Regulation of Canonical Wnt Signalling Pathway during Cementum Regeneration

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Abstract

The tooth is a common research model used in the study of molecular mechanisms of organ development. The canonical Wnt signalling pathway is known to play an imperative role in tooth pattern development and cell differentiation, which is responsible for tooth root formation. Research in the area of periodontal regeneration has also revealed similar cellular interactions and signalling activation. Cementum regeneration is one of the most difficult and challenging phases during periodontal tissue regeneration. Although there are increasing studies on cementum regeneration using various techniques including novel cell-based approaches, cementum regeneration is still not established or fully understood, nor are the molecular mechanisms. The purpose of this project was to address the question whether canonical Wnt signalling pathway regulates cementum regeneration and repair *in vitro* and *in vivo*. This research also endeavoured to develop suitable biomaterials for the cementum/periodontal tissue engineering *in vitro* with respect to the regulation of canonical Wnt signalling pathway.

For this purpose, a rat periodontal defect model was created to investigate the periodontal wound healing process. Micro-computed tomography (Micro-CT) scanning and histology studies further confirmed the periodontal defect and the healing process. Afterwards, the activation of canonical Wnt signalling at the periodontal defect was performed by local delivery of either neutralized anti-sclerostin antibody (Scl-Ab) or a Wnt enhancer (lithium chloride, LiCl) or lentivirus particles with gene of β-catenin (LV-Ctnnb). The results showed that activation of canonical Wnt signalling could induce formation of new cementum and well-oriented periodontal ligament fibres compared to the non-treatment group. This study further conducted *in vitro* experiments to explore whether canonical Wnt signalling regulates cementogenic differentiation of human periodontal ligament cells (hPDLCs) with gain-of-function analysis in respect to canonical Wnt signalling pathway. Western blotting and qRT-PCR assays were utilized to investigate the effect of canonical Wnt signalling pathway in cell proliferation and osteogenic/cementogenic differentiation in hPDLCs. The data demonstrated that canonical Wnt signalling pathway was involved in the progress of hPDLCs

osteogenic differentiation towards cementoblast-like cells. These findings thus provide clear evidence that canonical Wnt signalling pathway plays an important role during cementum regeneration *in vivo* and *in vitro*, suggesting that canonical Wnt signalling could be a potential target for periodontal regeneration.

Subsequently, canonical Wnt signalling activator of LiCl was incorporated into mesoporous bioactive glass (MBG) scaffolds. This approach yielded scaffolds that had a favourable composition, microstructure and mesopore properties for cell attachment, proliferation, and cementogenic differentiation of human periodontal ligament-derived cells (hPDLCs). The results showed that Li^+ ions, when incorporated into MBG scaffolds at the concentration of 5%, enhanced the proliferation and cementogenic differentiation of hPDLCs.

Subsequent experiments were performed to also determine whether Li-containing β-tricalcium phosphate (Li-β-TCP) bioactive ceramics could effectively induce this differentiation for both hPDLCs and human bone marrow stromal cells (hBMSCs). β-TCP is a widely accepted bone substitute material compared with MBG even though its bioactivity is lower than MGB. The results showed that Li-β-TCP bioceramic disks supported cell attachment and proliferation, and significantly enhanced bone/cementum-related gene expression and canonical Wnt signalling pathway activation in both hPDLCs and hBMSCs. These findings demonstrated that Li-β-TCP bioceramics possess excellent *in vitro* osteogenic/cementogenic stimulation properties, indicating that they could act as a promising biomaterial for bone/cementum regeneration.

In conclusion, the body of work presented in this thesis has demonstrated that activation of canonical Wnt signalling pathway can lead to cementum regeneration *in vivo* and *in vitro*. Activation of canonical Wnt signalling pathway by bioactive materials may provide potential therapeutic strategies for periodontal tissue regeneration.

