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Wnt/ β -catenin pathway regulates Bmp2-mediated differentiation of dental follicle cells

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Abstract

Background and Objectives—Bmp2-induced osteogenic differentiation has been shown to occur through the canonical Wnt/ β -catenin pathway, whereas factors promoting canonical Wnt signaling in cementoblasts inhibited cell differentiation and promoted 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.

Materials and Methods—SVF4 cells were stimulated with Bmp2, and the following assays were carried out: 1) Wnt/ β -catenin pathway activation assessed by western blot, β -catenin/TCF reporter assay, and gene expression of lymphoid enhancer-binding factor-1 (Lef1), transcription factor 7 (Tcf7), Wnt inhibitor factor 1 (Wif1) and Axin2, and 2) cementoblast/osteoblast differentiation assessed by mineralization *in vitro*, and mRNA levels of runt-related transcription factor 2 (Runx2), osterix (Osx), alkaline phosphatase (Alp), osteocalcin (Ocn) and bone sialoprotein (Bsp) by qPCR after Wnt3a treatment and knockdown of β -catenin.

Results—Wnt3a induced β -catenin nuclear translocation and upregulated the transcriptional activity of a canonical Wnt-responsive reporter, suggesting 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 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 to untreated cells. In contrast, Bmp2 induction of Bsp transcripts occurred independent of Wnt/ β -catenin signaling.

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Conclusions—These data suggest that stabilization of β -catenin by Wnt-3a treatment inhibits Bmp2-mediated induction of cementoblast/osteoblast differentiation in SVF4 cells, although Bmp2 requires endogenous Wnt/ β -catenin signaling to promote cell maturation.

Keywords

dental follicle cells; Wnt; cementoblast; maturation; BMP

Introduction

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 (PDL) 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*, toward a cementoblast/osteoblast phenotype characterized by expression of markers associated with both early (Cbfa-1/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 was reported that human dental follicle cells (HDFC) exposed to Bmp2 exhibit an increase in alkaline phosphatase and calcium deposition when compared to untreated controls(7).

Although existing data suggest that Bmp2 can promote follicle cell differentiation toward 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 Bmp2-induction of follicle cells differentiation, 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 an important for modulation of developmental and post-developmental 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 (Fzd) transmembrane receptors and the low-density lipoprotein related protein co-receptors (Lrp-5/6) 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, *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 toward an osteoblast phenotype, while *Wnt10b* and *Lrp5* are required for the expansion of these cells (12, 13). Furthermore, *in vitro* studies have shown that alkaline phosphatase (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 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). Further, 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 *Dkk1*, arrests tooth morphogenesis at an early stage of tooth development (20,22), and inactivating familial mutations in 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 formation tooth, following transplantation to a kidney capsule (24).

Little is known about the role of Wnt signaling during tooth root development and in 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-PDL 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 evidences 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 toward a cementoblast/osteoblast phenotype.

Materials 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 served 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 including BMP2 addition. SVF4 cells were seeded in 6-well culture plates at a density of 2×10^4 cells/cm² and incubated overnight in DMEM with 10% FBS supplemented with or without Wnt3a (25 ng/ml, Millipore). To harvest proteins, cells were lysed on ice for 20 min in RIPA buffer (20mM Tris, 150mM NaCl, 1% Triton, 0.25% sodium deoxycholate, 0.1% SDS; Sigma-Aldrich) supplemented with a protease inhibitor cocktail – P2714 (AEBSF 2mM, Aprotin 0.3 μ M, Bestatin 130 μ M, EDTA 1mM, E-64 14 μ M, Leupeptin 14 μ M; Sigma-Aldrich). Con A Sepharose 4B microbeads (GE Healthcare) were added to lysates and incubated for 1h to remove membrane fraction. Afterwards, lysates were centrifuged to spin beads down and remaining cytosolic and nuclear fractions were separated on Nupage 4–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membrane (Bio Rad). The membrane was blocked with 4% milk for 20 min, and then incubated overnight with anti- β -

catenin polyclonal antibody (1:1000; Cell Signaling). The membrane was then incubated with goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:10000; Cell Signaling) for 30 min. The membrane was developed using a Pierce ECL Western Blotting Substrate (Thermo Scientific).

