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Wnt/β-catenin pathway regulates bone morphogenetic protein (BMP2)-mediated differentiation of dental follicle cells

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Background and Objective: Bone morphogenetic protein 2 (BMP2)-induced osteogenic differentiation has been shown to occur through the canonical Wnt/βcatenin pathway, whereas factors promoting canonical Wnt signaling in cementoblasts inhibit cell differentiation and promote cell proliferation *in vitro*. The aim of this study was to investigate whether putative precursor cells of cementoblasts, dental follicle cells (murine SVF4 cells), when stimulated with BMP2, would exhibit changes in genes/proteins associated with the Wnt/β-catenin pathway.

Material and Methods: SVF4 cells were stimulated with BMP2, and the following assays were carried out: (i) Wnt/β-catenin pathway activation assessed by western blotting, β-catenin/transcription factor (TCF) reporter assays and expression of the lymphoid enhancer-binding factor-1 (*Lef1*), transcription factor 7 (*Tcf7*), Wnt inhibitor factor 1 (*Wif1*) and Axin2 (*Axin2*) genes; and (ii) cementoblast/osteoblast differentiation assessed by mineralization *in vitro*, and by the mRNA levels of runt-related transcription factor 2 (*Runx2*), osterix (*Osx*), alkaline phosphatase (*Alp*), osteocalcin (*Ocn*) and bone sialoprotein (*Bsp*), determined by quantitative PCR after treatment with wingless-type MMTV integration site family, member 3A (WNT3A) and knockdown of β-catenin.

Results: WNT3A induced β-catenin nuclear translocation and up-regulated the transcriptional activity of a canonical Wnt-responsive reporter, suggesting that the Wnt/β-catenin pathway functions in SVF4 cells. Activation of Wnt signaling with WNT3A suppressed BMP2-mediated induction of cementoblast/osteoblast maturation of SVF4 cells. However, β-catenin knockdown showed that the BMP2-induced expression of cementoblast/osteoblast differentiation markers requires endogenous β-catenin. WNT3A down-regulated transcripts for *Runx2*, *Alp* and *Ocn* in SVF4 cells compared with untreated cells. In contrast, BMP2 induction of *Bsp* transcripts occurred independently of Wnt/β-catenin signaling.

Conclusion: These data suggest that stabilization of β-catenin by WNT3A inhibits BMP2-mediated induction of cementoblast/osteoblast differentiation in SVF4 cells, although BMP2 requires endogenous Wnt/β-catenin signaling to promote cell maturation.

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Considerable attention has been paid to defining the key regulators of dental follicle cells because of the major role of these cells in the process of tooth eruption (1–3) as well as the substantial evidence that these cells are progenitors of periodontal cells (i.e. cementoblasts, periodontal ligament fibroblasts, and alveolar bone osteoblasts] (4,5). To date, the specific mechanisms and factors controlling follicle cell behavior during tooth eruption and formation of the periodontium remain undefined.

The ability of bone morphogenetic protein 2 (BMP2) to promote osteogenic differentiation suggests that this protein is a promising candidate for stimulating follicle cell maturation along a "cementoblast/osteoblast" pathway. Previous studies showed that BMP2 induced differentiation of murine follicle cells *in vitro*, towards a cementoblast/osteoblast phenotype characterized by the expression of markers associated with both early [Core-binding factor alpha-1/runt-related transcription factor 2 (RUNX2)] and later [bone sialoprotein (BSP) and osteocalcin (OCN)] phases of maturation, and by promoting mineral nodule formation in a time- and dose-dependent manner (6). More recently, it has been reported that human dental follicle cells exposed to BMP2 exhibit an increase in alkaline phosphatase (ALP) and calcium deposition when compared with untreated controls (7).

Although existing data suggest that BMP2 can promote follicle cell differentiation towards the cementoblast/osteoblast phenotype, the molecular mechanisms by which this occurs are unclear. In a previous investigation, it was suggested that phosphorylation of SMAD1, SMAD5 and/or SMAD8 may play a direct and critical role in the BMP2 induced differentiation of follicle cells, and that this process requires participation of the MAPK pathway (6).

Wnt glycoproteins form a large family of secreted ligands that activate several receptor-mediated signal transduction pathways (8,9). Activation of Wnt pathways has been shown to be important for modulation of developmental and postdevelopmental physiology by regulating cellular processes, including proliferation, differentiation and apoptosis (8,9). In the canonical Wnt/β-catenin pathway, binding of Wnt ligands to the Frizzled transmembrane receptors and to the low-density-lipoprotein-related protein co-receptors (LRP5/6) inhibits a protein complex responsible for degradation of the cytosolic effector protein β-catenin (CTNNB1). Following inactivation of the CTNNB1 destruction complex, CTNNB1 accumulates in the cytosol and translocates to the nucleus, where it associates with several transcription factors. The resulting complexes bind and regulate the promoter sequences of Wnt/β-catenin target genes (8,9).

The canonical Wnt signaling pathway has been implicated in promotion of bone formation. During embryonic development, wingless-type MMTV integration site family, member 1 (*Wnt1*), *Wnt4*, *Wnt9a*/*14* and *Wnt7b* are essential for osteoblast lineage differentiation (10,11). Postnatally, activation of the Wnt/β-catenin pathway is pivotal for the differentiation of mesenchymal stem cells towards an osteoblast phenotype, while *Wnt10b* and *Lrp5* are required for the expansion of these cells (12,13). Furthermore, *in vitro* studies have shown that ALP activity in osteogenic cultures of mouse cell lines C3H10T1/2 and C2C12 is induced by BMP2 through the canonical Wnt/β-catenin pathway (14,15).

It is well established that Wnt/β-catenin signaling plays a critical role in the early stages of tooth development. For example, stabilization of β-catenin in the dental epithelium is associated with mesenchymal expression of signaling molecules such as BMP4, BMP2, BMP7, fibroblast growth factor-3 (FGF3), activin and follistatin (16,17). Furthermore, Wnt10a has been implicated as a key molecule for dentinogenesis, acting as a regulator of cell–matrix interactions during odontoblast differentiation (18–21). Inhibition of canonical Wnt signaling, either by deleting *Lef1* function or overexpressing the Wnt inhibitor dickkopf homolog 1 (DKK1), arrests tooth morphogenesis at an early stage of tooth development (20,22), and inactivating familial mutations in Axin2 (AXIN2), a negative regulator of β-catenin, causes decreased tooth number because of the lack of tooth renewal (23). Conversely, activation of the Wnt/β-catenin pathway by exogenous expression of active CTNNB1 promotes ectopic tooth formation, following transplantation to a kidney capsule (24).