Keywords: canonical Wnt signalling pathway, rat periodontal defect model, periodontal tissue engineering, human periodontal ligament cells, Li-containing mesoporous bioactive glass, Li-containing β-tricalcium phosphate

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List of Publications and Presentations

The following is a list of awards granted during the PhD candidature:

1. Award: Australasian Society for Biomaterials and Tissue Engineering (ASBTE) conference award, 2014
   Presentation title: Application of Wnt canonical signalling enhancer in cementum regeneration
2. Award: Third Prize for Best Oral Presentation, Asia-Pacific Tissue Engineering and Regenerative Medicine International Society (TERMIS-AP) conference, 2013
   Presentation title: Lithium-containing β-tricalcium phosphate (β-TCP) inducing cementogenic/ osteogenic differentiation
3. Award: The Australian Dental Industry Association (ADIA) Research Award, 2011
   Project title: Investigation of hedgehog signalling pathway during cementum regeneration in rat root wood healing model

The following is a list of published, accepted or submitted manuscripts during the PhD candidature ([1] co-first author):

1. **Pingping Han**, Chengtie Wu, Jiang Chang, Yin Xiao. The cementogenic differentiation of periodontal ligament cells via the activation of Wnt/β-catenin signalling pathway by Li^+ ions released from bioactive scaffolds. **Biomaterials.** 2012, 33(27): 6370-6379. (IF: 8.3, Cited time: 20)

2. **Pingping Han**, Chengtie Wu, Yin Xiao. The effect of silicate ions on proliferation, osteogenic differentiation and cell signalling pathways (WNT and SHH) of bone marrow stromal cells. **Biomaterial Science.** 2013, 1: 379–392 (IF: new journal by **Royal Society of Chemistry**, Cited time: 29)

3. **Pingping Han**, Mengchi Xu, Jiang Chang, Nishant Chakravorty, Chengtie Wu, Yin Xiao. Lithium release from β-tricalcium phosphate inducing cementogenic and osteogenic differentiation for both hPDLCs and hBMSCs.

Biomaterials Science. 2014, 2 (9), 1230 – 1243 (IF: new journal by **Royal Society of Chemistry**)

4. **Pingping Han**, Saso Ivanovski, Ross Crawford, Yin Xiao. Activation of the canonical Wnt signaling pathway induces cementum regeneration. Major revision from **Journal of Bone and Mineral Research** (IF: 6.58)

5. **Pingping Han**, Saso Ivanovski, Ross Crawford, Yin Xiao. Pro-inflammatory cytokines regulates cementogenic differentiation of periodontal ligament cells by Wnt/Ca^{2+} signalling pathway. Under review by **Journal of Molecular Medicine** (IF: 4.73)

6. Xufang Zhang[1], **Pingping Han[1]**, Anjali Jaiprakash, Chengtie Wu, Yin Xiao. Stimulatory effect of $Ca_3ZrSi_2O_9$ bioceramics on cementogenic/osteogenic dif ferentiation of periodontal ligament cells. **Journal of Materials Chemistry B.** 2014, 2, 1415-1423 (IF: 6.62)

7. Chengtie Wu[1], **Pingping Han[1]**, Xiaoguo Liu, Mengchi Xu, Tian Tian, Jiang Chang, Yin Xiao. Mussel-inspired bioceramics with self-assembled Ca-P/polydopamine composite nanolayer: Preparation, formation mechanism, improved cellular bioactivity and osteogenic differentiation of bone marrow stromal cells. **Acta Biomaterialia.** 2014, (10): 428–438 (IF: 5.68, cited time: 2)

8. Chengtie Wu[1], **Pingping Han[1]**, Mengchi Xu, Xufang Zhang, Yinghong Zhou, Guangda Xue, Jiang Chang, Yin Xiao. Nagelschmidtite bioceramics with osteostimulation property: material chemistry activating osteogenic genes and WNT signalling pathway of human bone marrow stromal cells. **Journal of Materials Chemistry B.** 2013, 1: 876-885. (IF: 6.62, Cited time: 12)
9. Yinghong Zhou, Chengtie Wu, Xufang Zhang, **Pingping Han**, Yin Xiao. The ionic products from bredigite bioceramics induced cementogenic differentiation of periodontal ligament cells via activation of Wnt/β-catenin signalling pathway. **Journal of Materials Chemistry B,** 2013, 1: 3380-3389 (IF: 6.62, Cited time: 3)