Wnt3a treatment of SVF4 cells

SVF4 cells were plated in 12-well culture plates, at a density of 20,000 cells/cm² in DMEM with 10% FBS. At confluence, media were changed to osteogenic medium (OM), containing DMEM with 2% FBS, 50 µg/ml ascorbic acid and 5 mM β-glycerophosphate, supplemented with BMP2 recombinant protein (100 ng/ml; R&D Systems), +/- Wnt3a (25 ng/ml, Millipore) for 5 days. Media were 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/cm² in 12-well culture plates in DMEM containing 10% FBS. At confluence, designated day 0, media were removed and cells were incubated in OM with 100 ng/ml BMP2, +/- 25 ng/ml Wnt3a for 14 days. Media were changed every other day. On designated days up to 14 days, the ability of cells to promote mineral nodule formation was determined by von Kossa staining and alizarin red-S staining (29,30).

Gene silencing with siRNA

SVF4 cells were seeded at a density of 20,000 cells/cm² in 12-well culture plates, and transfected with validated mouse β-catenin (Ctnnb1) siRNA (31), or negative control siRNA1 (Invitrogen) at a final concentration of 20nM in the presence of a LipofectamineTM RNAiMAX according to manufacturer's instructions (Invitrogen). After 24 h of transfection, designated day 0, the culture medium was changed to OM supplemented with 100ng/ml of BMP2. On day 3, the transient transfection was repeated and total RNA was isolated at day 5, and then 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 6-well cultured plate 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 [27]) that had been concentrated 50X, and cultured in DMEM with 10% FBS for 24 h. The media were replaced with fresh DMEM with 10% FBS and 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). The resulting sorted cell lines (SVF4-pBARV and SVF4-pfuBARV) were seeded in 12-well cultures plates and incubated in OM supplemented with 100ng/ml BMP2, associated or not with 25ng/ml Wnt3a for 24 hours. Then, Venus expression was confirmed by fluorescence microscopy (Carl Zeiss LSM-510), 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 greater than SVF4-pfuBARV cells. Data were analyzed using FlowJo Software.

β-catenin/Tcf reporter assay

SVF4-pBARV cells were seeded in 60mm culture dishes at a density of 20,000 cells/cm² and cultured in DMEM with 10% FBS. At confluence, media were changed to OM with 100 ng/ml BMP2, and 25% Wnt3a conditioned medium (Wnt3a-CM) for 5 days; Wnt3a-CM

was employed because the scale of the experiment precluded use of commercially acquired Wnt3a for all conditions. Also, a pilot experiment to compare cell response to conditioned media vs. recombinant human Wnt3a (25 ng/ml) indicated these treatments induced a similar level response (Supplemental Figure 1). Wnt3a-CM was generated according to Willert et al. (32). Briefly, mouse L Wnt-3a cells (American Type Culture Collection, CRL-2647) were cultured in DMEM 10% FBS and 1% antibiotics (100units/ml penicillin, 100µg/ml streptomycin). At confluence, media were changed and incubated for 48 h. Next, media were collected, and the debris was removed passing through a 0.2µm filter. These steps were repeated, and a pool of three collections of Wnt3a-CM was maintained at 4°C, and used for experiments. Media of SVF4-pBARV cells were changed on days 2 and 4. After 5 days of culture, Venus-positive and 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) according to the manufacturer's instructions, and was DNase-treated (Turbo DNA-free[®], Ambion, Inc.). Single-stranded cDNA was synthesized from 1µg of total RNA using 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 and 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 time with the LC480 Fast Start DNA SYBR Green Kit (Roche Diagnostic Co.). PCR conditions were as follows: 50 cycles of amplification with 10s denaturation at 95°C, 10s annealing at 58°C, and 30s 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 (glyceraldehyde3-phosphate dehydrogenase) as the reference gene.

Statistical analysis

Mean and standard deviation (S.D.) were obtained to establish statistical comparisons. Intragroup comparison was performed using one-way ANOVA test ($\alpha = 0.05$) and pair-wise multiple comparison test (Bonferroni test) was used to identify the difference among the groups; whereas the Student's t-test ($\alpha = 0.05$) was performed for the intergroup analysis (BioEstat 5.0).