Little is known about the role of Wnt signaling during tooth root development and in the maintenance of the periodontium. A possible role for canonical Wnt signaling in root development was suggested by expressing a reporter of canonical Wnt signaling in mice. Reporter activity was observed in the region of cementum–periodontal ligament interface, an area of active cell proliferation (25). Furthermore, the canonical Wnt pathway has been shown to promote proliferation and to inhibit differentiation of cementoblasts *in vitro* (26).

Considering the evidence that follicle cells are periodontal progenitor cells, and these cells respond to BMP2 by differentiating along a cementoblast/osteoblast pathway (6), the aim of this study was to determine the role of the canonical Wnt pathway on BMP2-mediated differentiation of dental follicle cells towards a cementoblast/osteoblast phenotype.

Material and methods

Reporter plasmids

The β-catenin activated-reporter (BAR) is a lentiviral plasmid that contains 12 TCF-binding sites separated by distinct 5-based pair linkers upstream of a minimal thymidine kinase promoter that drives the transcription of β-globin intron-linked Venus (pBARV) (27). The equivalent control plasmid, "found unresponsive" BAR (pfuBARV), containing mutated TCF-binding sites, serves as a negative control for BAR (27). Both vectors also contain DsRed fluorescent protein, constitutively driven by the ubiquitin promoter, as a selectable marker for transduced cells.

Western blotting analysis

Methods used to obtain and characterize dental follicle cells (SVF4 cells)

have been previously published (6,28). It was demonstrated that SVF4 cells are induced to a cementoblast/osteoblast cell phenotype by BMP2 (6). Therefore, in order to study Wnt signaling during follicle cell differentiation, most assays were carried out in conditions that included the addition of BMP2. SVF4 cells were seeded in six-well culture plates at a density of 2×10^4 cells/cm^2 and incubated overnight in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with or without WNT3A (25 ng/mL; Millipore, Temecula, CA, USA). To harvest proteins, cells were lysed on ice for 20 min in RIPA buffer (20 mM Tris, 150 mM NaCl, 1% Triton, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with the protease inhibitor cocktail, P2714 [2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich, Saint Louis, MO, USA), 0.3 μM aproptin, 130 μM bestatin, 1 mM EDTA, 14 μM E-64 and 14 μM leupeptin; Sigma-Aldrich). Con A Sepharose 4B microbeads (GE Healthcare, Piscataway, NJ, USA) were added to lysates and incubated for 1 h to remove the membrane fraction. Afterwards, lysates were centrifuged to spin down the beads and the cytosolic and nuclear fractions were separated by electrophoresis on a Nupage 4–12% Bis–Tris gel (Invitrogen, Carlsbad, CA, USA) and then electrotransferred to a nitrocellulose membrane (Bio Rad, Benicia, CA, USA). The membrane was blocked with 4% milk for 20 min, then incubated overnight with polyclonal anti-β-catenin IgG (1:1000 dilution; Cell Signaling). The membrane was then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dlution; Cell Signaling) for 30 min. The membrane was developed using a Pierce ECL Western Blotting Substrate (Thermo Scientific, San Jose, CA, USA).

WNT3A treatment of SVF4 cells

SVF4 cells were plated in 12-well culture plates, at a density of 20,000 cells/cm^2, in DMEM containing 10% FBS. At confluence, the medium was changed to osteogenic medium (OM), containing DMEM, 2% FBS, 50 μg/mL of ascorbic acid, 5 mM β-glycerophosphate and 100 ng/mL of BMP2 recombinant protein (R&D Systems), with or without WNT3A (25 ng/mL; Millipore), for 5 d. The medium was changed on days 2 and 4, and total RNA was isolated on day 5.

Mineralization assay *in vitro*

SVF4 cells were seeded at a density of 20,000 cells/cm^2 in 12-well culture plates in DMEM containing 10% FBS. At confluence, designated day 0, the medium was removed and cells were incubated in OM containing 100 ng/mL of BMP2, with or without 25 ng/mL of WNT3A, for 14 d. The medium was changed every other day. On designated days up to day 14, the ability of cells to promote mineral nodule formation was determine by von Kossa staining and Alizarin Red S staining (29,30).

Gene silencing with small interfering RNA

SVF4 cells were seeded at a density of 20,000 cells/cm^2 in 12-well culture plates and transfected with validated mouse β-catenin (CTNNB1) small interfering RNA (siRNA) (31) or negative-control siRNA1 (Invitrogen) at a final concentration of 20 nM in the presence of Lipofectamine™ RNAiMAX, according to the manufacturer's instructions (Invitrogen). After 24 h of transfection, designated day 0, the culture medium was changed to OM supplemented with 100 ng/mL of BMP2. On day 3, the transient transfection was repeated and total RNA was isolated on day 5 and subjected to quantitative real-time PCR for determination of efficacy of target gene silencing.

Lentiviral transductions and reporter assays

To analyze whether BMP2 treatment activated a β-catenin reporter in SVF4 cells, cells were seeded in six-well culture plates at a density of 2×10^4 cells per well and were exposed to 200 μL of pBARV or pfuBARV lentivirus [prepared as described in Ref. (27)], concentrated ×50, and cultured for 24 h in DMEM containing 10% FBS. The medium was replaced with fresh DMEM containing 10% FBS and the cells were incubated for an additional 24 h. Then, transduced cells were selected based on DsRed expression via fluorescence-activated cell sorting using a FACS Aria (BD Bioscience, Miami, FL, USA). The resulting sorted cell lines (SVF4-pBARV and SVF4-pfuBARV) were seeded in 12-well culture plates and incubated in OM supplemented with 100 ng/mL of BMP2, with or without 25 ng/mL of WNT3A, for 24 h. Then, Venus expression was confirmed by fluorescence microscopy (Carl Zeiss LSM-510; Carl Zeiss LSM-510 GmbH, Mannheim, Germany), and the percentage of Venus-positive cells was determined by flow cytometry (FACS Canto; BD Bioscience). Venus-positive SVF4–pBARV cells were determined, via flow cytometry, as cells that expressed Venus at levels higher than SVF4-pfuBAR cells. Data were analyzed using FlowJo Software, Ashland, OR, USA.

β-catenin/Tcf reporter assay

SVF4-pBARV cells were seeded in 60-mm culture dishes at a density of 20,000 cells/cm^2 and cultured in DMEM containing 10% FBS. At confluence, the medium was changed to OM containing 100 ng/mL of BMP2 and 25% WNT3A-conditioned medium (WNT3A-CM), and incubation was continued for a further 5 d; WNT3A-CM was employed because the scale of the experiment precluded the use of commercially acquired WNT3A for all conditions. Also, a pilot experiment to compare the cell response to conditioned medium vs. recombinant human WNT3A (25 ng/mL) indicated that both treatments induced a similar level of response (Fig. S1). WNT3A-CM was generated according to Willert *et al.* (32). Briefly, mouse L Wnt-3a cells (American Type Culture Collection, CRL-2647) were

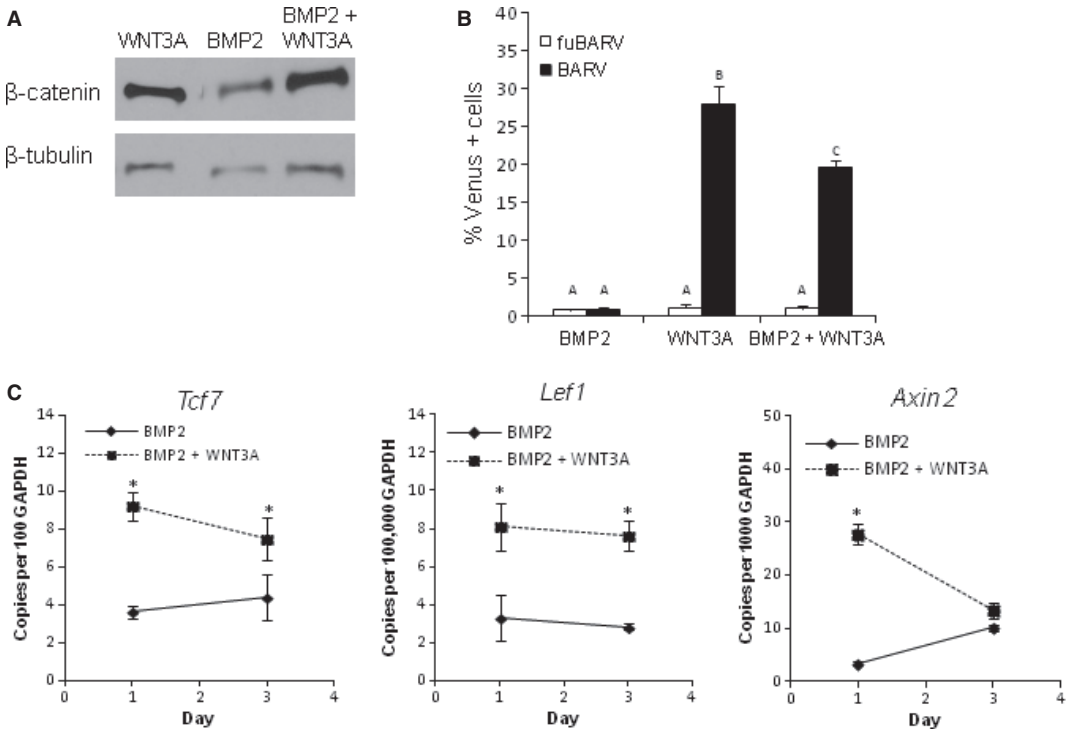

Fig. 1. The Wnt pathway is intact during bone morphogenetic protein 2 (BMP2)-mediated differentiation of SVF4 cells along a cementoblast/osteoblast pathway. (A) Western blot analysis confirmed increased β-catenin levels in SVF4 cells 24 h after the addition of wingless-type MMTV integration site family, member 3A (WNT3A). (B) β-catenin/transcription factor (TCF) transcription reporter activation was stimulated (Venus-positive cells) 24 h after the addition of WNT3A, with or without BMP2. (BARV, β-globin intron-linked Venus; fuBARV, 'found unresponsive' β-globin intron-linked Venus.) (C) Real-time PCR analysis showed that WNT3A (25 ng/mL) enhanced the expression of *Tcf7*, lymphoid enhancer-binding factor-1 (*Lef1*) and AXIn2 (*Axin2*) mRNAs in SVF4 cells after 24 h of treatment. Experiments were performed in triplicate three times, with comparable results obtained on each occasion, and data from one representative experiment are shown. Bars in (B) represent mean ± standard deviation (SD) where intergroup analysis statistical differences are indicated by different capital letters. Line graphs in (C) represent mean ± SD where intergroup analysis statistical differences are indicated by *($p < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cultured in DMEM containing 10% FBS and 1% antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin). At confluence, the mediim was changed and incubation continued for a further 48 h. Next, the medium was collected, and the debris was removed by passing the medium through a 0.2-μm filter. These steps were repeated, and a pool of three collections of WNT3A-CM was maintained at 4°C and used for experiments. The culture medium of SVF4-pBARV cells was changed on days 2 and 4. After 5 d of culture, Venus-positive and Venus-negative cells were sorted using fluorescence-activated cell sorting (FACS Aria; BD Bioscience). Total RNA for sorted cells was isolated and cementoblast/osteoblast gene expression was analyzed by quantitative PCR.