10. Chengtie Wu, Yinghong Zhou, Mengchi Xu, **Pingping Han**, Lei Chen, Jiang Chang, Yin Xiao. Copper-containing mesoporous bioactive glass scaffolds with multifunctional properties of angiogenesis capacity, osteostimulation and antibacterial activity. **Biomaterials.** 2013, 34 (2): 422-33 (IF: 8.3, Cited time: 38)

11. Chengtie Wu, Yinghong Zhou, Wei Fan, **Pingping Han**, Jiang Chang, Jones Yuen, Meili Zhang, Yin Xiao. Hypoxia-mimicking mesoporous bioactive glass scaffolds with controllable cobalt ion release for bone tissue engineering. **Biomaterials.** 2012, 33(7): 2076-85 (IF: 8.3, Cited time: 56)

12. Wei Fan, Chengtie Wu, **Pingping Han**, Yinghong Zhou, Yin Xiao. Porous Ca–Si-based nanospheres: A potential intra-canal disinfectant-carrier for infected canal treatment. **Materials Letters.** 2012, 18: 16-19 (IF: 2.26, Cited time: 3)

The following is a list of conference presentations during the PhD candidature:

1. **Pingping Han**, Chengtie Wu, Saso Ivanovski, Ross Crawford, Yin Xiao. Application of Wnt canonical signalling enhancer in cementum regeneration. **Europe Tissue Engineering and Regenerative Medicine International Society (TERMIS-EU).** 10^{th} -13^{rd} June 2014, Genova, Italy. Oral Presentation

2. **Pingping Han**, Chengtie Wu, Zetao Chen, Saso Ivanovski, Ross Crawford, Yin Xiao. Wnt enhancer of lithium chloride in cementum regeneration application. **Asia-Pacific Tissue Engineering and Regenerative Medicine International Society (TERMIS-AP).** 24^{th}-27^{th} September 2014, Daegu, Republic of Korea. Oral Presentation

3. **Pingping Han**, Chengtie Wu, Saso Ivanovski, Ross Crawford, Yin Xiao. Application of Wnt canonical signalling enhancer in cementum regeneration. **Australasian Society for Biomaterials and Tissue Engineering (ASBTE).** 22^{nd} -24^{th} April 2014, Victoria, Australia. Oral Presentation

4. **Pingping Han**, Mengchi Xu, Jiang Chang, Nishant Chakravorty, Anjali Tumkur Jaiprakash, Ross Crawford, Chengtie Wu, Yin Xiao. Lithium-

containing β-tricalcium Phosphate Inducing Cementogenic/osteogenic Differentiation of Human Periodontal Ligament Cells and Human Bone Marrow Stromal Cells. **Asia-Pacific Tissue Engineering and Regenerative Medicine International Society (TERMIS-AP).** 23rd-26th October 2013, Shanghai, China. Oral Presentation

5. **Pingping Han**, Chengtie Wu, Jiang Chang, Yin Xiao. Lithium-containing bioactive scaffolds improved cementogenic differentiation of periodontal ligament cells. **Australian & New Zealand Orthopaedic Research Society.** 30th August-1th September 2012, Perth, Australia. Oral presentation

6. **Pingping Han**, Chengtie Wu, Jiang Chang, Ross Crawford, Yin Xiao. Lithium (Li)-containing bioactive scaffolds improved cementogenic differentiation of periodontal ligament cells via the activation of Wnt/β-catenin signalling pathway by released Li^+ ions. **The Prince Charles Hospital Research Forum**. 18th-19th October 2012, Brisbane, Australia. Oral presentation

7. **Pingping Han**, Chengtie Wu and Yin Xiao. The Effect of silicate ions on proliferation, osteogenic differentiation, and cell signalling pathways (Wnt and Shh) of bone marrow stromal cells. **Asia-Pacific Bone & Mineral Research Meeting.** 2nd-4th September 2012, Perth, Australia. Poster presentation

8. **Pingping Han**, Chengtie Wu, Jiang Chang, Ross Crawford, Yin Xiao. Cementogenic differentiation of periodontal ligament cells via the activation of Wnt/β-catenin signalling pathway. **2012 International Conference on Biomaterial and Bioengineering.** 19th-20th December 2012, Hong Kong, China.

9. **Pingping Han**, Wei Fan, Ross Crawford, Saso Ivanovski, Yin Xiao. Activation of Wnt/β-catenin signalling stimulates cementum regeneration in rat periodontal defect model. **Brisbane Cell & Development Meeting.** 1st-2nd September 2012, Brisbane, Australia. Poster presentation.