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 Wnt target genes Lef1, Tcf7 and Axin2 were assessed. Treatment of SVF4 cells with Wnt3a increased 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 Western blot and reporter assay indicated relatively low levels of Wnt activity in SVF4 cells not receiving exogenous Wnt3a stimulation. Wnt3a additionally upregulated Lef1, Tcf7 and Axin2 by day 1 (Fig. 1C), confirming that 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 interrogate the role of Wnt/ β -catenin during BMP2-induced differentiation of SVF4 cells toward a cementoblast/osteoblast phenotype *in vitro*, follicle cells were cultured with osteogenic medium (OM), OM with Wnt3a (25 ng/ml), OM with BMP2 (100 ng/ml), or OM containing both Wnt3a and BMP2, for 5 days. As previously published (6), BMP2 treatment of SVF4 cells for 5 days led to increased expression of transcripts for Runx2, Osx, Alp, Ocn and Bsp (Fig. 2B), confirming cell differentiation. Gene expression of Runx2, Osx, and Alp induced by BMP2 was not altered by the treatment with Wnt3a (Fig. 2B). In contrast, Wnt3a inhibited BMP2-induced Ocn gene expression, and upregulated Bsp mRNA (Fig. 2B). After 14 days 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 ($\approx 20\text{--}30\%$) of SVF4-pBARV cells respond to Wnt3a treatment by expressing Venus (Fig. 1B), we next investigated whether there were differences in expression of cementoblast/osteoblast genes in cells with active (Venus positive) versus inactive (Venus negative) Wnt/ β -catenin signaling. At 5 days after treatment with OM containing 25% Wnt3a-CM with BMP2, SVF4-pBARV Venus-positive and negative cells were sorted and mRNA levels for Runx2, Alp, Ocn and Bsp 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). RT-PCR demonstrated an increase in expression of Runx2, Alp and Ocn in Venus-negative cells compared to Venus-positive cells (Fig. 3), whereas expression of Bsp was similar in both populations (Fig. 3). These results demonstrate an inverse relationship between 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 Bmp 2-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 days (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 at 5 days (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.

BMP2 induces Wif1 gene expression

In order to further elucidate potential BMP-Wnt interactions regulating differentiation of SVF4 cells, PCR array analysis was employed. The most dramatic finding was a BMP2-induced 700-fold increase in Wnt inhibitor factor 1 (Wif1; data not shown). This was confirmed by qPCR, which demonstrated an 8-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 toward a cementoblast/osteoblast phenotype. Additional Wnt regulatory genes modulated by BMP2 in the PCR array experiment, including dickkopf-related protein 1 (Dkk1; reduced 3-fold) and secreted frizzled related protein 4 (Sfrp4; increased 2-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, though 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 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.

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 toward a cementoblast/osteoblast phenotype, and included an increase in transcripts for Runx2, Osx, Alp, Ocn, 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 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, expression that was partially restored by addition of 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 Wnt3a addition inhibited BMP2 driven cell differentiation, manifested by suppression of Ocn transcripts and a reduction in mineralized nodule formation. Intriguingly, Bsp, a marker of mature cementoblast/osteoblast, was increased by Wnt3a addition.

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 measuring fluorescence. In contrast to other reporter systems where reporter activity is measured by luciferase activity, cells transduced with pBARV vector can be sorted by their fluorescence color, GFP 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 β -catenin/Tcf reporter activity induced by Wnt3a treatment was associated with reduced expression of mRNA levels 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, an 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 mRNA 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 Wnt/ β -catenin signaling pathway could potentially decrease BMP2-mediated differentiation would be by enhancing Lef1 expression, which exhibited more than a 2-fold increase 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), effects of Wnt signaling on cell differentiation markers can vary greatly depending on cell type and stage of differentiation.

Wnt/ β -catenin regulation of Bsp, a marker of early cementoblast/osteoblast differentiation, provided an intriguing result contrasting with 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 BMP2 treated controls, although both conditions exhibited repressed Runx2 expression. Additionally, we found comparable expression of Bsp in the cells with activation or lack of activation of β -catenin/Tcf reporter by Wnt3a. While speculative, these findings suggest that BMP regulation of Bsp may not be solely via Runx2 in follicle cells. Also, the interplay of the Wnt pathway with BMP signaling may be affecting transcriptional regulation of cementoblast/osteoblast genes in a non-uniform way. 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 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 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 activation of Wnt/ β -catenin signaling as measured by β -catenin reporter gene assay (Fig. 1B).

BMP2, like other TGF β superfamily members, binds to a serine/threonine kinase receptor that activates a pathway that includes Smad protein phosphorylation and nuclear

