Activation of the Canonical Wnt Signaling Pathway Induces Cementum Regeneration

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ABSTRACT
Canonical Wnt signaling is important in tooth development but it is unclear whether it can induce cementogenesis and promote the regeneration of periodontal tissues lost because of disease. Therefore, the aim of this study is to investigate the influence of canonical Wnt signaling enhancers on human periodontal ligament cell (hPDLCs) cementogenic differentiation in vitro and cementum repair in a rat periodontal defect model. Canonical Wnt signaling was induced by (1) local injection of lithium chloride; (2) local injection of sclerostin antibody; and (3) local injection of a lentiviral construct overexpressing β-catenin. The results showed that the local activation of canonical Wnt signaling resulted in significant new cellular cementum deposition and the formation of well-organized periodontal ligament fibers, which was absent in the control group. In vitro experiments using hPDLCs showed that the Wnt signaling pathway activators significantly increased mineralization, alkaline phosphatase (ALP) activity, and gene and protein expression of the bone and cementum markers osteocalcin (OCN), osteopontin (OPN), cementum protein 1 (CEMP1), and cementum attachment protein (CAP). Our results show that the activation of the canonical Wnt signaling pathway can induce in vivo cementum regeneration and in vitro cementogenic differentiation of hPDLCs. © 2015 American Society for Bone and Mineral Research.

KEY WORDS: CANONICAL WNT SIGNALING PATHWAY; REGENERATIVE MEDICINE; CEMENTUM REGENERATION; TISSUE ENGINEERING

Introduction
The tissues forming the tooth-supporting apparatus of the periodontium include alveolar bone, periodontal ligament (PDL), cementum, and gingiva. Once periodontal tissues are destroyed by the commonly occurring disease periodontitis, it is a major challenge for dental clinicians to restore the original structure and function via tissue regeneration.11 Regeneration of the lost periodontium requires the formation of multiple tooth-supporting structures, including new cementum, PDL, alveolar bone, and gingival tissue. In addition, it is important to promote appropriate PDL tissue orientation, involving the perpendicular insertion of collagen fibers into both cementum and alveolar bone. Furthermore, appropriate mechanical loading is essential for the development of highly organized functional PDL fibers.2,3 In periodontal tissue engineering and regeneration, cementum regeneration is a critical and challenging phase of the formation of functional PDL formation; however, the molecular mechanisms underlying the process of cementum regeneration are still poorly understood.3,4 There are few, if any, reports describing the interaction between cell signaling pathways and periodontal tissue engineering, especially cementum regeneration.

Cementum is a thin layer of mineralized tissue covering the tooth root surface and provides a mineralized interface for PDL anchorage to the tooth.5 Increasing evidence has shown that dental mesenchymal progenitor cells in the PDL differentiate to cementoblasts to form cementum.6,7 PDL cell sheets have been shown to form new periodontal-cementum complexes, with or without the addition of growth factors, in various animal models.8–11 However, the capacity of PDLCs to differentiate into cementoblast-like cells is not fully established, and nor are the molecular mechanisms responsible for this process.

Understanding the critical regulators associated with cementogenesis is of great importance for developing molecular therapies for cementum regeneration. It is well known that canonical Wnt signaling is involved in multiple stages of tooth morphogenesis by mediating the transcription of multiple target genes, such as c-myc, Axin2, and cyclin D.12,13 Recently, it has been shown that the activation of canonical Wnt signaling in osteoblasts and odontoblasts leads to aberrant dento-alveolar complex formation with hypoplastic cementum and periodontal ligament in mice.14,15 Furthermore, constitutive β-catenin stabilization in the dental mesenchyme leads to the excessive formation of dentin and cellular cementum in mice.16 These data raise the question of whether activation of canonical Wnt
signaling can assist in the regeneration of cementum/periodontal tissues in vivo. Indeed, although its essential role in tooth morphologies is well documented, little is known about the involvement of the canonical Wnt signaling pathway in the differentiation of PDL cells, and its ability to promote cementum regeneration.

Experiments using neutralizing antibodies have shown that inhibition of sclerostin (Scl-Ab) could promote bone formation via activation of canonical Wnt signaling. A similar effect is also seen with the administration of lithium chloride (LiCl), which by inhibiting GSK3β enhances bone healing by enhancing canonical Wnt signaling. In the present study, we test the hypothesis that activation of canonical Wnt signaling using either neutralizing Scl-Ab, lithium, or overexpression of β-catenin can have a favorable impact on rat periodontal wound healing. We sought to activate the canonical Wnt pathway during in vivo cementum regeneration in a rat periodontal defect model using the following approaches: (1) local injection of LiCl; (2) local injection of neutralizing Scl-Ab; and (3) local injection of a lentiviral construct overexpressing of β-catenin (LV-Ctnnb). Furthermore, in vitro activation of the canonical Wnt pathway (by either LiCl, LV-Ctnnb, or Wnt3a conditional media) in human periodontal ligament cells (hPDLCs) was also performed to investigate the mechanism underlying cementoblast-like differentiation. Our data demonstrates that activation of the canonical Wnt signaling pathway can enhance cementum regeneration as evidenced by the enhanced expression of cementum-related markers.

**Subjects and Methods**

**Rat periodontal defect model**

All animal procedures for this study were performed under guidelines approved by the Queensland University of Technology Animal Ethics Committee (approval no. 1100000141). Twelve-week-old adult male Lewis rats (*Rattus norvegicus*, Strain-LEW/CrlArc; Animal Resources Centre, Murdoch, WA, Australia) were anaesthetized by isoflurane (Attane; Bomac Animal Health Pty Ltd, Hornsby, NSW, Australia) inhalation. A rat periodontal defect model was created according to a previously published protocol. Briefly, a full-thickness skin incision was made through the skin along the inferior border of the mandible. Mucoperiosteal flaps were subsequently elevated by dissection of the underlying masseter muscle and the periosteum covering the alveolar bone adjacent to the buccal aspect of the first and second mandibular molars. The alveolar bone covering the root surfaces was removed with a dental bur under saline irrigation. A periodontal window defect of approximately 1.5 mm in width, 3 mm in length, and 2 mm in depth was created. All periodontal ligament tissues and cementum on the tooth root surface were removed (Fig. 1A–D). The flap was repositioned against the root surface and sutured. The rats were euthanized 1, 2, and 4 weeks after the surgery and the mandibles (*n* = 4 for each group at each time point) were harvested and fixed in 4% paraformaldehyde (PFA) solution overnight at room temperature and then washed in phosphate buffered saline (PBS).

**Local administration of Scl-Ab and LiCl in rat periodontal defect**

To investigate the regenerative potential of canonical Wnt signaling during periodontal regeneration, the periodontal defects were treated on a daily basis for 2 weeks with either vehicle (*n* = 4), Scl-Ab (humanized monoclonal Scl-Ab III; Amgen, Thousand Oaks, CA, USA; *n* = 4) or LiCl (Sigma-Aldrich, Castle Hill, NSW, Australia; *n* = 6). Scl-Ab and LiCl was administered locally into the periodontal defect site between the right mandible first and second molars and near the base of the interproximal gingival papillae (at doses of 25 mg/kg and 149 mg/kg, respectively). Local injection of phosphate-buffered saline (1 × PBS; Life Technologies, Mulgrave, VIC, Australia) served as the vehicle control in the study. The treatment commenced 12 hours after the surgery for each group.

**Local injection of LV-Ctnnb in rat periodontal defect**

To further confirm the effect of activation of canonical Wnt signaling on periodontal regeneration, lentivirus particles containing the rat gene for β-catenin (Ctnnb) were produced by a 293 T cell line that can be efficiently transduced by calcium phosphate DNA precipitation. The HIV-1 lentivirus was produced by cotransfection of 293 T cells with 10 μg plenti-Ctnnb (Thermo Fisher Scientific, Scoresby, VIC, Australia) gene expression vector, 5 μg of envelope vector pCMV-VSVG and 5 μg of packaging plasmid pCMV-drR8.2. The medium was changed 12 hours after the transfection and then the lentivirus containing medium was collected. LV-GFP without the inserted gene was used as a control in this study. Virus concentration was determined by transduction of 293 T cells and quantification of transduced cells by flow cytometry for GFP expression. Lentivirus particles (1 × 10^5) were locally administrated into the periodontal defect area with either LV-GFP (*n* = 4) or LV-Ctnnb (*n* = 4) for 2 weeks. Local injection of 1 × PBS (*n* = 3) and LV-GFP (*n* = 4) served as the vehicle control.

**Micro–computed tomography scanning analysis**

Mandibular bone specimens including first, second, and third molars were collected at the designated end points, placed in 4% paraformaldehyde for 24 hours and transferred to 1 × PBS for micro–computed tomography (μCT) scanning. Tissues were scanned by μCT (μC40; SCANCO Medical AG, Brüttisellen, Switzerland) with high resolution of 12 μm, voltage of 45 kVp, and current of 177 mA. The three-dimensional (3D) images were reconstructed from the scans by the μCT system software.

**Histomorphometric analysis**

Following μCT analysis, all the same specimens were rinsed with 1 × PBS buffer and decalcified in 10% EDTA/PBS solution over a period of 4 to 8 weeks, and then embedded in paraffin for histological analyses. The samples were sectioned to a thickness of 5 μm. Serial sections were stained with hematoxylin and eosin (H&E) staining. Heidenhain’s AZAN trichrome (AZAN) staining was also carried out to determine newly formed cementum. AZAN staining provides optimal contrast, with cell nuclei stained dark red by azocarmine and collagen stained blue by aniline blue. Histological images were captured with a Zeiss Axio Scope A1 Microscope (Carl Zeiss Pty, Ltd, Macquarie Park, NSW, Australia).

Newly-formed cementum was defined as new mineralized tissue covering the exposed root surface within the defect. The percentage of newly-formed cementum was calculated as a proportion of newly-formed cementum over the full length of the defect on the root surface. Percentage of new alveolar bone
Fig. 1. Rat periodontal defect model. (A) Schematic illustration of rat mandibular first molar. (B) Schematic illustration of periodontal fenestration defect. (C) Macroscopic observation of the periodontal defect during surgery. (D) The periodontal defect was successfully established. (E, F) Confirmation of periodontal defect after the surgery. μCT scanning was applied to confirm periodontal defect immediately after the surgery. The μCT of 3D construct (E) and 2D images (F) indicate all the periodontal ligament tissues were removed for this study. D = dentin; PDL = periodontal ligament; C = cementum; M1 = mandibular first molar; AB = alveolar bone.
formulation was calculated as a proportion of new bone over the total alveolar bone defect area.

In situ hybridization and immunohistochemistry

RNA-RNA in situ hybridization was used to localize mRNA within the tissue sections. All samples were fixed overnight in 4% formaldehyde solution at 4 °C, decalcified in 1% EDTA, and then embedded in paraffin. Antisense riboprobes labeled with digoxin for the canonical Wnt signaling antagonist Axin 2 were used for in situ hybridization.

Immunohistochemical staining was carried out after dewaxing, hydration, and neutralization of endogenous peroxidase activity. The following primary antibodies were used: cementum protein 1 (CEMP1, 1:100, goat anti-rat; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cementum attachment protein (CAP, 1:100, rabbit anti-rat; Santa Cruz Biotechnology), wingless-type mouse mammary tumor virus (MMTV) integration site family, member 3a (Wnt3a, 1:100, rabbit anti-rat; Abcam, Melbourne, Australia), axis inhibition protein 2 (Axin2, 1:500, rabbit anti-rat; Cell Signaling Technology, Beverly, MA, USA), beta-cadherin-associated protein (β-catenin, 1:500, rabbit anti-rat; Cell Signaling Technology). The sections were incubated with the primary antibodies overnight at 4 °C, followed by incubation at room temperature with a biotinylated universal swine-anti-mouse, rabbit, goat secondary antibody (DAKO, Carpinteria, CA, USA) for 15 min, and then with horseradish peroxidase-conjugated avidin-biotin complex (DAKO) for another 15 min. The antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 minutes. Mayer’s hematoxylin (HD Scientific Pty Ltd.) was used for counterstaining.

Cell culture, proliferation, and differentiation assay

The isolation and culture of hPDLCs and human alveolar bone-derived osteoblasts (hOBs) was performed according to published protocols. Informed consent was given by all patients involved and the research protocol was approved by the Human Ethics Committees of Queensland University of Technology, Brisbane, Australia. hPDLCs and hOBs were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco-Invitrogen) supplemented with 10% vol/vol fetal bovine serum (FBS; Thermo Scientific) and 50 U/mL penicillin and 50 mg/mL streptomycin (P/S; Gibco-Invitrogen) at 37 °C in a humidified CO2 incubator. For osteogenic differentiation, cells were cultured in osteogenic DMEM medium containing 10% FBS, 50 μg/mL ascorbic acid, 3 mM β-glycerophosphate, and 10 mM dexamethasone in the absence or presence of LiCl (Sigma-Aldrich) and LV-Ctnnb.

To further investigate activation of canonical Wnt signaling, a murine cell line with stable overexpression of Wnt3a was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The L-Wnt3a cell line (ATCC CRL2647) was maintained in modified DMEM (ATCC 30–2002) containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. The Wnt3a conditional media was collected according to a published protocol. After culturing for 4 days, the media was obtained and sterile-filtered and fresh media was added to the plates and cultured for an additional 3 days. The two media samples were added to the osteogenic differentiation medium for hPDLCs. Control-conditioned medium was obtained in a similar way using the parental L-cell line (ATCC CRL-2648).

Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the methods as described in our protocols. Briefly, 0.5 mg/mL of MTT solution (Sigma-Aldrich) was added to each well and incubated at 37 °C for 4 hours to form formazan crystals. The formazan was solubilized with dimethyl sulfoxide and the absorbance read at 495 nm in a 96-well plate reader.