List of Abbreviations

AB	Alveolar bone
ACP	Amorphous calcium phosphate
ALP	Alkaline phosphatase
APC	Adenomatous polyposis coli
ASCs	Adipose-derived stem cells
AXIN	Axis inhibition proteins
AZAN	Heidenhain's AZAN trichrome staining
bp	Basepair
β-catenin/CTNNB	Beta-cadherin-associated protein
β-TCP	Beta-tricalcium phosphate
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
^{o}C	Degrees Celsius
CaMKII	Calcium calmodulin kinase II
CAP	Cementum attachment protein
CLSM	Confocal laser scanning microscopy
COL	Collagen
CDS	Coding sequence
cDNA	Complementary dioxyribonucleic acid
CEMP1	Cementoblastoma-derived protein cementum protein 1
ChIP	Chromatin immunoprecipitation
DAB	3, 3'-diaminobenzidine
DE	Dental epithelium
DM	Dental mesenchyme
DP	Dental papilla
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
Dex	Dexamethasone
Dhh	Desert hedgehog
Dkks	Dickkopf family

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dspp	Dentin sialophosphoprotein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EK	Enamel knot
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
Fz	Frizzled
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF	Growth factors
GSK3β	Glycogen synthase kinase 3β
GTR/GBR	Guided tissue/bone regeneration
HAp	Hydroxylapatite
hBMSCs	Human bone marrow stromal cells
HCl	Hydrogen chloride
H&E staining	Hematoxylin & Eosin staining
HERS	Hertwig's epithelial root sheath
HMDS	Hexamethyldisilizane
Hhs	Hedgehog protein family
hOBs	Human alveolar bone derived osteoblasts
hPDLCs	Human periodontal ligament cells
ICP-AES	Inductive coupled plasma atomic emission spectrometry
Ihh	Indian hedgehog
iPSCs	Induced pluripotent stem cells
ISH	*In situ hybridization*
JNK	Jun n-terminal kinase
Kb	Kilobases
kDa	Kilodalton
KO	Knockout
LDL	Low-density lipoprotein

LEF	Lymphoid enhancer factor
LiCl	Lithium chloride
LRP	Low-density-lipoprotein receptor related protein
MBG	Mesoporous bioactive glasses
MERF	Medical Engineering Research Facility
mg	Milligram
Micro-CT	Micro-computed tomography
mL	Milliliter
mM	Millimolar
MMLV	Moloney murine leukemia virus
mOBs	Mandible osteoblasts
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal cell
MT T	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
NAB	Newly-formed alveolar bone
NC	Newly-formed cementum
NBG	Non-mesoporous bioactive glasses
OCN	Osteocalcin
OD	Optical density
OPN	Osteopontin
OVX	Ovariectomized
PBS	Phosphate buffered saline
PCP	Planar cell polarity pathway
PCR	Polymerase chain reaction
PDGF	Platelet-rich growth factor
PDL	Periodontal ligament
PDLC	Periodontal ligament cell
PFA	Paraformaldehyde
PIPAAm	Poly N-isopropylacylamide
PKC	Protein kinase C
PLA	Polylactic acid
PLC	Phospholipase C
P/S	Penicillin and streptomycin

qRT-PCR	Real time quantitative reverse transcription polymerase chain reaction
ROCK	Rho-associated kinase
RNA	Ribonucleic acid
SCAP	Stem cells from apical papilla of human teeth
Scl-Ab	Sclerostin antibody
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide
SEM	Scanning electron microscopy
SDS	Sodium dodecyl sulfate
SFRP	Secreted frizzled-related protein
SHED	Stem cells from exfoliated deciduous teeth
Shh	Sonic hedgehog
STG	Secondary tooth germ
TBST	Tris buffered saline-Tween
TCF	T cell-specific transcription factor
TEM	Transmission electron microscopy
TGF-β	Transforming growth factors β
TNF	Tumor necrosis factors
WB	Western blot
WIF-1	Wnt inhibitory factor
Wnt	Wingless-type MMTV integration site family member
XRD	X-ray diffraction

According to the official International Committee recommendation on Standardized Genetic Nomenclature, gene names are written in *italics* and protein names in CAPITALS in the text.

For example ***Wnt* gene-rat, *WNT* gene-human**

Wnt protein-rat, WNT protein-human

Wnt signalling pathway in general

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

Date: 19/08/14

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Chapter 1: Introduction

1.1 BACKGROUND

The tooth is a common research model used in the study of the molecular mechanisms of organ development [1-2]. Tooth formation is a typical epithelial-mesenchymal interaction with a series of cellular signals between oral epithelium and crest-derived mesenchyme [3]. Among all the signals, the canonical Wnt signalling pathway is known as an imperative guiding signal system in the embryonic course of pattern formation and differentiation responsible for tooth crown and root formation [4].

Periodontium consists of not only soft tissues (gingival and periodontal ligament) but also hard tissues (bone and cementum). Current non-surgical or surgical treatments cannot achieve regeneration of the lost tissues, including periodontal ligament, alveolar bone and cementum, thus resulting in inferior aesthetics and function [5-6]. The goal of regenerative periodontal therapy is to reform a tooth supporting tissues, which have been lost following dental trauma or periodontal disease. Therefore, novel techniques are required for successful regeneration, which is difficult as the periodontium complex structure consists of soft and hard tissues. The formation of new cementum is the most important event in periodontal regeneration, but is being a unique and poorly understood phenomenon. The regeneration of cementum is crucial because fibrous filaments from the periodontal ligament have to be inserted into the cementum, thus functionally anchoring the tooth. Although there are increasing research efforts concentrating on cementum regeneration using cell sheet techniques and other novel cell-based approaches, no establishment or molecular mechanisms of cementum regeneration have been reported to date [7]. Therefore, regenerating functional cementum *in vivo* still poses a challenge.

Along with developments in molecular and cellular biology, knowledge of the underlying mechanisms of periodontal tissue engineering techniques has thrown a new light on the repair of periodontal complex. The realm of periodontal tissue regeneration has a plethora of unanswered questions and challenges, due to the

complexity of restoring mineralised and fibrous connective tissues are ultimately to be covered by epithelium in a very specific spatial organisation. Wound healing of the periodontium follows a highly ordered sequence of events that guides cellular morphology, differentiation, migration and proliferation and comprises a series of cellular, extracellular and molecular reciprocal interactions [8-9].

In canonical Wnt signalling, the neutralized sclerostin (a negative Wnt pathway regulator) antibody (Scl-Ab) was proven to activate the canonical Wnt signalling to promote bone regeneration in fracture healing [10-11]. Lithium chloride (LiCl), known as a glycogen synthase kinase 3β (GSK3β) inhibitor, is capable of enhancing *in vivo* bone mass through activation of canonical Wnt signalling pathway in cultured calvarial osteoblasts from *Lrp5*$^{-/-}$ mice [12], suggesting that activation of the Wnt signalling pathway may assist in regeneration of bone and associated periodontal tissues. Therefore, it is reasonable to hypothesize that this current knowledge may further assist in investigating the regulation of Wnt signalling in periodontal regeneration application.

Biomaterials, such as natural biomaterials, synthetic polymers and bioceramics, have been use in clinical practise for treatment of damaged or lost bone for several decades [13]. These materials serve as carriers to deliver therapeutic cells and/or various related growth factors to restore the periodontium [14-16]. The biomaterials have been designed with suitable pore size and greater porosity to maximize the cell attachment with the materials [15]. Many methods have been developed to increase the efficiency of regenerative capability by combining the growth factors of bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) with the scaffold, as well as gene transfection [17-18]. However, due to difficulties associated with the controlled release of growth factors, the results to date have been disappointing, causing an unpredictable degree of vascularization and even oncogenesis. Thus, it is of great importance to design a material that can carry other easily controlled signalling molecules that are safer than growth factors.

It is speculated that understanding the role of activation of the Wnt signalling pathway in periodontal tissue engineering will enable us in deciphering the molecular basis of cementogenesis and cementum regeneration. Furthermore, lithium may be applied in the biomaterials to activate canonical Wnt signalling, which could be

another new approach for periodontal tissue engineering. However, some major concerns in relation to this concept exist in the reconstruction of periodontal tissues, namely insufficient understanding of cementogenesis and cementum regeneration, biomaterials designs and immunological concerns.