translocation (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 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 inhibits osteoblastic differentiation (47). 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 impact of loss of β -catenin on differentiating SVF4 cells (such as 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 *Wif1* induction by BMP2. BMP2 also regulated other Wnt pathway inhibitors, including *Dkk1* and *Sfrp4*, supporting this concept. While *Osx* has been shown to directly regulated Wnt inhibitors *Dkk-1* (51) and *sclerostin* (52), here we found that BMP2 did not induce changes in *Osx* mRNA levels, 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 as to the pathways that are of value to modulate in order to develop more robust and predictable regenerative therapies than currently available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The Wnt pathway is intact during 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 Wnt3a addition. (B) β -catenin/TCF transcription reporter activation occurred (Venus positive cells) by 24 h following addition of Wnt3a, with or without BMP2. (C) Real-time PCR analysis showed that Wnt3a (25 ng/ml) enhanced the expression of Tcf7, Lef1, and Axin2 in SVF4 cells after 24 h of treatment. Experiments were performed in triplicate three times with comparable results, and data from one representative experiment are shown. Bars in (B) represent mean $\pm$ SD where intergroup analysis statistical differences are indicated by different capital letters. Line graphs in (C) represent mean $\pm$ SD where intergroup analysis statistical differences are indicated by * ($p < 0.05$).

Figure 2. Wnt3a inhibits 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 mineralization assay. (B) SVF4 cells cultured in osteogenic medium (OM), OM + Wnt3a (25 ng/ml), OM + BMP2 (100 ng/ml), or OM + both Wnt3a and BMP2. Real-time PCR analysis at 5 days after treatment indicated Wnt3a did not alter expression patterns for Runx2, Osx, and Alp (all with or without BMP2). BMP2 induced Ocn expression was inhibited, and Bsp mRNA transcripts were upregulated by Wnt3a. (C) Von Kossa and alizarin red-S staining for mineral nodule formation at 14 days after treatment showed that inclusion of Wnt3a decreased mineralization. (D) Quantification of alizarin red-S staining confirmed that Wnt3a inhibited SVF4 mineralization at 14 days. Experiments were performed in triplicate three times with comparable results, and data from one representative experiment are shown. Bars in (B) and (D) represent mean $\pm$ SD where intergroup analysis statistical differences are indicated by different capital letters ($p < 0.05$).

Figure 3. Wnt responsive SVF4 cells express lower levels of cementoblast/osteoblast marker genes

SVF4 cells were transduced with BARV plasmids and treated with osteogenic medium (OM) supplemented with BMP2 (100ng/ml) and Wnt3a conditioned media (Wnt3a-CM) at 1:4 for 5 days. Real-time PCR analysis revealed significantly higher expression of Runx2, Alp and Ocn in SVF4-pBARV Venus negative cells compared to positive cells, whereas expression of BSP was similar in both groups. Experiments were performed in triplicate three times with comparable results, and data from one representative experiment are shown. Bars represent mean $\pm$ SD where intergroup analysis statistical differences are indicated by * ($p < 0.05$).

Figure 4. Endogenous β -catenin is required for BMP2-mediated differentiation of SVF4 cells SVF4 cells were transiently transfected with 20 nM of siRNA targeted against β -catenin (Ctnnb1) or control siRNA, and treated with osteogenic medium (OM) +/- 100 ng/ml BMP2. **(A)** β -catenin siRNA reduced gene expression by 70% at 5 days (as normalized to Gapdh). **(B)** Real-time PCR revealed that knockdown of β -catenin decreased mRNA levels for Runx2, Alp, and Ocn in BMP2-treated SVF4 cells, and increased expression of Bsp at 5 days. **(C)** BMP2 upregulated expression of Wif1, independently of the presence of Wnt3a. **(D)** Knockdown of β -catenin significantly reduced BMP2-mediated induction of Wif1. Experiments were performed in triplicate three times with comparable results, and data from one representative experiment are shown. Bars represent mean $\pm$ SD where intergroup analysis statistical differences are indicated by different capital letters, and intragroup analysis statistical differences are indicated by different lower-case letters ($p < 0.05$).

Table 1

Primer sequences used for real-time quantitative polymerase chain reaction amplifications.

Gene	Primer Forward	Primer Reverse
Runx2	CTTCATTGCGCTCACAAAC	GTCAGTGCCTGAAGA
Osx	CGGGTCAGGTACAGTG	ACCATGACGACAAGGG
Alp	GGGGACATGCAGTATGAGTT	GGCCTGGTAGTTGTTGTGAG
Bsp	GAGACGGCGATAGTTCC	AGTGCCGCTAACTCAA
Ocn	TGAACAGACTCCGGCG	GATACCGTAGATGCGTTTG
Tcf7	GGAGCTGCAGCCATATGATAG	TAGCCTCCTTCTCTGCCTTG
Lef1	TCCTGAAATCCCCACCTTCT	TGGGATAAACAGGCTGACCT
Wif1	TGTATTTGCCCTCCTGGA	TTTACCAATGCATTTACCTCCA
Axin2	GAGAGTGAGCGGCAGAGC	CGGCTGACTCGTTTCTCCT
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA