed neither C/EBPα nor PPARγ1 as compared with control cells expressing nontarget scrambled shRNAs. None of the constructs influenced expression of laminin (Fig. 5A).

To test cellular effects of C/EBPα and/or PPARγ deficiency, ST2 cells were induced to undergo adipogenesis (Fig. 5B) or osteoblastogenesis (Fig. 5C). As expected, cells expressing short hairpin C/EBPα undergo adipogenesis with reduced efficiency, as assessed by accumulation of lipid (Fig. 5B) or expression of FABP4, a molecular marker of adipogenesis (Fig. 5A). Loss of PPARγ1 alone or loss of both C/EBPα and PPARγ1 completely blocked accumulation of lipid and expression of FABP4 (Fig. 5, A and B). In parallel, control and shRNA-expressing cells were exposed to osteogenic media for 3 weeks (Fig. 5C). Suppression of C/EBPα or
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PPARγ1 alone caused a substantial amount of spontaneous osteoblastogenesis as assessed by von Kossa staining (Fig. 5C) or measurement of calcium deposition (Fig. 5D). Of note, the extracellular calcium content in the cells with knockdown of both C/EBPα and PPARγ was comparable with that caused by ectopic expression of Wnt10b (Figs. 1B and 5D).

To confirm and extend these observations, we introduced these shRNA constructs into MEFs and assessed their adipogenic and osteoblastogenic potential (Fig. 6). All of the constructs efficiently knocked down C/EBPα and PPARγ in this cell model (Fig. 6A). Suppression of C/EBPα, PPARγ, or both factors strongly reduced adipogenic potential (Fig. 6B) and, of note, stimulated osteoblastogenic capacity as assessed by mineralization of extracellular matrix and deposition of calcium (Fig. 6, C and D). Taken together, these results indicate that suppression of C/EBPα or PPARγ alone is sufficient to stimulate osteoblastogenesis in ST2 cells and MEFs.

DISCUSSION

We have found that multipotent cells express adipogenic and osteoblastogenic transcription factors at physiological levels and that constitutive overexpression of Wnt10b changes expression profile of these factors, with suppression of PPARγ and C/EBPα and up-regulation of Runx2, Dlx5, and Osterix (4). Considerable evidence suggests that balance between adipogenic and osteoblastogenic components might be a crucial mechanism for mesenchymal precursor cells to maintain their quiescence and to determine lineage commitment. The molecular balance is modulated by reciprocal inhibitory regulation between these two sets of factors. For example, PPARγ physically interacts with Runx2 and inhibits DNA binding ability of Runx2 (56) and transactivation ability on the osteocalcin promoter (57). Likewise, Msx2 inhibits transcriptional activity of PPARγ (58) and C/EBPα (59), and ectopic expression of Msx2 suppresses adipogenesis (58, 59). Therefore, either suppressing expression of adipogenic transcription factors or increasing expression of osteoblastogenic factors might be sufficient to
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FIGURE 6. Suppression of C/EBPα or PPARγ is sufficient to stimulate osteoblastogenesis in MEFs. A, MEFs were infected with retroviruses expressing shRNAs targeting mRNA sequences for C/EBPα (sh(Cα)), PPARγ (sh(Pγ)), or both CEBPα and PPARγ (sh(Cα + Pγ)). A scrambled RNA was included as a control (sh(-)). The effectiveness and specificity of shRNAs was evaluated by immunoblot analyses for CEBPα, PPARγ, and laminin proteins. Lanes 2 and 3 demonstrate knockdown achieved with two independent shRNAs for C/EBPα. Lanes 4 and 5 show the effects of two independent shRNAs for PPARγ. Two combinations of shRNAs for C/EBPα and PPARγ were achieved by sequential retroviral infection, and the results are shown in lanes 6 and 7. B, cells were stimulated with adipogenic media 1 day after confluence, and lipid accumulation was assessed with Oil Red-O staining 5 days later. At confluence, cells were induced with osteogenic media, and lipid accumulation was assessed with Oil Red-O staining 5 days later. C, cells were stimulated with adipogenic media, and cells were evaluated for mineralized matrix (see arrows) by phase-contrast microscopy (C) and by measuring calcium deposition (D) at day 14. A representative experiment is presented as mean ± S.D. (n = 5). Significant differences compared with controls are depicted with an asterisk (Student’s t test, p < 0.05).

shift mesenchymal cell fate toward the osteoblast lineage. However, which set of gene expression changes is a primary mechanism for Wnt/β-catenin to stimulate osteoblastogenesis has not been investigated prior to this study.

In the current study, we observed that transient activation of Wnt/β-catenin signaling with purified recombinant Wnt3a or a small molecular inhibitor of GSK3 (CHIR99021) rapidly repressed expression of both C/EBPα and PPARγ while not altering osteoblastogenic transcription factors, at least during this relatively early phase of osteoblastogenesis (Fig. 2). This observation suggests that suppression of adipogenic transcription factors by Wnt signaling precedes the induction of osteoblastogenic transcription factors. Increased Runx2, Dlx5, and osterix in cells with retroviral expression of Wnt10b could be the result of differences in strength or duration of signal transduction achieved by constitutive expression of Wnt10b. In fact, mineralization occurred more rapidly and to a larger extent in Wnt10b cells than in ST2 cells treated with CHIR99021 (data not shown).

Of interest, activation of Wnt/β-catenin signaling independently represses expression of C/EBPα and PPARγ even in the presence of constitutive expression of the other (Fig. 3). Consistent with this, enforced expression of either C/EBPα or PPARγ2 decreases mineralizing potential of ST2 cells expressing Wnt10b, suggesting that their repression is required for Wnt signaling to redirect precursor cells toward the osteoblast lineage (Fig. 4).

Although we did not observe that the GSK3 inhibitor CHIR99021 rapidly altered expression of osteoblastogenic transcription factors, Wnt/β-catenin signaling might stimulate osteoblastogenesis by directly affecting activity or expression of these factors at a later stage of differentiation. Supporting this hypothesis, β-catenin stimulates transcription of Runx2 by binding its promoter (7), and β-catenin-deficient embryos have decreased expression of osterix (60). Moreover, PPARγ inhibits DNA-binding activity of Runx2 through direct physical interaction (57). Hence, we tested whether suppression of C/EBPα or PPARγ per se is sufficient to stimulate osteoblastogenesis. Indeed, decreasing expression of either C/EBPα or PPARγ using shRNAs not only greatly reduces lipid accumulation but also causes spontaneous mineralization in ST2 cells and MEFs. Moreover, the extent of spontaneous mineralization by suppressing both C/EBPα and PPARγ is nearly equivalent to that caused by ectopic expression of Wnt10b (Figs. 1A and 5C). These results suggest that C/EBPα and PPARγ play a dominant repressive role in differentiation of multipotential cells into osteoblasts.

Consistent with our findings, the antiosteogenic effect of PPARγ has been evaluated in several in vivo and in vitro studies. For instance, PPARγ haploinsufficient mice exhibit a high bone mass with increased osteoblastogenesis (61). It should be noted that serum leptin levels were not changed in these mice compared with wild type mice, unlike in lipodystrophic PPARγ hyp/hyp mice, due to the absence of PPARγ in adipose tissue (62). Moreover, activation of PPARγ with chronic treatment of thiazolidinediones causes decreased bone mass in mice (63), and similar effects were observed in a small sample of human subjects (64). Furthermore, regulatory factors affecting PPARγ activity or expression are also implicated in osteoblast differentiation. In C3H10T1/2 cells, inhibition of PPARγ activity by pharmacological activation of the histone deacetylase, Sirt1, represses adipogenesis and stimulates osteoblastogenesis (65). Conversely, inhibition of Sirt1 activity causes the opposite cellular phenotypes (65). Suh et al. (66) demonstrated that hedgehog signaling inhibits fat body formation in Drosophila, and ectopic expression of its mammalian homolog in 3T3-L1 cells potently inhibits adipogenesis and induces expression of osteoblastogenic markers, such as Runx2 and osterix. Further, they found that ectopic expression of PPARγ bypasses inhibitory effects of hedgehog signaling (66). Finally, the transcription regulator, TAZ, was recently reported to act as a molecular switch between osteoblastogenesis and adipogenesis by inhibiting PPARγ activity and stimulating Runx2 activity in response to BMP2 (67). However, roles for these factors in mediating effects of Wnt/β-catenin signaling on mesenchymal precursor cell fate have not been reported.