1.2 PURPOSE OF THIS RESEARCH

The main purpose of this study was to investigate the role of canonical Wnt signalling pathway during cementogenic differentiation *in vitro* and cementum regeneration *in vivo*; a suitable biomaterial was designed by the activation of canonical Wnt signalling for periodontal tissue engineering *in vitro*.

1.3 QUESTIONS TO BE ANSWERED

1. Did activation of canonical Wnt signalling pathway stimulate periodontal tissue regeneration *in vivo* and human periodontal ligament cells (hPDLCs) differentiation into cementobolas-like cells *in vitro*?

2. Was it possible to activate canonical Wnt signalling in mesoporous bioactive glasses (MBG) for *in vitro* cementogenic differentiation of human periodontal ligament cells (hPDLCs)?

3. Could human bone marrow stromal cells (hBMSCs) be another alternative for periodontal tissue engineering? Could we design a new potential material of beta-tricalcium phosphate (β-TCP) for periodontal tissue engineering?

1.4 POSSIBLE OUTCOMES AND SIGNIFICANCE

Based on the role of canonical Wnt signalling pathway during cementogenesis, the concept of activation of Wnt signalling for periodontal regeneration, especially cementum regeneration, was emphasized in this project. More knowledge may be revealed throughout this project about canonical Wnt signalling pathway and cementum interactions. This project was believed to be the first to investigate the important role of canonical Wnt signalling during cementogenic differentiation *in vitro* and cementum regeneration *in vivo*. This project was also a pioneer in the design of a biological biomaterial with activation of canonical Wnt signalling for periodontal tissue engineering. It may reveal the potential molecular mechanism of

periodontal repair that could provide another approach for periodontal repair in the clinic. The newly designed biomaterial in this project could be manufactured and marketed as a medical product, thereby potentially providing a commercial outcome.

Chapter 2: Hypothesis & Aims of Research

2.1 HYPOTHESIS

Activation of canonical Wnt signalling could enhance cementum regeneration *in vivo* and *in vitro*; the suitable biomaterials can be designed by activation of canonical Wnt signalling for periodontal tissue engineering

2.2 AIMS OF RESEARCH

The specific objectives of the study were as follows:

Aim 1: To investigate regulation of canonical Wnt signalling in cementum regeneration using a periodontal defect model and cementogenic differentiation for hPDLCs

Aim 2: To design lithium-containing biomaterials to stimulate canonical Wnt signalling for periodontal tissue engineering (divided into two parts)

2.3 OVERALL RESEARCH DESIGN

The project was designed as three separate parts, based on the aims described above:

2.3.1 Part one (Chapter 4):

To understand the role of canonical Wnt signalling pathway during *in vivo* cementum repair and *in vitro* cementogenic differentiation for hPDLCs

Rat periodontal defect establishment

The periodontal defect was created as described according to the previous publication [19]. The mandibles of twelve female Lewis rats were harvested and fixed at 7, 14 and 28 days after surgery. Micro-CT was carried out to confirm the periodontal defect and analyse the alveolar bone formation. After decalcification, the tissues were performed with Hematoxylin & Eosin (H&E) staining. Heidenhain's AZAN trichrome (AZAN) staining was also used to assess the wound healing with

blue collagen fibres, cartilage matrix and red cell nuclei. Changes in the structure along with the root dentin were analyzed from different groups. Subsequent immunohistochemical staining was performed using several specific antibodies to identify the cell populations in different groups.

The activation of the canonical Wnt signalling in rat model

LiCl, Scl-Ab and lentivirus particles expression β-catenin (LV-Ctnnb) were locally injected at the periodontal defect. The mandible from eight female Lewis rats were harvested and fixed at 14 days after surgery. Micro-CT was carried out to confirm the periodontal defect and analyse the alveolar bone formation. After decalcification, the tissues were assessed with H&E and AZAN staining. Changes in the structure along with the root dentin were analyzed from different groups. Subsequent immunohistochemical staining was performed using several specific antibodies to identify the cell populations in different groups.

