Osteoblasts Directly Control Lineage Commitment of Mesenchymal Progenitor Cells through Wnt Signaling*

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Lineage commitment of mesenchymal progenitor cells is still poorly understood. Here we demonstrate that Wnt signaling by osteoblasts is essential for mesenchymal progenitor cells to differentiate away from a default adipogenic into an osteoblastic lineage. Dominant adipogenesis and reduced osteoblastogenesis were observed in calvarial cell cultures from transgenic mice characterized by osteoblast-targeted disruption of glucocorticoid signaling. This phenotypic shift in mesenchymal progenitor cell commitment was associated with reciprocal regulation of early adipogenic and osteoblastogenic transcription factors and with a reduction in Wnt7b and Wnt10b mRNA and β-catenin protein levels in transgenic versus non-transgenic cultures. Transwell co-culture of transgenic mesenchymal progenitor cells with wild type osteoblasts restored commitment to the osteoblast lineage. This effect was blocked by adding sFRP1, a Wnt inhibitor, to the co-culture. Treatment of transgenic cultures with Wnt3a resulted in stimulation of osteoblastogenesis and suppression of adipogenesis. Our findings suggest a novel cellular mechanism in bone cell biology in which osteoblasts exert direct control over the lineage commitment of their mesenchymal progenitor through Wnt signaling. This glucocorticoid-dependent forward control function indicates a central role for osteoblasts in the regulation of early osteoblastogenesis.

Mesenchymal stem cells are able to differentiate to multiple connective tissue and musculoskeletal cell types under the control of specific transcription factors directing their lineage commitment. For example, osteoblasts and adipocytes share a common mesenchymal cell progenitor that can be isolated from neonatal mouse or fetal rat calvaria (1). Primary cell cultures derived from these tissues can differentiate into osteoblasts or adipocytes. The differentiation of these mesenchymal progenitors into osteoblasts or adipocytes is governed by the expression of the master transcription factors runt-related gene 2 (Runx2) (2) or peroxisome proliferator-activated receptor γ (PPARγ) (3). However, the cellular interactions and mechanisms that must drive the expression of these transcription factors are poorly understood.

Glucocorticoid (GC) signaling through its cognate receptor is known to influence osteoblast and adipocyte lineage commitment both in vitro and in vivo and is, thus, likely to have a role in regulating cellular interactions driving cell commitment. In addition, specific enzymes modulate GC metabolism within the cell at the pre-receptor level (4, 5). Within certain tissues, two isoforms of 11β-hydroxysteroid dehydrogenase (11βHSD) vary intracellular GC concentrations independent of circulating GC levels. 11βHSD type 1 (11βHSD1) predominantly converts inactive cortisol to active cortisol to increase intracellular GC concentrations; in contrast, 11βHSD type 2 (11βHSD2) unidirectionally catalyzes the conversion of active GC to their inactive metabolites (4).

Kream and co-workers (6) generated a Col2.3–11βHSD2 transgenic (tg) mouse in which the rat gene for 11βHSD2 was linked to the 2.3-kilobase collagen type I (Col2.3) promoter to target transgene expression to mature osteoblasts (6). The Col2.3 promoter has been well characterized and specifically targets gene expression to mature osteoblasts (7–10). Green fluorescent protein (GFP) transgenic mice using the same fragment of the type I collagen promoter to drive GFP expression (2.3Col-GFP) demonstrated that only cells with features of mature osteoblasts show fluorescence in vivo and in vitro (7, 9).

Microarray analysis data on this population of GFP-positive cells show it to be highly enriched for expression of mature osteoblast-specific genes such as osteocalcin, bone sialoprotein ( BSP), and dentin matrix protein-1 (8). Similarly, immunohistochemistry studies localized transgenic 11βHSD2 protein expression to mature osteoblasts and osteocytes in the bones of transgenic mice with no expression in bones derived from wild type littermates. Transgenic 11βHSD2 enzyme activity was confirmed in vitro by measuring the conversion of [3H]cortisol to 11-dehydrocortisol (11). Because overexpression of HSD2 results in the inactivation of cytoplasmic GCs, GC signaling is, thus, effectively disrupted in mature osteoblasts (11, 12) in Col2.3–11βHSD2 transgenic mice.

Col2.3–11βHSD2 transgenic mice have been shown to exhibit vertebral osteopenia in females (11), reduced femoral cortical bone area and thickness, and impaired mineralized nodule formation in primary calvarial cultures. However, the mechanism underlying these phenotypic changes remained
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unclear (12). Others have targeted 11βHSD2 overexpression to osteoblasts/osteocytes with the osteocalcin gene 2 promoter. These transgenic mice had no discernible bone phenotype, but osteocyte viability was protected during GC treatment (13).

In the present study using primary calvarial cell cultures derived from the Col2.3–11βHSD2 tg mice, we discovered a novel mechanism in which fully differentiated osteoblasts control lineage commitment and differentiation of mesenchymal progenitor cells. We find that GC-regulated Wnt signaling is a strong candidate for mediating this function.

EXPERIMENTAL PROCEDURES

Transgenic Mice—Col2.3–11βHSD2 transgenic mice were generated as described previously (11) and were provided as a gift by Dr. Barbara Kream (Dept. of Medicine, University of Connecticut Health Center, Farmington, CT). Col2.3-GFP mice (7) were provided as a gift by Dr. David Rowe (Dept. of Genetics and Developmental Biology, University of Connecticut Health Center). Mice were maintained at the animal facilities of the ANZAC Research Institute (Sydney, Australia) in accordance with Institutional Animal Welfare Guidelines and according to an approved protocol.

Primary Calvaria Cell Culture—Primary calvaria cells were generated from 1-day-old wild type (WT) or Col2.3–11βHSD2 tg littermates. Cells were released by 4 sequential 10-min digestions with 0.1% collagenase (Worthington Biomedical Co., Lakewood, NJ) and 0.2% dispase (Invitrogen). Cell populations from digestions 2 to 4 were collected and pooled (P2–4). For mineralized nodule formation assays, cells were cultured in 24-well plates at a density of $1 \times 10^5$/well in α-minimum essential medium (Invitrogen) containing 10% fetal bovine serum (FBS) and cortisol at a concentration of $8 \times 10^{-8}$ M (derived from the FBS). Cells were allowed to attach for 24 h, and then osteogenic conditions were provided to induce osteoblast differentiation (designated day0) by adding ascorbic acid (50 μg/ml; Sigma) and β-glycerophosphate (10 mM; Sigma). Cells were incubated for 14 days with fresh medium provided three times weekly.

Transwell Co-culture—Calvaria cells were isolated by sequential enzyme digestion yielding four populations (1). Cells collected from the first digestion, designated population 1 (P1), were cultured in 24-well plates. Cells collected from third and fourth digestions were pooled and designated as populations 3 and 4 (P3–4). This population or cells was cultured in transwell inserts (pore size 3 μm) of the eluate were counted in each well in a standard area of 157 mm$^2$. Staining for mineralized nodules. Adipocyte lipid was assessed by staining cells with Oil Red O (Sigma) after fixation in 4% paraformaldehyde. The Oil Red O-stained adipocytes were counted in each well in a standard area of 157 mm$^2$. Staining by Oil Red O was further quantified by spectrophotometry. Briefly, the stained dishes were rinsed free of residue stain and eluted with 1 ml of 100% isopropanol for 20 min at room temperature. Three aliquots (100 μl) of the eluate were transferred to a 96-well plate and quantified by absorbance measurement at 490 nm (PerkinElmer Life Sciences). The linear range of detection was determined by standard solutions of Oil Red O.

Wnt3a-conditioned Medium—Cell lines of murine subcutaneous connective tissue-derived L-fibroblasts permanently transfected with Wnt3a (L-Wnt3a) or an empty vector (L) were obtained from American Type Culture Collection (ATCC; Manassas, VA). Control and Wnt3a-conditioned media were prepared from L- and L-Wnt3a cells, respectively, as described by the ATCC. Briefly, cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum for 3 days, after which conditioned medium was collected, filter-sterilized, and stored at −70 °C until further use.

RT-PCR and Real-time RT-PCR—Total RNA was isolated from primary mouse calvaria cell cultures of populations P1 and P2–4 using NucleoSpin (Machery-Nagel, Easton, PA) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 2 μg of total RNA by incubating for 1 h at 50 °C with Superscript III reverse transcriptase (Invitrogen) after oligo(dT) priming. The sequences of primers used are listed in Table 1. PCR was conducted for 20–30 cycles, each of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Ten microliters of each reaction mixture was mixed by 2% agarose gel electrophoresis. Real-time RT-PCR was carried out using IQ SYBR Green Supermix (Bio-Rad) according to manufacturer’s instructions using a Bio-Rad iCycler iQ5 real-time PCR detection system. 18 S was used for cDNA normalization. All primers sequences are listed in Table 1.

Western Blotting—The total cellular protein levels of β-catenin were detected as described (15) with minor modifications. Briefly, primary calvaria cells derived from 1-day-old WT and tg mice were cultured in osteogenic media for 7 days. Total cellular protein was isolated from cell lysates in lysis buffer (1% Triton X-100, 40 mM KCl, 25 mM Tris, pH 7.4, protease inhibitor, Roche Applied Science). To isolate nuclear extract (16), cells were lysed in ice-cold lysis buffer, cell lysates were passed through a 27-gauge needle and centrifuged at 8000 × g for 3 min, and the supernatant was recovered as the cytoplasmic fraction. The remaining pellet containing the nuclear fraction was spun through an additional 15 min at 15,000 × g, washed with lysis buffer, and resuspended in sucrose buffer (250 mM sucrose, 20 mM Tris, pH 7.4, and protease inhibitor). Both cellular and nuclear proteins were separated by SDS-PAGE (7.5% polyacrylamide) and transferred onto nitrocellulose filters. The filters were immunoblotted with anti-β-catenin antibody (BD Biosciences) at a 1:500 dilution or anti-β-actin antibody (BD Biosciences) at a 1:2000 dilution as an internal control. Protein analysis was performed using image analysis software (Image), National Institutes of Health) to determine the number and area of mineralized nodules. Adipocyte lipid was assessed by staining cells with Oil Red O (Sigma) after fixation in 4% paraformaldehyde. The Oil Red O-stained adipocytes were counted in each well in a standard area of 157 mm$^2$. Staining by Oil Red O was further quantified by spectrophotometry. Briefly, the stained dishes were rinsed free of residue stain and eluted with 1 ml of 100% isopropanol for 20 min at room temperature. Three aliquots (100 μl) of the eluate were transferred to a 96-well plate and quantified by absorbance measurement at 490 nm (PerkinElmer Life Sciences). The linear range of detection was determined by standard solutions of Oil Red O.
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TABLE 1
Mouse-specific primer pairs used for RT-PCR and real-time RT-PCR

| Gene      | Forward Primer                                      | Reverse primer                                      |
|-----------|------------------------------------------------------|------------------------------------------------------|
| 11βHSD2   | 5′−CTGACTGACCTCCAAATGCTGG-3′                       | 5′−CTTCCCGAGAGTCACATTT-3′                           |
| 11βHSD1   | 5′−GTCGGAGCTTCTGCACCGAG-3′                         | 5′−GGCTCCAGAGTAAGATGAG-3′                           |
| SGK       | 5′−GACAAGCTTCTCCAGACGGC-3′                         | 5′−CCAGCTTCGAGAGCTAT-3′                            |
| ALP       | 5′−TGAAATCGCTTTTCCGCAAGC-3′                        | 5′−TCTGTCGATCCACACTTTG-3′                          |
| BSP       | 5′−TTGCTGCTGACCACACGAC-3′                          | 5′−TCCAGGCAGGACACCG-3′                             |
| Osteocalcin| 5′−CAGGAGCTTGATGACATCTC-3′                        | 5′−CCAGGAGCTTGATGACATCTC-3′                        |
| Runx-2    | 5′−CGAAGGCTGGGAGGTGGTC-3′                          | 5′−GGGAGGACGAGCTCATCC-3′                           |
| PPARγ     | 5′−GCTGTCGATCCACACTTTG-3′                          | 5′−TCCAGGCAGGACACCG-3′                             |
| C/EBPα    | 5′−AGCACACCTTCGGAGAGAATCTT-3′                      | 5′−AGTAGAAGACCCTGAGAAC-3′                          |
| Wnt16b    | 5′−GCCAGCTTCGAGAGCTAT-3′                           | 5′−TTGGAAGCTGTCGAGACATTT-3′                        |
| TCF7      | 5′−AGTCAGTTGCTGAGACATTT-3′                         | 5′−AGAGTTGGAGGATGGTCTGC-3′                         |
| BMP2      | 5′−CTGACTGACCTCCAAATGCTGG-3′                       | 5′−TTTGGCAAGGAGACAGCTAA-3′                         |
| BMP4      | 5′−AGCAAGGCTGGGAGGTGGTC-3′                          | 5′−TTCTCCCAGAGGTTCACATT-3′                         |
| SOST      | 5′−CCAGGAGCTTGATGACATCTC-3′                        | 5′−TTTGGCAAGGAGACAGCTAA-3′                         |
| GR        | 5′−TTCTCCCAGAGGTTCACATT-3′                         | 5′−TTTGGCAAGGAGACAGCTAA-3′                         |
| 18 S      | 5′−CTGACTGACCTCCAAATGCTGG-3′                       | 5′−TTTGGCAAGGAGACAGCTAA-3′                         |

RESULTS

Osteoblast Differentiation Is Inhibited, and Adipocyte Differentiation Is Increased in Col2.3–11βHSD2 tg Culture—Primary osteoblast cultures were generated from the calvaria of 1-day-old Col2.3–11βHSD2 tg mice and WT littermates and grown under osteogenic conditions (see “Experimental Procedures”). Cultures derived from Col2.3–11βHSD2 tg mice exhibited a 60% reduction in mineralized nodule formation when compared with WT cultures (Fig. 1, A and B). In the same cultures, a significant increase in adipocyte numbers was observed when compared with WT cultures, in which only a few adipocytes formed (Fig. 1C). The Oil Red O content of cultures was found to be at least 2 times higher in tg cultures compared with WT cultures (Oil Red O elution A490; WT 0.21 ± 0.01, tg 0.47 ± 0.03, p < 0.001). In light of the fact that the transgene is exclusively expressed in mature osteoblasts (7, 11), these results indicate that early precursor cells derived from transgenic, 11βHSD2-overexpressing animals experience a major shift in lineage commitment from osteoblast to adipocyte.