Alizarin red staining was carried out as described. The medium was discarded and cells were fixed with ice-cold 100% methanol for 20 min and washed with PBS. Then the cells were stained with alizarin red for 5 min prior to observation. The relative ALP activity in the hPDLCs was assessed at days 7 and 14 after culture in osteogenic differentiation medium. Cells were lysed in a lysis buffer containing 0.2% Triton X-100 and 50 mM Tris-HCl. The whole cell lysis was measured using QuantiChrom Alkaline Phosphatase Kit (BioAssay Systems, Hayward, CA, USA) by adding para-nitrophenylphosphate (pNPP) as a substrate assay buffer containing MgCl2, and then the kinetic of absorbance was read at 405 nm. The enzyme activity was expressed as optical density (OD) value per minute per microgram (μg) of protein. The total protein in the cell lysis was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

The calcium concentration in hPDLCs was measured at days 7 and 14 in culture. Cells were washed twice by distilled water and incubated overnight in 0.6 N HCl at 4 °C. The dissolved calcium was assayed using a QuantiChrom Calcium Assay Kit (BioAssay Systems) following the manufacturer’s instructions.

qRT-PCR and Western blotting analysis

Total cellular RNA was extracted using Trizol reagent (Life Technologies) and reverse transcription was performed using a DyNAmo cDNA Synthesis Kit (Finzymes, Pittsburgh, PA, USA) according to the manufacturers’ instructions. The qRT-PCR primers for each gene are shown in Supporting Table 1. The qRT-PCR was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the relative gene expression was calculated using the following formula: 2−ΔΔCt × 10^4.

Western blotting analysis was performed for the detection of protein expression. The protein concentration was determined by the BCA Protein Assay Kit (Thermo Fisher Scientific) and then 10 μg of proteins from each sample were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Pall Corporation, East Hills, NY, USA). After blocking for 1 hour with Odyssey blocking buffer (Millennium Science, Mulgrave VIC, Australia), the membranes were incubated with primary antibodies against ALP (1:1000, rabbit anti-human; Abcam), OCN (1:2000, rabbit anti-human; Abcam), CAP (1:1000, mouse anti-human; Santa Cruz Biotechnology), β-catenin (1:1000, rabbit anti-human; Cell Signaling Technology), Axin2 (1:2000 rabbit anti-human; Abcam), and GAPDH (1:1000, mouse anti-human; Abcam) overnight at 4 °C and then with fluorescent secondary antibodies (1:4000; Cell Signaling Technology), and then targeted proteins were visualized using the Odyssey infrared imaging system.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS) version 21.0 software (IBM, Armonk, NY, USA). Data were pooled by experimental groups and the mean
and standard deviation (SD) were calculated. One way analysis of variance (ANOVA) followed by Student’s t-test were performed for measuring statistically significant differences between groups for new alveolar bone formation and cementum formation; p values <0.05 were considered to be statistically significant.

**Results**

No cementum formation takes place during periodontal wound healing in control samples. μCT scanning was applied to confirm periodontal defect immediately after the surgery as shown in Fig. 1(E&F), indicating all the periodontal ligament tissues were removed in this study. Four weeks after surgery, no visible adverse reactions, such as root exposure, infection, or suppuration, were observed. Initial inflammation immediately after surgery was comparable across all experimental groups. No acute inflammatory reaction was observed during the healing period. 3D μCT images were reconstructed to evaluate the bony healing of the defect at different time points. As shown in Fig. 2A, mineralized tissue covering the defect could be seen to increase with time.

Periodontal tissue biopsies including gingiva, alveolar bone and tooth were obtained at the different time points. The representative images of H&E and AZAN staining showed that new osteoblasts were formed along the surface of the old bone fragments at weeks 2 and 4 after surgery (Fig. 2B, C). At week 2, a high density of newly deposited connective tissue fibers was formed along the root dentin surface, although the fibers were not well-oriented. At week 4, new bone formation could be seen mainly at the edge of the existing native bone. There was no new cementum found during this process (Fig. 2B, C).

![Fig. 2. Periodontal wound healing in untreated (control) defects. (A) Representative μCT reconstruction images showing increased new bone formation (yellow arrow) over time. (B, C) The representative H&E and AZAN staining images showed the periodontal healing process at 1, 2, and 4 weeks after the surgery. New osteoblasts (black or yellow arrows) were formed along the old bone fragments. There was no cementum formation during the 4-week wound healing period. M1 = mandibular first molar; D = dentin; AB = alveolar bone; PDL = periodontal ligament.](image-url)
Local activation of canonical Wnt signaling with LiCl and Scl-Ab in surgically created periodontal defect

To determine the role of canonical Wnt signaling in alveolar bone repair, the Wnt signaling activators LiCl and anti-sclerostin primary antibody were injected locally at the periodontal defect area for 2 weeks. After 2 weeks, μCT analysis of samples from rats with LiCl and Scl-Ab injections showed an increase in alveolar bone compared with the PBS injected control group (Fig. 3A, B and 4A).

Histological analysis demonstrated that in all groups the original margin of the defect was still identifiable, and this made it possible to distinguish new bone from the old bone. Two weeks after injections, new bone formation could be observed in all groups, with the LiCl and Scl-Ab groups having enhanced new bone formation in the defect site (Fig. 3C, D). There was a significantly higher percentage of bone coverage for LiCl and Scl-Ab groups compared to the PBS injection groups (Fig. 3E) (p < 0.05).

LiCl and Scl-Ab injections enhanced cementum formation

H&E (Fig. 4B) and AZAN (Fig. 4C) histological staining showed that the LiCl and Scl-Ab injection groups had a significant increase of new cementum formation, whereas the control groups showed no cementum formation. After 2 weeks of Scl-Ab injections, newly formed cementum was seen on the denuded root surface with well-orientated PDL collagen fibers inserted (Fig. 4B, C). After 2 weeks, the LiCl injection group also showed obvious cementum formation with cementocytes entrapped and well-oriented Sharpey’s collagen fibers inserted (Fig. 4B, C). There was a significantly higher percentage of cementum coverage for LiCl and Scl-Ab groups compared to the PBS injection group (Fig. 4D) (p < 0.0001).

Overexpression of β-catenin facilitates periodontal regeneration

To further confirm whether the activation of canonical Wnt signaling has an enhanced effect on cementum regeneration, lentivirus particles for overexpression of Ctnnb were injected locally in the periodontal defect area for 2 weeks. Confocal laser scanning microscopy (CLSM) images provided evidence of newly-formed cementoblast-like cells (Fig. 5A, B, yellow triangles) and well-oriented PDL fibers with strong GFP expression in LV-Ctnnb injected animals compared to controls. The representative H&E (Fig. 5C) and AZAN (Fig. 5D) staining images showed that LV-Ctnnb had enhanced cementum formation with well-ordered PDL fibers.

Regeneration mode: detection of canonical Wnt signaling in periodontal regeneration tissues

In order to confirm the presence of newly-formed cementum, immunohistochemical staining was carried out to detect the expression of cementogenesis and osteogenesis related markers. As shown in Fig. 6, both of the cementum markers CEMP1 and CAP were expressed in cementocytes, cementoblasts, and PDL cells in normal and regenerated periodontium; whereas no expression of CEMP1 and CAP was detected in dentin tissue. Compared to the vehicle group, activation of canonical Wnt signaling by LiCl, Scl-Ab, and LV-Ctnnb induced CEMP1 and CAP expression in newly-formed cementocytes and cementoblasts in the new cementum. The bone-related marker ALP was more prominently expressed in newly-formed bone and osteoblasts (Fig. 6).

In situ hybridization and immunohistochemical analysis of Axin2 expression was carried out to investigate whether canonical Wnt signaling was activated in the defect area. In situ hybridization showed weak Axin2 expression in the PBS injection control group. However, there was strong expression of Axin2 detected in newly-formed cementoblast-like cells (Fig. 7A, black triangles) and newly-formed periodontal cells in the LiCl, Scl-Ab, and LV-Ctnnb groups, indicating the activation of canonical Wnt signaling in the periodontal defect area. Immunohistochemistry (IHC) data confirmed that protein expression pattern of Axin2 was observed locally in the periodontal defect area (Fig. 7B). It is noted that activation of canonical Wnt signaling with LiCl, Scl-Ab, and LV-Ctnnb significantly increased Axin2-positive cementoblasts cell numbers when compared to the PBS injection control group (Fig. 7E).

Furthermore, the expression of Wnt3a (Fig. 7C) and β-catenin (Fig. 7D) was also investigated to confirm the activation of canonical Wnt signaling. It was noted that both Wnt3a and β-catenin were strongly expressed in cementocytes, cementoblasts, PDL cells, osteoblasts and osteocytes (Supporting Fig. 1A, B), suggesting the canonical Wnt signaling is required for periodontal regeneration. It is clear that both Wnt3a and β-catenin have increased expression in cementocytes and cementoblasts of newly-formed cementum in the Scl-Ab treatment group compared to the vehicle group. Additionally, LiCl and LV-Ctnnb treatment induced Wnt3a and β-catenin expression in new cementoblasts compared to the control group (Fig. 7C, D).

Activation of canonical Wnt signaling pathway during hPDLCs osteogenic/cementogenic differentiation

LiCl induced activation of canonical Wnt signaling was carried out to investigate whether it can regulate osteogenic/cementogenic differentiation of hPDLCs in vitro. The effect of different concentrations of LiCl on the proliferation of hPDLCs was assessed by MTT assay. The MTT assay results showed that LiCl significantly stimulated proliferation of hPDLCs compared to control groups after 1, 3, and 7 days of culture, with 5 mM of LiCl being the optimum concentration for proliferation (Supporting Fig. 2). Mineralization was examined using Alizarin Red staining, relative ALP activity, and intracellular calcium assay. As shown in Supporting Fig. 2A, the cultures treated with LiCl for 14 days showed a dose-dependent increase of calcium precipitation and matrix mineralization. In parallel with this result, LiCl also increased relative ALP activity and intracellular calcium release (Supporting Fig. 2C).

qRT-PCR and Western blot analysis were carried out to investigate the effect of LiCl treatment on cementoblastic/osteogenic differentiation marker expression by hPDLCs (Fig. 8). The results showed that gene expressions of the osteogenic markers OCN and OPN increased in the presence of LiCl over time compared to the control group (Fig. 8A), as did the expression of the cementogenic markers CEMP1 and CAP (Fig. 8B).

The analysis of the expression of the canonical Wnt-related genes AXIN2 and CTNNB was performed by qRT-PCR after culturing hPDLCs with LiCl (5 mM) for 3, 7, and 14 days. The results showed that the expression of AXIN2 and CTNNB by hPDLCs in the presence of LiCl was significantly higher compared to control groups at days 3, 7, and 14 (Fig. 8C, D).
Fig. 3. Enhanced alveolar bone formation via LiCl and Scl-Ab local injections. (A, B) After 2 weeks of injections, LiCl and Scl-Ab groups had induced bone formation between the first molar roots (yellow arrow) compared to the PBS injection group. (C, D) Representative H&E and AZAN stained sections of the defects after 2 weeks. At the second week, new bone was observed in the LiCl and Scl-Ab injection groups, with PDL fibers inserted. (E) Scl-Ab and LiCl injections enhanced new bone coverage compared to the control group. *Significant difference between Scl-Ab group and PBS group. **Significant difference between LiCl group and PBS group. D = dentin; NAB = newly-formed alveolar bone; PDL = periodontal ligament; NC = newly-formed cementum; C = cementum; AB = alveolar bone.
Western blot analysis was performed on the protein expression of hPDLCs cultured with LiCl for 7 days. The protein expression of bone/cementum markers (OPN, CAP) and Wnt-related proteins (AXIN2, β-CATENIN) increased in the presence of LiCl (Fig. 8E).

The effect of Ctnnb and Wnt3a overexpression on hPDLCs differentiation

hPDLCs were transfected with LV-Ctnnb lentivirus to explore the effect of Ctnnb overexpression in these cells. Strong GFP expression demonstrated that the hPDLCs had been successfully infected by lentivirus (Fig. 9A). Ctnnb gene/protein expression assays further confirmed that LV-Ctnnb infected hPDLCs activated canonical Wnt signaling, resulting in enhanced CAP and β-catenin protein expression (Fig. 9B, C). To further examine the effect of canonical Wnt signaling activation on hPDLCs differentiation, Wnt3a conditioned media was added to osteogenic media in the hPDLC cultures. It was noted that Wnt3a CM induced the activation of canonical Wnt signaling (Supporting Fig. 3), leading to increased gene/protein expression of the osteogenic differentiation markers OCN and OPN, and cementogenic differentiation markers CAP and CEMP1 (Fig. 9D, E).

Fig. 4. Activation of canonical Wnt signaling inducing periodontal regeneration. (A) Representative μCT images of the periodontal healing process at week 2 via activation of canonical Wnt signaling. (B, C) Representative images of H&E (B) and AZAN (C) staining of histological sections of periodontal defect 2 weeks after the local injection in the surgical area. The Scl-Ab and LiCl injection groups had de novo cementum formation with perpendicularly orientated PDL fiber (black arrow) insertion; vehicle injected group had no cementum formation. Black triangles indicate new cementocytes present on the de novo formed cementum. (D) Scl-Ab and LiCl significantly enhanced new cementum coverage compared to the control group. *Significant difference between Scl-Ab group and PBS group. **Significant difference between LiCl group and PBS group. D = dentin; AB = alveolar bone; PDL = periodontal ligament; NC = newly-formed cementum; C = cementum.
Canonical Wnt signaling in differentiation of osteoblasts

To investigate the role of canonical Wnt signaling in the cementogenic differentiation of alveolar bone derived osteoblasts, LiCl were applied to the culture medium. qRT-PCR results showed a failure of osteoblasts to express the CAP gene and protein in response to LiCl treatment (Supporting Fig. 4B). However, LiCl treatment did result in enhanced AXIN2 and β-catenin protein expression by the osteoblasts (Supporting Fig. 4C).

Discussion

This study was conducted to investigate the interaction between the activation of the canonical Wnt signaling pathway and cementum repair/regeneration in vivo and in vitro. To the best of our knowledge, this is the first study to investigate the effect of local activation of canonical Wnt signaling in a healing periodontal defect. The results showed that the activation of canonical Wnt signaling stimulates de novo alveolar bone formation, with perpendicularly-orientated inserting PDL fibers and cementum formation. In addition, the regulation of canonical Wnt signaling was also investigated in vitro in relation to hPDLC proliferation and osteogenic/cementogenic differentiation. These data indicate that the canonical Wnt signaling pathway is an important regulator for cementum regeneration and suggest that this signaling pathway can be a promising target for the treatment of periodontal disease.

The rat is frequently used as a model for studies of periodontal disease and tissue regeneration due to cost effectiveness and ease of handling; however, the typical defect size is relatively small, which makes defect creation a challenging task. It is particularly difficult, during the bone and cementum removal process, to irrigate with saline due to the small defect size, and
thin bone and cementum.\(^{27,28}\) We can report that in this study, the rat periodontal defect model was successfully established (Fig. 1), with the exposed tooth root surface documented by macroscopic and \(\mu\)CT assessment immediately following surgery. Although some periodontal tissues were formed 4 weeks after the surgery, including periodontal ligament fibers and alveolar bone, cementum formation was not observed in this model for the 4-week duration of the study. There is much evidence to support the essential role of canonical Wnt signaling in tooth development and the transition of tooth morphogenesis from bud to cap stage, suggesting the potential use of Wnt activation in strategies for tooth regeneration.\(^{13}\) However, the molecular mechanism of canonical Wnt signaling in cementum regeneration is largely unknown. For this reason, it is important to elucidate the involvement of the canonical Wnt signaling pathway in new cementum formation in order to establish its role during periodontal regeneration.

Many studies have suggested that LiCl and anti-sclerostin neutralizing antibody could enhance in vitro cell proliferation and improve bone mass in mice in vivo by activating the canonical Wnt signaling pathway.\(^{20,29,30}\) The pharmacological inhibition of sclerostin using neutralizing monoclonal antibodies has been studied in clinical trials for postmenopausal osteoporosis, and in preclinical models of osteoporosis, bone repair, and fracture healing.\(^{31–33}\) Moreover, oral use of lithium has been used to target the Wnt signaling pathway for the treatment of humans with bipolar disease for over a half-century, with substantial benefit.\(^{34–36}\) Few studies have specifically addressed the effect of lithium therapy on bone metabolism. Lithium therapy has been shown to not only increase bone mass and density clinically, but also restore bone mass in LRPS knockout mice and increase fracture healing in mice, indicating a targeted effect on the Wnt pathway.\(^{19,20}\) In both the preclinical and clinical settings, administration of Scl-Ab and LiCl is consistently associated with increased bone formation and bone density at several sites throughout the body and has been linked to activation of canonical Wnt signaling; however, effects within the periodontal complex have never been studied. Our study provides early preclinical evidence that Scl-Ab and LiCl have a significant role in periodontal regeneration. We can report that within 2 weeks of local administration of Scl-Ab and LiCl in periodontal defect sites, alveolar bone showed significantly greater bone volume and mineral density relative to a vehicle control. More importantly, it was observed that the Scl-Ab and LiCl treatment groups had significantly enhanced de novo cementum regeneration with well-organized periodontal ligament fibers inserted compared to the control group. Similar results were obtained in terms of newly-formed cementum, alveolar bone, and periodontal fibers, suggesting that both LiCl and Scl-Ab act in a similar fashion to induce new cementum/periodontal ligament fibers inserted compared to the control group. Axin2 is recognized as the most accurate reporter gene in the canonical Wnt pathway because it is a direct target gene of Wnt ligand binding and activation of the Wnt signaling pathway.\(^{37,38}\) Overexpression of \(\beta\)-catenin in this study enhanced cementum formation with perpendicularly-oriented inserted PDL fibers.

Axin2 is recognized as the most accurate reporter gene in the canonical Wnt pathway because it is a direct target gene of Wnt ligand binding and activation of the Wnt signaling pathway\(^{39}\) and has been found to be expressed during tooth formation, including both crown and root development\(^{12,39}\). It is known that Axin2 is expressed in the periodontal ligament during cementum formation, as described\(^{40,41}\). In this study, the in situ hybridization and IHC results demonstrated that Axin2 transcript/protein expression was increased in response to Scl-Ab, LiCl, and LV-Ctnnb treatments compared to the controls, whereas there was only weak Axin2 expression in the control group. The Axin2 expression coincided with newly-formed cementum formation, as described.\(^{40,41}\) In this study, the in situ hybridization and IHC results demonstrated that Axin2 transcript/protein expression was increased in response to Scl-Ab, LiCl, and LV-Ctnnb treatments compared to the controls, whereas there was only weak Axin2 expression in the control group. The Axin2 expression coincided with newly-formed cementum formation, as described.\(^{40,41}\)

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**Fig. 6.** The expression pattern of bone/cementum-related markers during periodontal regeneration. After injection of Scl-Ab, LiCl, and LV-Ctnnb for 2 weeks, CEMP1 and CAP expression was detected in newly-formed cementoblasts (black triangles) and cementocytes (red triangles). Bone-related marker ALP was expressed in new osteoblasts (black arrows) along the newly-formed bone with activation of canonical Wnt signaling treatments. D = dentin; PDL = periodontal ligament; NC = newly-formed cementum; NAB = newly-formed alveolar bone; AB = alveolar bone; C = cementum.
Sharpey’s fibers and cementoblasts in the LiCl, Scl-Ab and LV-Ctnnb treatment groups, indicating that activation of canonical Wnt signaling is correlated with periodontal regeneration. Moreover, activation of canonical Wnt signaling by LiCl, Scl-Ab and LV-Ctnnb also induced strong expression for both Wnt3a and β-catenin in cementoblasts within the newly-formed cementum compared to the control. It appears that upregulation of canonical Wnt signaling can have a similar cementogenic effect during periodontal wound healing as it does in cementum development, which may provide the rationale for an entirely new approach to cementum regeneration.

A number of experimental studies have previously demonstrated that hPLDCs can be differentiated into osteoblasts, fibroblasts, and cementoblast-like cells in vivo and in vitro, a process that is mediated by BMP-like molecules. Recent findings demonstrated that the Wnt/β-catenin signaling pathway stimulates cell proliferation and osteoblastic differentiation in hPDLCs. However, it is unknown whether canonical Wnt signaling is involved with hPDLC osteogenic/cementogenic differentiation. In this study, LiCl, a GSK-3β inhibitor, was used to examine the possible involvement of canonical Wnt signaling in regulating hPDLC behavior. In agreement with many recent reports, our results showed that activation of canonical Wnt signaling could enhance the expression of the late osteoblast makers OPN and OCN, and the cementoblast-related marker CAP. As a control group, human alveolar bone–derived osteoblasts showed no expression of CAP in the presence of activated canonical Wnt signaling (Supporting Fig. 2). To further investigate activation of canonical Wnt signaling, Wnt3a conditioned media significantly enhanced the cementogenesis-related marker CAP at both gene and protein levels. These findings indicate that hPDLCs, rather than hOBs, are the desirable target cell type for the induction of cementogenic differentiation.
We put forward the hypothesis that activation of the canonical Wnt signaling pathway could be a beneficial therapy for in vivo cementum repair and regeneration; however, this concept needs to be further investigated with other regulators of the Wnt signaling pathway. Additionally, future evaluation of cementum forming capacity is required in large animal models to demonstrate that canonical Wnt signaling can initiate bone/cementum regeneration as well as its crosstalk with other signaling pathways in relation to the differentiation of PDLCs to cementoblasts.
Fig. 9. Overexpression of Ctnnb and Wnt3a for cementum differentiation of hPDLCs. (A) GFP expression is visible following infection of hPDLCs with lentivirus containing Ctnnb gene. (B) LV-Ctnnb infection results in enhanced gene expression of Ctnnb. *Significant difference between LV-Ctnnb group and Osteo group at day 3. **Significant difference between LV-Ctnnb group and Osteo group at day 7. (C) LV-Ctnnb increased CAP and β-catenin protein expression. Noninfected cells and LV-GFP–infected cells were used as controls. (D) Wnt3a CM led to enhanced OCN, OPN, CEMP1, and CAP gene expression. (E) Protein expression of OCN, OPN, and CAP was increased after 7-day culture with Wnt3a CM compared to that of non-treatment. *Significant difference between Wnt3a CM group and non-treatment group at day 3. **Significant difference between Wnt3a CM group and non-treatment group at day 7. CM = conditional media; L = lentivirus; O = Osteo; Osteo = osteogenic differentiation.
Conclusion

In summary, local injections of LiCl, Sc1-Ab, and LV-Ctnnb in a rat periodontal defect model resulted in de novo cementum formation with well-oriented PDL fibers, via the regulation of the canonical Wnt signaling pathway. Canonical Wnt signaling activation by LiCl and Wnt3a conditioned media significantly enhanced bone/cementum-related gene/protein expression in hPDLcs. Targeting the canonical Wnt signaling pathway may represent a novel strategy for promoting periodontal regeneration.

Disclosures

All authors state that they have no conflicts of interest.

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