RNA isolation, reverse transcription and quantitative PCR

Total RNA from cultured cells was isolated using Trizol® reagent (Gibco BRL, Carlsbad, CA, USA) according to the manufacturer's instructions, and was treated with DNase (Turbo DNA-free®; Ambion, Inc., Austin, TX, USA). Single-stranded complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a First Strand cDNA Synthesis Kit (Roche Diagnostic Co., Indianapolis, IN, USA), following the manufacturer's recommendations. Primer sequences for *Runx2*, *Osx*, *Alp*, *Ocn*, *Bsp*, *Lef1*, *Tcf7*, *Axin2,* Wnt inhibitor factor 1 (*Wif1*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) are shown in Table 1. Quantitative PCR (qPCR) was performed with the LightCycler480 and software (Roche Diagnostic Co.). PCR was performed twice in triplicate with the LC480 Fast Start DNA SYBR Green Kit (Roche Diagnostic Co.). PCR conditions were as follows: 50 cycles of amplification with 10 s of denaturation at 95°C, 10 s of annealing at 58°C and 30 s of elongation at 72°C. Water (no template) was used as a negative control for all experiments. Relative quantification of reaction products was accomplished using *Gapdh* as the reference gene.

Statistical analysis

Mean and standard deviation (SD) were calculated to establish statistical comparisons. Intragroup comparison was performed using one-way analysis of variance ($\alpha = 0.05$), and a pairwise multiple-comparison test (Bonferroni

Table 1. Primer sequences used for real-time quantitative PCR amplifications

Gene	Primer forward	Primer reverse
Runx2	CTTCATTCGCCTCACAAAC	GTCACTGCGCTGAAGA
Osx	CGGGTCAGGTACAGTG	ACCATGACGACAAGGG
Alp	GGGGACATGCAGTATGAGTT	GGCCTGGTAGTTGTTGTGAG
Bsp	GAGACGGCGATAGTTCC	AGTGCCGCTAACTCAA
Ocn	TGAACAGACTCCGGCG	GATACCGTAGATGCGTTTG
Tcf7	GGAGCTGCAGCCATATGATAG	TAGCCTCCTTCTCTGCCTTG
Lef1	TCCTGAAATCCCCACCTTCT	TGGGATAAACAGGCTGACCT
Wif1	TGTATTTGCCCTCCTGGACT	TTTACCAATGCATTTACCTCCA
Axin2	GAGAGTGAGCGGCAGAGC	CGGCTGACTCGTTTCTCCT
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Alp, alkaline phosphatase; *Axin2*, Axin2; *Bsp*, bone sialoprotein; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Lef1*, lymphoid enhancer-binding factor-1; *Ocn*, osteocalcin; *Osx*, osterix; *Tcf7*, transcription factor 7; *Runx2*, runt-related transcription factor 2; *Wif1*, Wnt inhibitor factor 1.

test) was used to identify the difference among the groups; the Student's *t*-test ($\alpha = 0.05$) was performed for the intergroup analysis (BioEstat 5.0, Belém, Brazil).

Results

The Wnt pathway is intact during BMP2-mediated differentiation of SVF4 cells

To verify whether the Wnt/β-catenin pathway was intact during BMP2-mediated differentiation of SVF4 cells, western blot analysis for β-catenin and β-catenin reporter activation was performed, and transcripts of the Wnt target genes *Lef1*, *Tcf7* and *Axin2* were assessed. Treatment of SVF4 cells with WNT3A increased the stability of β-catenin (Fig. 1A) and enhanced *Tcf/Lef*-dependent transcriptional activity, as demonstrated by the presence of Venus-positive cells (Fig. 1B). Both the western blot and the reporter assay indicated relatively low levels of Wnt activity in SVF4 cells that did not receive exogenous WNT3A stimulation. WNT3A additionally up-regulated *Lef1*, *Tcf7* and *Axin2* by day 1 (Fig. 1C), confirming that the Wnt/β-catenin pathway is intact and responsive during the early phase of BMP2-induced cementoblast/osteoblast differentiation.

Exogenous WNT3A inhibits BMP2-mediated differentiation of SVF4 cells

Figure 2A outlines the culture conditions used for these experiments. To investigate the role of Wnt/β-catenin during BMP2-induced differentiation of SVF4 cells towards a cementoblast/osteoblast phenotype *in vitro*, follicle cells were cultured for 5 d with OM alone, or with OM + WNT3A (25 ng/mL), OM + BMP2 (100 ng/mL), or OM + WNT3A + BMP2. As previously published (6), treatment of SVF4 cells with BMP2 for 5 d led to increased expression of transcripts for *Runx2*, *Osx*, *Alp*, *Ocn* and *Bsp* (Fig. 2B), confirming cell differentiation. Co-treatment with WNT3A + BMP2 did not significantly alter the expression of *Runx2*, *Osx* and *Alp* compared with expression in the presence of BMP2 alone (Fig. 2B). In contrast, WNT3A inhibited BMP2-induced expression of *Ocn*, and up-regulated the expression of *Bsp* mRNA (Fig. 2B). After 14 d in culture, Von Kossa staining showed that WNT3A blocked BMP2-induced mineralization (Fig. 2C). This result was also confirmed and quantified by Alizarin Red S staining (Fig. 2D). Follicle cells without BMP2 do not show a capacity to promote mineralization (data not shown).

Wnt-responsive SVF4 cells express lower levels of cementoblast/osteoblast marker genes

Having demonstrated that only a subpopulation (≅ 20–30%) of SVF4-pBARV cells respond to WNT3A treatment by expressing Venus (Fig. 1B), we next investigated whether there were differences in the expression of cementoblast/osteoblast genes in cells with active (Venus positive) vs. inactive (Venus negative) Wnt/β-catenin signaling. Five days after treatment with OM containing 25% WNT3A-CM + BMP2, SVF4-pBARV Venus-positive and Venus-negative cells were sorted and the mRNA levels for *Runx2*, *Alp*, *Ocn* and *Bsp* were assessed by RT-PCR. Cell sorting was performed with the following criteria: SVF4-pBARV Venus-positive cells were identified by high expression of DsRed, while Venus-negative cells were defined as cells exhibiting no signal (data not shown). The RT-PCR amplification results demonstrated an increase in expression of *Runx2*, *Alp* and *Ocn* in Venus-negative cells compared with Venus-positive cells (Fig. 3), whereas expression of *Bsp* was similar in both populations (Fig. 3). These results demonstrate an inverse relationship between the levels of Wnt/β-catenin signaling and the expression of several genes associated with BMP2-induced differentiation of SVF4 cells into cementoblasts/osteoblasts.

Endogenous β-catenin is required for BMP2-mediated differentiation of SVF4 cells

Given that exogenous WNT3A inhibits BMP2-mediated differentiation of follicle cells along the cementoblast/osteoblast pathway, we next investigated whether endogenous β-catenin was also an inhibitor of this process. SVF4 cells were transiently transfected with β-catenin siRNA, which resulted in a 70% reduction of β-catenin transcripts at 5 d (Fig. 4A). In SVF4 cells treated with BMP2 as well as β-catenin siRNA, we observed a reduction in the cementoblast/osteoblast markers *Runx2*, *Alp* and *Ocn* after 5 d of culture (Fig. 4B). In contrast, knockdown of β-catenin transcripts resulted an increase in *Bsp* transcripts relative to cells treated with BMP2 alone. These results indicate that endogenous Wnt/β-catenin signaling is required for BMP2-induced expression of some, but not all, cementoblast/osteoblast markers.

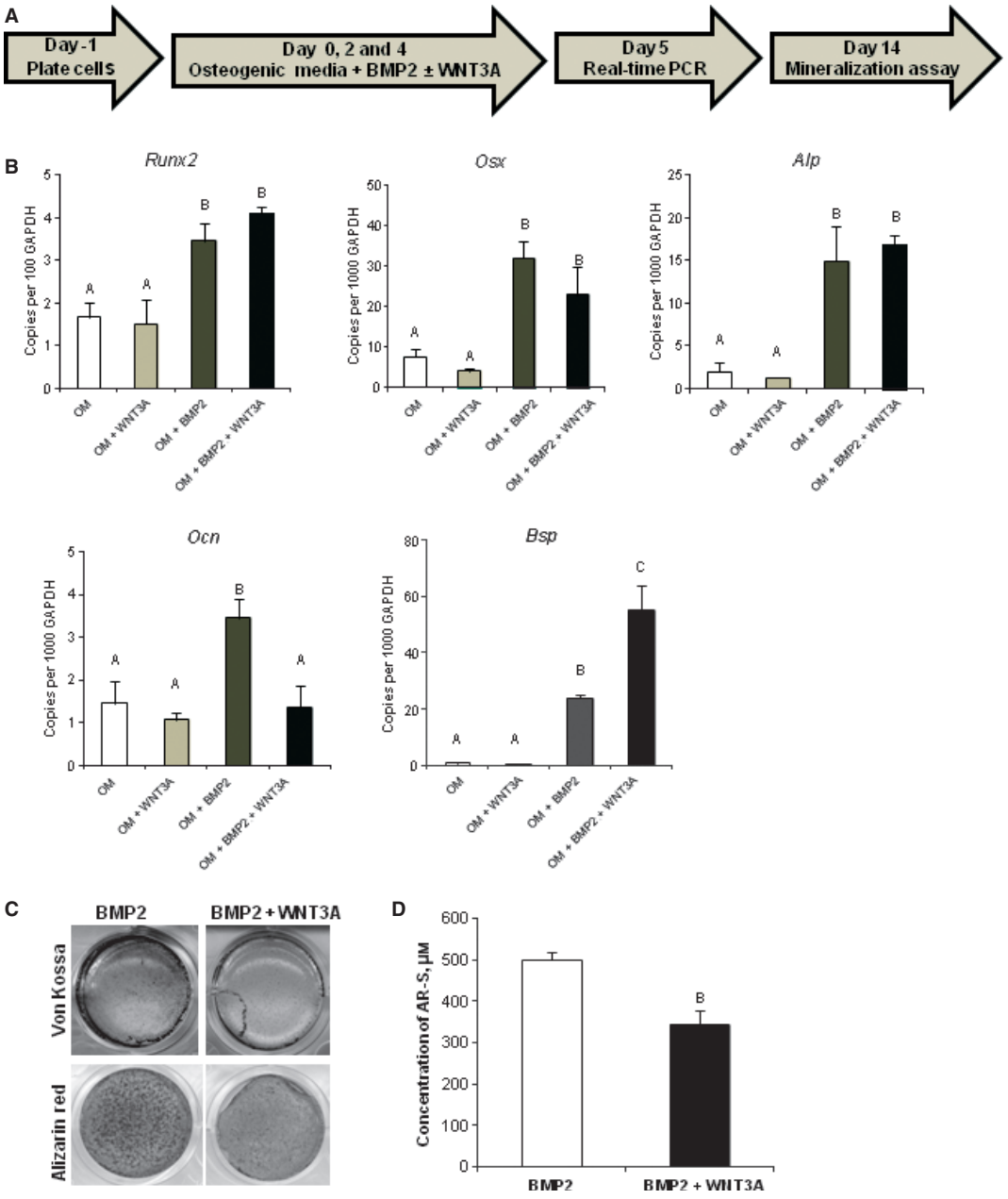

Fig. 2. Wingless-type MMTV integration site family, member 3A (WNT3A) inhibits bone morphogenetic protein 2 (BMP2)-mediated differentiation of SVF4 cells along a cementoblast/osteoblast pathway. (A) Schematic diagram describing the culture conditions used for BMP2-mediated differentiation of SVF4 cells along a cementoblast/osteoblast pathway, and the time course of real-time PCR analysis and the mineralization assay. (B) SVF4 cells were cultured in osteogenic medium (OM) alone, or in OM + WNT3A (25 ng/mL), OM + BMP2 (100 ng/mL), or OM + WNT3A + BMP2. Real-time PCR analysis 5 d after treatment indicated that WNT3A did not alter the expression pattern of mRNAs for runt-related transcription factor 2 (*Runx2*), osterix (*Osx*) and alkaline phosphatase (*Alp*) (all with or without BMP2). The BMP2-induced expression of ostocalcin (*Ocn*) was inhibited by WNT3A, whereas that of bone sialoprotein (*Bsp*) mRNA transcripts was up-regulated by WNT3A. (C) Von Kossa and Alizarin Red S staining for mineral nodule formation at 14 d after treatment showed that inclusion of WNT3A decreased mineralization. (D) Quantification of Alizarin Red S (AR-S) staining confirmed that WNT3A inhibited SVF4 mineralization at 14 d. The experiments were performed in triplicate three times with comparable results obtained on each occasion, and therefore the data from one representative experiment are shown. The bars in (B) and (D) represent mean ± standard deviation where intergroup analysis statistical differences are indicated by different capital letters ($p < 0.05$).