The Shift in Lineage Commitment Is Due to Disrupted GC Signaling in Mature Osteoblasts—11βHSD2 mRNA expression was only detected in Col2.3–11βHSD2 tg cultures. WT cultures expressed neither rat transgene nor native mouse 11βHSD2 (primers used could detect both rat transgene and native mouse 11βHSD2). Increased 11βHSD2 expression levels were observed in tg cultures from day 3 to day 7; this finding is consistent with the expected increase in the number of differentiated osteoblasts during culture (Fig. 2, A and B). mRNA for 11βHSD1 was expressed at very low levels and similarly in tg and WT cultures, with a minor increase at day 7. Thus, 11βHSD2 is the major enzyme regulating GC signaling in cultures derived from Col2.3–11βHSD2 animals, and the expression of 11βHSD1 is not regulated by 11βHSD2. mRNA for the glucocorticoid receptor was similarly expressed in both WT and tg cultures and was not regulated by 11βHSD2 (data not shown). The increase in 11βHSD2 mRNA was accompanied by a reciprocal decrease in mRNA for serum and glucocorticoid-induced kinase, a GC target gene (17), indicating that GC signaling is attenuated in Col2.3–11βHSD2 tg cultures (Fig. 2C).

Thiram, a dithiocarbamate that inhibits 11βHSD2 but not 11βHSD1 enzyme activity (18) was added to WT and tg cultures. At a concentration of 10−10 M, Thiram increased mineralized nodule formation to levels seen in WT cultures. In the same cultures, Thiram dose-dependently inhibited adipocyte formation (Fig. 2F). Thiram had no effect on WT cultures at any concentration used (Fig. 2, D–F). Hence, the Col2.3–11βHSD2 tg culture phenotype could be completely reversed by blocking 11βHSD2 enzyme activity in the culture system. Taken together, these results indicate that the shift in lineage commit-

expression was visualized with a peroxidase-labeled sheep antimal mouse secondary antibody by enhanced chemiluminescence detection reagents (Amersham Biosciences) and by exposure to x-ray film.

Statistical Analysis—Data are represented as the means ± S.E. of the mean, and statistical analysis was performed with Student’s t test. For multiple comparisons the p value was adjusted using the Bonferroni method. A p value of less than 0.05 was considered statistically significant.
ment from osteoblast to adipocyte in tg cultures is due to disrupted GC signaling in mature osteoblasts overexpressing 11βHSD2.

Corticosterone or dexamethasone, substrates for 11βHSD2 (19, 20), were added to tg and WT cultures at concentrations of 10^{-10}–10^{-7} M. As expected, neither steroid had any effect at low concentrations (10^{-12}–10^{-8} M), but each inhibited mineralized nodule formation at a high concentrations (10^{-7} M) in both WT and tg cultures (data not shown).

Transcription Factors for Osteoblasts and Adipocytes Are Reciprocally Regulated in Col2.3–11βHSD2 Transgenic Culture—

RNA was isolated from primary calvaria cell cultures (populations 2–4) in osteogenic media at the indicated times for RT-PCR analysis of the osteoblast markers alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OC). RT-PCR and real-time RT-PCR analyses were performed for osteoblast (Runx2) and adipocyte (PPARγ and C/EBPα) transcription factors (*, p < 0.05). A and B, RT-PCR analysis. C, real-time PCR quantitation of relative mRNA expression levels, normalized to 18 S expression, for Runx2, PPARγ, and C/EBPα. D, relative mRNA expression levels for Twist1, Twist2, Msx2, Dlx5, and Osterix (Osx) in WT and tg cultures after 7 days of osteogenic differentiation (*, p < 0.05).

A–C, primary calvaria cells (populations 2–4) derived from WT and tg mice were cultured in osteogenic media at the indicated times. A, RT-PCR analysis of 11βHSD1 and 11βHSD2 mRNA expression in WT and tg cultures. B and C, real-time PCR analysis of 11βHSD2 (B) and serum and glucocorticoid-induced kinase (SGK) (C) mRNA expression of relative mRNA expression levels normalized to 18 S expression. Serum and glucocorticoid-induced kinase (SGK) expression in tg cultures are shown as percentage of SGK expression in WT cultures (*, p < 0.05). D, primary calvaria cells derived from WT and tg mice were cultured in osteogenic media in the absence or presence of Thiram at a dose range of 10^{-12}–10^{-9} M for 14 days. Me_{2}SO (0.1%) treatment was carried out as a vehicle control. Cells were stained with alizarin red to visualize mineralized nodule formation. E, quantitative analysis of mineralized nodule formation assays (*, p < 0.05 versus tg control). F, Oil Red O-stained adipocytes were counted in each well in a standardized area of 157 mm², and results were expressed as number of adipocyte/mm² (*, p < 0.05 versus tg control).