An important question is how Wnt signaling suppresses expression of C/EBPα and PPARγ. Based upon our time course experiment in which transient activation of Wnt signaling suppresses mRNAs for both C/EBPα and PPARγ within 4–8 h (Fig. 2), we speculate that Wnt regulates these adipogenic fac-
tors at the transcriptional level. One possibility is that Wnt inhibits activity of C/EBPβ, which is an early inducible adipogenic transcription factor, and activates transcription of C/EBPα and PPARγ. Phosphorylation is one mechanism to regulate activity of transcription factors, including C/EBPβ. Tang et al. (68) suggest that phosphorylation of C/EBPβ by extracellular signal-regulated kinases and GSK3 is required for transactivation of C/EBPα and/or PPARγ genes. Although the pool of GSK3 that mediates Wnt signaling is thought to be separate from the one transducing signals from growth factors (69), recent studies suggest a connection between the two pools (70–72).

Another possibility is that Wnt/β-catenin inhibits activity of PPARγ. Liu et al. reported that PPARγ physically interacts with β-catenin and facilitates its degradation (73). In addition, they showed that ectopic expression of a β-catenin mutant that avoids phosphorylation by GSK3 inhibits a set of adipocyte genes, including perilipin, adiponectin, and C/EBPα, but not PPARγ (73). As postulated in their work, it is possible that β-catenin inhibits activity of PPARγ, which results in a decrease in transcription of its own expression as well as that of C/EBPα. However, in our study, ectopic expression of PPARγ2 did not prevent suppression of C/EBPα or PPARγ1 (Fig. 3), suggesting that even if this is the case, there is an additional mechanism to suppress transcription of PPARγ. Although it is conceivable that Wnt signaling blocks PPARγ by increasing expression of a transcriptional repressor, this idea is not supported by the decline in PPARγ observed with Wnt3a in the presence of a protein synthesis inhibitor (Fig. 2C).

Although β-catenin is generally considered a transactivator, another possible mechanism is that β-catenin directly represses transcription of C/EBPα and PPARγ. To address this hypothesis, we have tried reporter gene assays after transient transfection; however, we paradoxically observed stimulatory effects of Wnt on C/EBPα and PPARγ promoters (data not shown). This might be because this assay does not adequately reflect chromatin structure of these genes or the promoter fragments analyzed do not contain the Wnt-regulatory regions. Typically, β-catenin activates transcriptional events by disengaging repressors, including the HDAC and TLE complexes, from TCF/LEF and instead recruits co-activators, including p300/CPB and Brg1 (74–76). Therefore, it is possible that Wnt signaling suppresses C/EBPα and PPARγ in part through a TCF/LEF-independent mechanism. From our analyses, ectopic expression of dominant negative TCF is not able to rescue adipogenesis blocked by Wnt10b in in vitro models, although it causes spontaneous preadipocyte differentiation in 3T3-L1 and other preadipocyte models (77). To support this notion, several studies reported that β-catenin regulates transcriptional events by interacting with specific transcription factors other than TCF/LEF, including steroidogenic factor-1 (78, 79), Prop1 (80), androgen receptor (81), and retinoic acid receptor-α (82).

The reciprocal regulation between regulatory factors appears to be an important mechanism for progenitor cells to specify their lineage commitment. When external cues tip the balance among molecular components, precursor cells tilt toward their favored cell lineage. Here, we report that Wnt signaling is an external cue that stimulates osteoblast differentiation at the expense of adipocyte differentiation through inhibition of C/EBPα and PPARγ. Although Wnt/β-catenin signaling has received considerable attention as a potential therapeutic target for osteoporosis, pharmacological activation of this pathway may cause side effects, including increasing susceptibility to cancer. However, further investigation of molecular mechanisms by which Wnt/β-catenin signaling regulates bone formation may identify targets such as C/EBPα and PPARγ for evaluation.

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