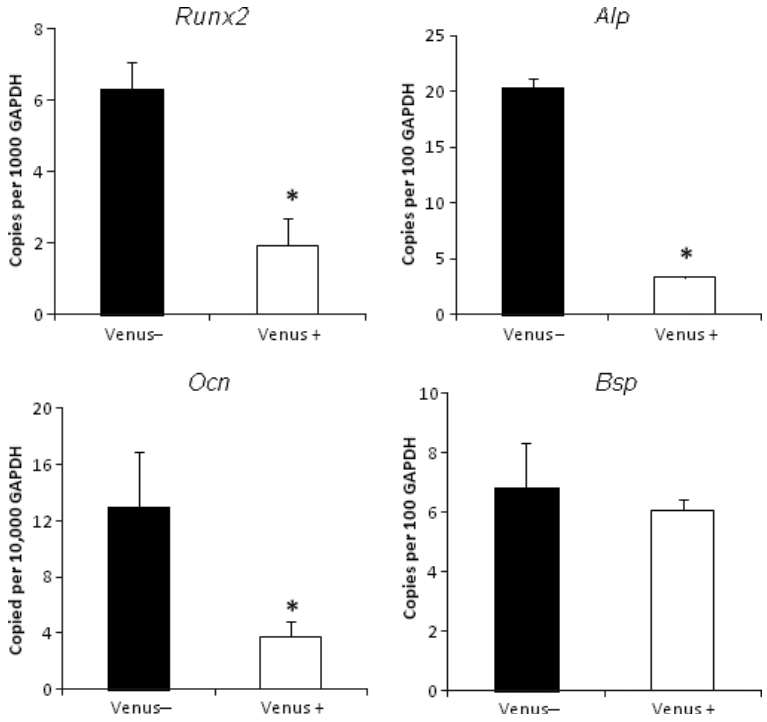

Fig. 3. Wnt-responsive SVF4 cells express lower levels of cementoblast/osteoblast marker genes. SVF4 cells were transduced with β-globin intron-linked Venus (BARV) plasmids and treated with osteogenic medium (OM) supplemented with bone morphogenetic protein 2 (BMP2; 100 ng/mL) and WNT3A-conditioned medium (WNT3A-CM; 1:4 ratio) for 5 d. Real-time PCR amplification revealed significantly higher expression of mRNA for runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*) and ostocalcin (*Ocn*) in SVF4-pBARV Venus-negative cells compared with Venus-positive cells, whereas expression of BSP was similar in both groups. Experiments were performed in triplicate three times, with comparable results obtained on each occasion, and therefore data from one representative experiment are shown. Bars represent mean ± standard deviation where intergroup analysis statistical differences are indicated by *($p < 0.05$).

BMP2 induces expression of the *Wif1* gene

In order to further elucidate potential BMP–Wnt interactions involved in regulating the differentiation of SVF4 cells, PCR array analysis was employed. The most dramatic finding was a BMP2-induced 700-fold increase in *Wif1* (data not shown). This was confirmed by qPCR, which demonstrated an eight-fold increase in *Wif1* expression in BMP2-treated SVF4 cells, independent of the presence of WNT3A (Fig. 4C). In parallel, we observed that the effect of BMP2 on the expression of *Wif1* was dependent on the presence of endogenous β-catenin/*Wnt* signaling. Knockdown of β-catenin significantly abrogated BMP2-mediated *Wif1* expression (Fig. 4D) ($p < 0.05$). These data suggest that BMP2 initiates a negative Wnt feedback loop which may be important in controlling the maturation of follicle cells towards a cementoblast/osteoblast phenotype. Additional Wnt regulatory genes modulated by BMP2 in the PCR array experiment, including *Dkk1* (reduced three-fold) and secreted frizzled related protein 4 (*Sfrp4*; increased two-fold), support the concept of BMP2–Wnt interactions during follicle cell differentiation and will be explored further in future studies.

Discussion

BMP and Wnt signaling pathways have been examined extensively with regard to their role in the regulation of early embryonic development and the control of cell differentiation and proliferation (33–36). Studies have reported that these two signaling pathways link to biological responses via the formation of a nuclear transcription factor complex that acts on target genes, resulting in Wnt signaling repression of BMP-induced activation (37,38). Because WNT3A plays a key role in the development of the axial and appendicular skeleton and appears to regulate stem cell proliferation and differentiation (39,40), we explored the potential role of Wnt signaling during BMP2-driven dental follicle cell differentiation. Exogenous WNT3A inhibited the differentiation of follicle cells, although endogenous Wnt/β-catenin signaling was required for cell maturation to a cementoblast/osteoblast phenotype. These findings of Wnt–BMP crosstalk during follicle cell differentiation were underscored by the powerful induction of the Wnt regulator WIF1 by BMP2. These data support the interpretation that a balance between Wnt and BMP signaling pathways is necessary for proper maturation and function of dental follicle cells.

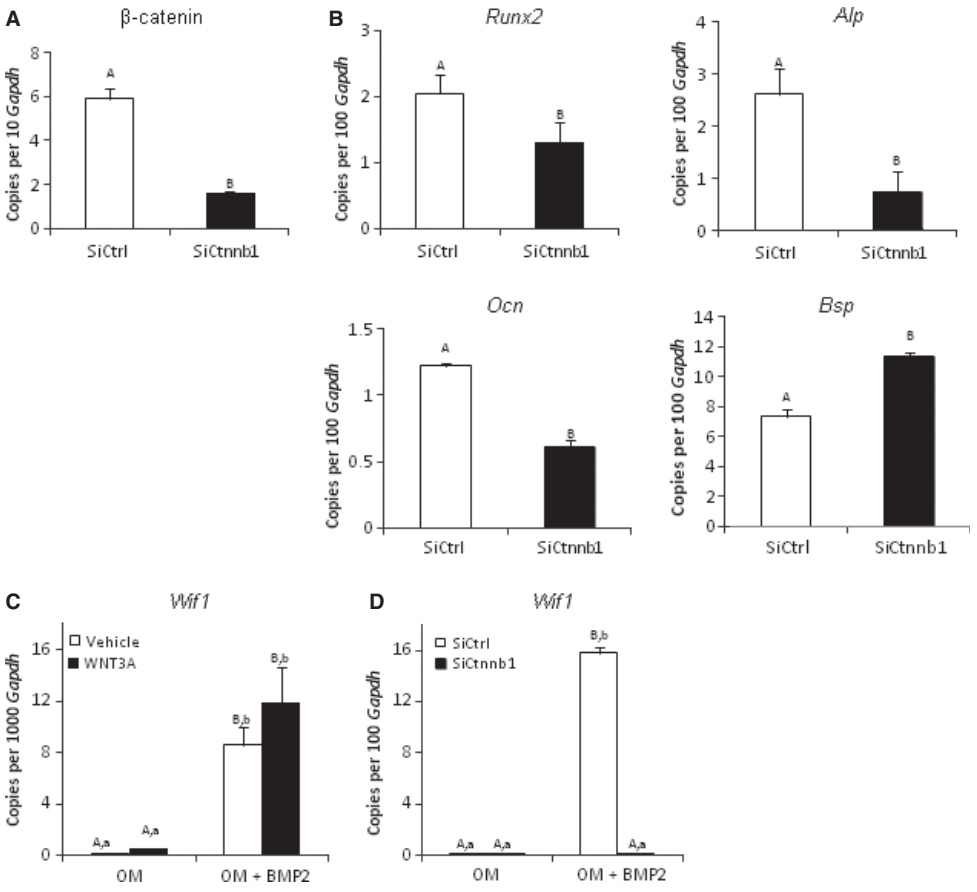

Fig. 4. Endogenous β-catenin is required for bone morphogenetic protein 2 (BMP2)-mediated differentiation of SVF4 cells. SVF4 cells were transiently transfected with 20 nM of small interfering RNA (siRNA) targeted against β-catenin (siCtnnb1) or control siRNA (siCtrl), and treated with osteogenic medium (OM) ± 100 ng/mL of BMP2. (A) β-catenin siRNA reduced gene expression by 70% at 5 d [after normalization to the expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)]. (B) Real-time PCR revealed that knockdown of β-catenin decreased the levels of mRNA for runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*), and ostocalcin (*Ocn*) in BMP2-treated SVF4 cells, and increased the expression of bone sialoprotein at 5 d. (C) BMP2 up-regulated the expression of Wnt inhibitor factor 1 (*Wif1*), independently of the presence of Wnt3a. (D) Knockdown of β-catenin significantly reduced the BMP2-mediated induction of *Wif1*. Experiments were performed in triplicate three times, with comparable results obtained on each occasion, and data from one representative experiment are shown. Bars represent mean ± standard deviation, where intergroup analysis statistical differences are indicated by different capital letters, and intragroup analysis statistical differences are indicated by different lower-case letters ($p < 0.05$).

Canonical Wnt signaling antagonizes BMP-induced follicle cell differentiation

In agreement with our previous investigation (6), the present study showed that BMP2 induced differentiation of SVF4 dental follicle cells towards a cementoblast/osteoblast phenotype, and included an increase in transcripts for *Runx2*, *Osx*, *Alp*, *Ocn* and *Bsp*, as well as increased *in vitro* mineralization (Figs 1, 2). Active Wnt signaling was detected during this differentiation process (Fig. 1). Canonical Wnt signaling has been reported to positively participate in BMP2-mediated osteoblastic differentiation, as measured by induced gene expression of *Alp* and *Runx2* in C3H10T1/2 and C2C12 murine mesenchymal progenitor cells (14,15). Conversely, there have also been reports of negative effects of Wnt/β-catenin signaling on osteoblast differentiation. WNT3A inhibited BMP2-dependent induction of *Osx*, *Alp* and *Ocn* in C2C12 cells, gene expression that was partially restored by addition of the Wnt antagonist DKK1, suggesting that Wnt/β-catenin signaling inhibited BMP signaling in these cells (35,36).

Little is known about the role of the canonical Wnt pathway in cementoblast differentiation. In an interesting investigation using a murine cementoblast cell line (OCCM-30), WNT3A was shown to suppress the expression of genes encoding *RUNX2, OSX, ALP, BSP and OCN*, suggesting that canonical Wnt signaling inhibits the behavior of the differentiated cementoblast phenotype (26). In the present study of dental follicle cells, we observed that the addition of WNT3A inhibited BMP2-driven cell differentiation, manifested by the suppression of *Ocn* transcripts and a reduction in mineralized nodule formation. Intriguingly, the transcript level of *Bsp*, a marker of mature cementoblast/osteo-

blasts, was increased by the addition of WNT3A.

To further examine the effect of WNT3A on BMP2-induced cementoblast/osteoblast markers, we employed a β-catenin activated-reporter plasmid (pBARV) and assessed reporter activity using flow cytometry to measure fluorescence. In contrast to other reporter systems where reporter activity is measured by luciferase activity, cells transduced with pBARV can be sorted by their fluorescence color – green fluorescence protein or dsRed – which is associated with either activation or lack of activation of the transduced β-catenin/TCF reporter, respectively. Using this approach, we observed, in BMP2-treated cells, that the β-catenin/TCF reporter activity induced by treatment with WNT3A was associated with reduced levels of expression of mRNA for *Runx2*, *Alp* and *Ocn* vs. cells lacking reporter activity (Fig. 3). These findings further confirm that WNT3A suppresses BMP2-dependent cementoblast/osteoblast differentiation by a β-catenin/TCF-dependent mechanism. Thus, during *in vitro* differentiation of dental follicle cells along a cementoblast/osteoblast pathway, exogenous stimulation of Wnt/β-catenin is not required, and in fact, suppresses cementoblast gene expression and mineralization ability.