FIGURE 2. 11βHSD2 transgene was expressed and functional in Col2.3–11βHSD2 tg primary calvarial cell cultures. A–C, primary calvaria cells (populations 2–4) derived from WT and tg mice were cultured in osteogenic media at the indicated times. A, RT-PCR analysis of 11βHSD1 and 11βHSD2 mRNA expression in WT and tg cultures. B and C, real-time PCR analysis of 11βHSD2 (B) and serum and glucocorticoid-induced kinase (SGK) (C) mRNA expression of relative mRNA expression levels normalized to 18 S expression. Serum and glucocorticoid-induced kinase (SGK) expression in tg cultures are shown as percentage of SGK expression in WT cultures (*, p < 0.05). D, primary calvaria cells derived from WT and tg mice were cultured in osteogenic media in the absence or presence of Thiram at a dose range of 10^{-12}–10^{-9} M for 14 days. Me_{2}SO (0.1%) treatment was carried out as a vehicle control. Cells were stained with alizarin red to visualize mineralized nodule formation. E, quantitative analysis of mineralized nodule formation assays (*, p < 0.05 versus tg control). F, Oil Red O-stained adipocytes were counted in each well in a standardized area of 157 mm², and results were expressed as number of adipocyte/mm² (*, p < 0.05 versus tg control).

FIGURE 3. Transcription factors for osteoblast and adipocytes were reciprocally regulated in Col2.3–11βHSD2 tg primary calvarial cell cultures. RNA was isolated from primary calvaria cell cultures (populations 2–4) in osteogenic media at the indicated times for RT-PCR analysis of the osteoblast markers alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OC). RT-PCR and real-time RT-PCR analyses were performed for osteoblast (Runx2) and adipocyte (PPARγ and C/EBPα) transcription factors (*, p < 0.05). A and B, RT-PCR analysis. C, real-time PCR quantitation of relative mRNA expression levels, normalized to 18 S expression, for Runx2, PPARγ, and C/EBPα. D, relative mRNA expression levels for Twist1, Twist2, Msx2, Dlx5, and Osterix (Osx) in WT and tg cultures after 7 days of osteogenic differentiation (*, p < 0.05).
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We then isolated the P1 cell population from Col2.3–11βHSD2 tg mice and their WT littermates and found that these cells did not form mineralized nodules in either WT or Col2.3–11βHSD2 tg cultures after 21 days (Fig. 4, B and C). In contrast, cells from P3–4 populations formed many mineralized nodules in WT and tg cultures, although numbers were significantly lower in tg cultures (data not shown). P1 cells were then co-cultured with P3–4 cells of the same or reciprocal genotype seeded in the transwell inserts (Fig. 4B). P3–4 cells from WT mice induced nodule formation in cultures of both WT and Col2.3–11βHSD2 tg P1 cells. P3–4 cells from Col2.3–11βHSD2 tg mice failed to induce mineralized nodules in either WT or tg P1 cells (Fig. 4, B and C). P1 cells, without co-culture, readily differentiated into adipocytes at comparable levels in both WT and Col2.3–11βHSD2 tg cultures (Fig. 4D). These results clearly demonstrate that osteoblastic cells from WT mice produce soluble factor(s) that act on the precursor cells to promote osteoblastogenesis and inhibit adipogenesis. Although P3–4 cells from Col2.3–11βHSD2 tg mice failed to induce osteoblast differentiation (Fig. 4, B and C), a significant reduction in the number of adipocytes was observed in both WT and Col2.3–11βHSD2 tg P1 cells (Fig. 4D), although this effect was reduced relative to that seen with WT P3–4 cells.

P1 cells derived from WT or Col2.3–11βHSD2 tg mice cultured alone formed similar numbers of adipocytes and had similar numbers of mineralized nodules induced by WT P3–4 cells (Fig. 4, C and D). This again suggests that both WT and Col2.3–11βHSD2 tg cultures consisted of similar cell populations and that P1 cells from WT and tg mice had the same potential to differentiate into either adipocytes or osteoblasts, depending on the strength of the signals received.

Osteoblasts Control the Fate of Their Mesenchymal Precursors via the Canonical Wnt Signaling Pathway—The Wnt family consists of a large number of secreted glycoprotein-signaling molecules that are involved in regulating cell differentiation (22, 23) and plays an important role in regulating osteoblast differentiation (24–27). In particular, Wnt10b has recently been reported to promote osteoblastogenesis and inhibit adipogenesis (28, 29). These data led us to investigate proteins of the Wnt family as potential signals between osteoblasts and their mesenchymal progenitors. We assessed mRNA expression of the known Wnts, and except for Wnt10b and Wnt7b, all were found to be either not expressed (e.g. Wnt1, Wnt3a, Wnt9a, and Wnt16) or not regulated (e.g. Wnt5a) during osteoblast differentiation (data not shown).

The expression of Wnt10b and Wnt7b mRNA increased with time in WT cultures, reaching high expression levels at day 7 (Fig. 5, A and B). In contrast, mRNA expression levels of Wnt10b and Wnt7b remained at basal levels in Col2.3–11βHSD2 tg cultures. Interestingly, the expression level of sFRP1, a decoy receptor for Wnt proteins and competitive Wnt inhibitor, was increased in Col2.3–11βHSD2 tg cultures when compared with WT cultures (Fig. 5B). Taken together, Wnt-mediated signaling in Col2.3–11βHSD2 tg cultures was decreased by both reduced expression of Wnts and increased expression of a Wnt inhibitor.
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FIGURE 5. Wnt expression and signaling is attenuated in tg cultures. A and B, RNA was isolated from primary calvaria cell cultures (populations 1 and 2–4) under osteogenic conditions at the indicated times for RT-PCR and quantitative RT-PCR analysis. A, RT-PCR analysis of Wnt10b expression. B, real-time PCR quantitation of relative mRNA expression levels for Wnt7b and sFRP1 after normalization for 18S expression (*, p < 0.05). C, real-time PCR quantitation of relative mRNA expression levels for Wnt10b in population 1 (P1) and populations 3–4 (P3–4) after normalization for 18S expression (*, p < 0.05). D, Western blot analysis of β-catenin protein in the total cellular and nuclear levels in primary calvaria cell cultures at day 7 under osteogenic conditions. β-Actin is used for total protein loading normalization. E, real-time RT-PCR analysis of TCF1 mRNA in primary calvaria cultures (P2–4 cells) under osteogenic conditions (*, p < 0.05).

To compare the mRNA levels of Wnt7b and Wnt10b in P1 cells and P3–4 cells, RNA was isolated from P1 and P2–4 cells cultured under osteogenic conditions. In both tg and WT cultures, Wnt10b mRNA was expressed at low and similar levels in P1 cells, with further decreasing levels from day 3 onwards. In contrast, Wnt10b mRNA levels increased over time in WT but not tg P3–4 cells. At day 7, mRNA for Wnt10b was 13-fold higher in P3–4 cells compared with P1 cells in WT cultures (Fig. 5C).

Using real-time PCR, Wnt7b mRNA was not detected in P1 cells of both WT and tg cultures at any time point (data not show). These results indicate that in the co-culture system employed here, mature osteoblast served as a source for Wnts. Wnt7b and Wnt10b are both canonical Wnts that have the ability to stabilize β-catenin and to activate lymphoid enhancer binding factor/T cell factor (TCF)-mediated gene transcription (30–32). The total cellular and nuclear protein levels of β-cate-

FIGURE 6. Evidence that osteoblastic cells directly regulate precursor cell commitment via canonical Wnt signaling, promoting osteoblastogenesis and inhibiting adipogenesis. A and B, primary calvaria cell cultures (populations 2–4) were derived from WT andtg mice and cultured in osteogenic media in the presence of 5% control CM or 5% Wnt3a CM for 14 days. Cultures were stained with alizarin red for mineralized nodules or with Oil-Red-O for adipocytes. A, mineralized nodules were quantified using ImageJ (*, p < 0.05 WT versus tg cultures; #, p < 0.05 tg cultures treated with control CM versus Wnt3a treatment). There is no statistically significant difference in the number of mineralized nodules formed in response to Wnt3a treatment when comparing WT and tg cultures. B, adipocyte numbers were counted in a standard field (*, p < 0.05, comparisons as indicated). C and D, P1 cells from WT mice were cultured in 24-well plates alone or co-cultured with P3–4 cells from WT in transwell inserts and treated with or without 200 ng/ml recombinant sFRP1. The transwell inserts were removed after 3 weeks of culture in osteogenic medium, and the P1 cells cultured at the bottom of the wells were stained with alizarin red for mineralized nodules (C) or with Oil Red O for adipocytes (not shown). D, quantitative analysis of adipocyte numbers (*, p < 0.05, comparisons as indicated).

To assess whether tg cultures can be “rescued” by activating canonical Wnt signaling, exogenous Wnt3a was added into WT and tg cultures. The addition of 5% Wnt3a-conditioned medium (CM) to tg cultures increased mineralized nodule formation 10-fold, whereas adipocyte formation was strongly inhibited (Fig. 6, A and B). In contrast, the addition of 5% Wnt3a CM to WT cultures resulted in a non-significant increase in mineralized nodule formation when compared with control media (Fig. 6A). To further demonstrate that the soluble factors produced by mature osteoblasts are indeed Wnts, we treated the transwell co-cultures with added recombinant sFRP1, a Wnt inhibitor. As shown in Fig. 6, C and D, WT P3–4 cells induce osteoblastogenesis and bone nodule formation and inhibit adipogenesis in P1 cells. However, the addition of sFRP1 blocked osteoblastogenesis and bone nodule formation and partially restored adipogenesis in P1 co-cultured with transwell, whereas it had no effect on P1 cells without transwell. Conversely, we have treated P1 cells with Wnt3a and find that this inhibits adipogenesis and stimulates mineralized nodule formation (data not shown). These results strongly support our conclusion that the soluble factors produced by mature osteo-
blasts to control mesenchymal lineage commitment are Wnt proteins.