It has been suggested that the master transcription factor *RUNX2* drives expression of *Alp*, *Bsp* and *Ocn* during cementoblast and osteoblast differentiation (6,41). In cementoblasts, addition of WNT3A suppressed *Runx2*, as well as *Alp*, *Bsp* and *Ocn* (26). The sensitive assay employing pBARV-transfected follicle cells revealed that the reduction in expression of *Runx2*, *Alp* and *Ocn* mRNAs was associated with activated Wnt signaling. WNT3A enhanced SVF4 expression of the Wnt-responsive transcription factor, LEF1, as observed previously in cementoblasts (26). It has been reported that LEF1 represses RUNX2-dependent activation of the OCN promoter in osteoblastic cells (42,43). Although an association between LEF1 and cementoblast differentiation has not been reported, one mechanism by which the Wnt/β-catenin signaling pathway could potentially decrease BMP2-mediated differentiation would be by enhancing LEF1 expression, which exhibited an increase of more than two-fold in follicle cells exposed to WNT3A (Fig. 1). Ongoing studies aim to confirm this proposed mechanism and elucidate documented differences in Wnt–BMP interactions in other cell types. As reported previously (44), the effects of Wnt signaling on cell-differentiation markers can vary greatly depending on the cell type and the stage of differentiation.

Wnt/β-catenin regulation of BSP, a marker of early cementoblast/osteoblast differentiation, provided an intriguing result that contrasted with the regulation of other markers described above. In OCCM-30 cementoblasts, WNT3A-repressed *Runx2* expression was associated with the down-regulation of *Bsp* expression (26). In contrast, WNT3A treatment of follicle cells, as well as the knockdown of the β-catenin gene, enhanced *Bsp* mRNA levels beyond those of BMP2-treated controls, although both conditions exhibited repressed expression of *Runx2*. Additionally, we found comparable expression of *Bsp* in cells with or without activation of the β-catenin/TCF reporter by WNT3A. While speculative, these findings suggest that BMP regulation of BSP may not occur solely via RUNX2 in follicle cells. Also, the interplay of the Wnt pathway with BMP signaling may affect transcriptional regulation of cementoblast/osteoblast genes in a nonuniform manner. Additional investigations are warranted to identify other pathways that may be involved.

A role for endogenous Wnt signaling during dental follicle cell differentiation

It has been reported that mutations in Wnt-related proteins such as LRP5, a Wnt co-receptor, affect both the proliferation and the osteogenic differentiation of osteoblastic progenitor cells *in vitro* and *in vivo* (12). We assessed the role of endogenous Wnt/β-catenin signaling in dental follicle cell differentiation by employing siRNA-mediated knockdown of β-catenin (Fig. 4). The data showed that, in the presence of BMP2, dental follicle cells require endogenous β-catenin in order to up-regulate the expression of *Runx2*, *Alp* and *Ocn*, suggesting that BMP2-mediated osteoblast/cementoblast differentiation in these cells partially relies on the canonical Wnt signaling pathway. However, while BMP2-mediated differentiation of SVF4 cells requires endogenous β-catenin, this protein did not lead to the activation of Wnt/β-catenin signaling, as measured by the β-catenin reporter gene assay (Fig. 1B).

BMP2, like other transforming growth factor-beta superfamily members, binds to a serine/threonine kinase receptor that activates a pathway which includes phosphorylation and nuclear translocation of SMAD protein (46). While these signaling components are distinct from the known Wnt/β-catenin signal transduction pathway, they may stimulate processes that cooperate with activated β-catenin to promote osteoblast differentiation (14). In another system, Xenopus β-catenin and LEF/TCF form a complex with SMAD4 and participate in the synergistic activation of the twin gene during formation of the Spemann's organizer (47). Based on our data, we propose that β-catenin and SMAD proteins are needed to activate cementoblast/osteoblast gene expression and promote full differentiation.

Investigations using murine mesenchymal cells demonstrated that β-catenin knockdown may also affect the function of adherens junctions by α-catenin knockdown, and through this mechanism indirectly inhibit osteoblastic differentiation (45). In our study, the siRNA sequence used was specific for β-catenin (CTNNB1), as demonstrated previously (31), confirming the specific need for Wnt pathway effector β-catenin during the differentiation process. However, additional experiments must be performed to clarify both the impact of loss of β-catenin on differentiating SVF4 cells (such as the effects of β-catenin knockdown on BMP2-induced mineral nodule formation by SVF4 cells), as well as the precise role of endogenous Wnt signaling during cementoblast/osteoblast differentiation.

Our observation that canonical β-catenin was required for BMP2-mediated expression of several early cementoblast/osteoblast markers raises the question about the timing and precise mechanism by which endogenous β-catenin and BMP signaling pathways may be linked in order to mediate differentiation of dental follicle cells. In agreement with previous studies using different cell types (48–50), we observed that BMP2 induced increased expression of WIF1, an antagonist of Wnt signaling, suggesting that one method by which BMP2 controls follicle cell maturation is by promoting a negative Wnt-feedback loop. Intriguingly, the Wnt pathway itself is implicated in the negative feedback, as silencing β-catenin abrogated the induction of *Wif1* by BMP2. BMP2 also regulated other Wnt pathway inhibitors, including DKK1 and SFRP4, supporting this concept. While Osx has been shown to directly regulate the Wnt inhibitors DKK1 (51) and sclerostin (52), here we found that BMP2 did not induce changes in the level of *Osx* mRNA, suggesting alternate mechanisms for generating BMP2-induced Wnt inhibition.

In conclusion, we report for the first time that the Wnt/β-catenin signaling pathway has putative physiological significance for BMP-mediated cell differentiation of murine dental follicle cells. These data reveal that increased Wnt/β-catenin pathway activation, as well as β-catenin knockdown, inhibits differentiation, suggesting that precise levels of Wnt/β-catenin signaling are critical for regulation of BMP-induced differentiation. These findings not only increase our understanding of the processes governing periodontal development, but also give insight into the pathways that are of value to modulate in order to develop more robust and predictable regenerative therapies than those currently available.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pilot experiment to compare cell response to conditioned media vs. recombinant human Wnt3a. SVF4 cells were transduced with BARV plasmids, and cultured in DMEM 2% FBS supplemented with 25% Wnt3a conditioned medium (Wnt3a-CM) or recombinant human Wnt3a (25 ng/ml) by 24 h. β-catenin/TCF transcription reporter activation (Venus positive cells) was induced at similar level for both treatment. Bars represent mean ± SD where intergroup analysis statistical differences are indicated by different capital letters ($p < 0.05$).

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