**DISCUSSION**

In this paper we describe the discovery of a previously unknown regulatory function of mature osteoblasts in directing lineage commitment of early mesenchymal progenitor cells via activation of the canonical Wnt signaling pathway. These findings provide clear evidence for the pivotal role of mature osteoblasts in regulating early osteoblastogenesis.

We have demonstrated that, among cells isolated from neonatal mouse calvaria, osteoblasts play a central role in directing cell lineage commitment of early mesenchymal progenitors. The P1 population of calvarial cells (from either WT or Col2.3–11βHSD2 tg mice) formed numerous adipocytes but no mineralized nodules when cultured under osteogenic conditions even though osteoblast precursors were present. In WT cultures containing a mixed population of immature precursors and mature osteoblasts, mineralized nodules formed readily under osteogenic conditions, with very few adipocytes present. In contrast, cultures from transgenic mice, in which intracellular GC signaling was disrupted exclusively in mature osteoblasts, mineralized nodule formation was profoundly impaired, and adipocyte formation was enhanced. These results suggest that calvarial cell cultures, by default, mesenchymal precursors differentiate into adipocytes; however, in the presence of mature osteoblasts, progenitor lineage commitment is changed with promotion of osteoblast and inhibition of adipocyte differentiation. These findings explain the earlier observation that calvarial cells derived from Runx2−/− mice do not differentiate into osteoblasts under osteogenic conditions but instead form numerous adipocytes (35).

We further demonstrated that in transwell co-culture experiments, osteoblasts from WT mice drive precursor differentiation toward the osteoblast phenotype while inhibiting adipogenesis; in contrast, osteoblasts from Col2.3–11βHSD2 tg mice, in which GC signaling is abrogated, failed to elicit this response. These findings provide evidence for the existence of a direct signaling pathway between osteoblasts and their precursors; specifically, the differentiated cell population appears to produce a soluble factor that induces differentiation of precursors along the osteoblast lineage while simultaneously suppressing programmed differentiation to adipocytes.

Notably, the mineralized nodules induced by cells cultured in the transwell were located in close spatial proximity to the center of the transwell (Fig. 4B). Conversely, adipocyte formation was suppressed most profoundly under the center of the transwell, whereas this effect diminished toward the edges of the culture well. These observations point to a concentration gradient of the soluble factor(s) released from the transwell-resident cells into the culture.

There is good evidence for the important role of Wnt proteins in the regulation of bone formation (24–27). For example, loss-of-function mutations in the Wnt co-receptor low density lipoprotein receptor-related proteins 5 (LRP5) are associated with osteoporosis pseudoglioma syndrome in humans (36) and LRP5−/− mice exhibit low bone mass due to suppressed osteoblast proliferation and activity (37). Activation of canonical Wnt signaling by Wnt1, Wnt3a, or Wnt10b or by an activating mutation in low density lipoprotein receptor-related proteins 5 stimulates osteoblastogenesis (26, 28, 38–40).

Inactivation of this pathway by the Wnt antagonists sFRP1 or dickkopf 1 (Dkk1) reduces osteoblastogenesis (41). Furthermore, during early skeletal development Wnt/β-catenin signaling induces osteoblastic and suppresses chondrocytic differentiation (25, 42). Importantly, activation of Wnt/β-catenin signaling also inhibits adipogenesis of mesenchymal precursors (28, 29). Despite all the evidence of a role for Wnts in osteoblastogenesis, the specific cellular source of Wnts remained unknown. Our data now identify the mature osteoblast as an important source of Wnt7b and Wnt10b, indicating that a primary role for these secreted canonical Wnts is to control mesenchymal precursor lineage commitment.

Compared with WT cultures, the expression of both Wnt10b and Wnt7b mRNA was reduced in tg cultures, whereas at the same time the expression of sFRP1, a Wnt inhibitor, was increased. Furthermore, the total cellular and nuclear β-catenin protein levels and TCF1 mRNA expression were lower in tg than in WT cultures, indicating that Wnt canonical signaling was attenuated in tg cultures. These changes were associated with impaired mineralized nodule formation in tg cultures. Activation of canonical Wnt signaling by the addition of Wnt3a to tg cultures restored normal mineralized nodule formation while simultaneously inhibiting adipocyte formation. Furthermore, treatment with recombinant sFRP1 completely blocked osteoblast-induced mineralized nodule formation of P1 cells in transwell culture, confirming that GC-dependent Wnt expression is a major signaling pathway in osteoblasts. Taken together, these results clearly support a role for Wnts acting through the canonical pathway in the regulation of precursor lineage commitment by mature osteoblasts.

Bone morphogenetic proteins (BMPs) and sclerostin (SOST) are also candidate soluble regulators of osteoblast differentiation (43); BMPs promote osteoblast differentiation (44, 45), and SOST is a circulating Wnt inhibitor expressed almost exclusively in osteocytes (46). In the present study BMP2 and BMP4 expression levels were not significantly different in WT and Col2.3–11βHSD2 tg cultures, and the addition of Noggin, a specific BMP inhibitor, did not block nodule formation in WT cultures (data not shown). SOST mRNA was expressed at extremely low levels with, again, no detectable difference between WT and tg cultures at day 7 (data not shown). We, therefore, believe that in the present culture system, BMPs and SOST are unlikely to mediate the actions of WT mature osteoblasts on precursor differentiation.

The impairment of mineralized nodule formation and the increase in adipogenesis seen in Col2.3–11βHSD2 tg cell cultures indicates that the regulatory actions of mature osteoblasts on their mesenchymal progenitor cells are glucocorticoid-dependent. The source of GC in our calvaria cell cultures is cortisol at a concentration of $8 \times 10^{-9}$ M, derived from 10% fetal bovine serum added to culture medium. Treatment with added corticosterone or dexamethasone, both substrates for 11βHSD2, did not restore nodule formation in tg cultures. This is not unexpected, as low GC concentrations ($10^{-10}$–10$^{-8}$ M) may not overcome inactivation by the overex-
pressed enzyme, 11βHSD2, whereas we found that higher concentrations inhibited mineralized nodule formation in both WT and tg cultures, indicating the effect of GC at concentration-dependent. In this respect our findings are consistent with those of Leclerc et al. (47), demonstrating that dexamethasone was inhibitory to osteoblast only at pharmacological levels (10^{-7}, 10^{-6} M), whereas concentrations in the physiological range (10^{-8} M) were stimulatory. Second, pharmacological levels of GC impact all cells at all stages of differentiation, whereas we have modulated GC signaling only in more differentiated cells. Thus, GC may have divergent effects on mesenchymal cells depending on their stage of differentiation. In contrast, specifically blocking transgenic enzyme activity in tg osteoblasts with Thiram dose-dependently reversed the tg culture phenotype with no effect in WT culture, confirming that the tg culture phenotype was due to disrupted GC signaling in mature osteoblasts and was not an epiphenomenon of transgene insertion. It is interesting to note that the inhibition of mineralized nodule formation by dexamethasone at high concentrations parallels the clinical situation where sustained exposure to pharmacologic levels of GC in patients is associated with impaired bone formation (48, 49).

Based on our observations, we propose a model for osteoblast-directed lineage commitment of mesenchymal progenitor cells via glucocorticoid-dependent Wnt signaling (Fig. 7A). In this model, glucocorticoids stimulate osteoblasts to increase secretion of Wnts and to decrease secretion of the Wnt inhibitor, sFRP1; both actions would advance canonical Wnt signaling in mesenchymal progenitor cells. As a result, β-catenin enters the nucleus and increases Runx2 while inhibiting PPARγ expression, promoting osteoblastogenesis but inhibiting adipogenesis. When glucocorticoid signaling is disrupted in osteoblasts, as is the case in Col2.3–11βHSD2 tg cultures, canonical Wnt signaling is attenuated by decreased Wnt and increased sFRP1 levels. This in turn will direct lineage commitment of mesenchymal progenitor cells toward the adipocyte lineage (Fig. 7B). The mechanisms of maintenance and regulation of stem cell and early progenitor cell populations and their commitment to specific lineages is an important area of research with implications for many diseases. The theme identified in this study of active regulation of these processes by more differentiated cells of the same lineage bears a resemblance to mechanisms found in other systems. For example, in growth plate cartilage, cross-talk between mature hypertrophic chondrocytes and early chondrocyte precursors via parathyroid hormone-related protein (PTHrP) and Indian Hedgehog has been shown essential for normal limb growth (50, 51). Osteoprogenitors and adipocyte progenitors originate from common mesenchymal cells, and their differentiation is regulated reciprocally (52, 53). In vivo, an inverse relationship between the number of osteoblasts and bone marrow adipocytes has been demonstrated with age-related bone loss often associated with increased adipogenesis (54, 55). These observations are consistent with the clinical finding that bone loss and osteoporosis are associated with increased marrow adiposity (53). Our new observations may identify the relevant mechanism for the reciprocal changes in adipocyte and osteoblast numbers seen in aged bone. Because numbers of mature osteoblasts decrease with aging, the strength of their paracrine signal would decline, further decreasing osteoblast differentiation and permitting enhanced adipogenesis. These mechanisms would suggest that osteoporosis and bone fracture repair could be limited by insufficient osteoblast-mediated signaling in addition to inadequate recruitment of mesenchymal precursors to sites of bone formation and fracture. Treatment of non-union of bone fractures using stem cell treatments may be more effective if coupled
with gene therapies or treatments with paracrine factors to produce commitment to the osteoblast lineages.

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