Regulation of Wnt signalling in human periodontal ligament cells (hPDLCs)

Isolation and culture of human PDLCs were performed according to previously published protocols [20]. Teeth were obtained from six systemically healthy patients (18–25 years old) undergoing third molar extraction surgery. Informed consents were given to all patients involved and the research protocol was approved by the Human Ethics Committees of Queensland University of Technology, Brisbane, Australia. Periodontal ligament tissues were separated from the middle third of the root surface using a scalpel and were cultured in a T25 flask in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS; Thermo Scientific) and 50 U/mL penicillin and 50 mg/mL streptomycin (P/S; Gibco-Invitrogen) at 37^{o}C in a humidified CO_2 incubator. The medium was changed after five days and the outgrown cells growing around the PDL tissues were passaged at approximately 80% confluence and expanded through two passages to obtain a sufficient number of cells for the *in vitro* assays. Cells at passages P2-P5 were used for the study.

For osteogenic differentiation, cells were cultured in osteogenic differentiation DMEM medium which was supplemented with 10% FBS, 50μg/ml ascorbic acid (Sigma, Australia), 3mM β-glycerophosphate (Sigma, Australia) and 10nM

dexamethasone (Sigma, Australia). To activate canonical Wnt signalling, different concentrations of lithium chloride (LiCl at 0, 2.5, 5, 10 and 20 mM) were supplemented into osteogenic media in hPDLCs cultures. To further investigate the effect of canonical Wnt signalling during this process, LV-Ctnnb was supplemented in the osteogenic medium.

The effect of canonical Wnt signalling in gene/protein expression for hPDLCs

After 7 days culture under osteogenic differentiation, the expression of relevant cementogenic markers of *cementum attachment protein (CAP)*, canonical Wnt signalling molecules of *axis inhibition protein 2 (AXIN2)* and *beta-cadherin-associated protein (β-catenin, CTNNB)* was determined by RT-qPCR, western blot and immunohistochemistry staining, and compared between differentiated hPDLCs and human osteoblasts (hOB) from jaw bone without LiCl or LV-Ctnnb.

2.3.3 Part two (Chapter 5):

<u>To design mesoporous bioactive glass (MBG) scaffolds for periodontal tissue engineering</u>

Preparation of Li-MBG scaffolds

Porous lithium-containing mesopore-bioglass (Li-MBG) scaffolds were prepared by incorporating Li (molar: 0, 2 and 5%) into MBG. The large-pore structure and inner microstructure of the calcined Li-MBG scaffolds were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Cell attachment and proliferation on Li-MBG scaffolds

hPDLCs at passages P2–P5 were used for the study. At an initial density of 1×10^5cells/scaffold, hPDLCs were cultured on 5×5×5 mm Li-MBG scaffolds placed in 48-well culture plates with osteogenic differentiation medium. After culturing for 7 days, SEM analysis, MTT assay, relative ALP activity and calcium assay were performed to investigate the cell morphology, proliferation and differentiation, respectively.

The effect of Li-MBG scaffolds on cementogenic differentiation via Wnt signalling

The cells on MBG and Li-MBG scaffolds were collected after culturing for 3 and 7 days. The gene expression for cementogenic markers (*CEMP1* and *CAP*) and Wnt signalling molecules (*AXIN2* and *CTNNB*) was measured by qRT-PCR.

2.3. 4 Part three (Chapter 6):

Li-β-TCP ceramics as another potential material for periodontal tissue engineering

Preparation and characterization of 5Li-β-TCP bioceramic powders and disks

The Li-containing β-TCP (5Li-β-TCP) powders and ceramic disks were synthesized by a chemical precipitation method in which 5% molar Ca was substituted with Li. The particle size of powder and crystal phase and surface morphology of the sintered ceramic discs were characterized by X-ray diffraction (XRD) and SEM.

The cell morphology and proliferation of hPDLCS and hBMSCs on 5Li-β-TCP bioceramic disks

hPDLCs and hBMSCs were seeded on 5Li-β-TCP ceramic disks at the initial seeding density of 10^5 cells per disk in 48-well plates and cultured under osteogenic induction medium. The cells cultured in β-TCP ceramics and blank wells were used as a control in this study. The cell morphology on the disks was observed by SEM and confocal laser scanning microscopy (CLSM). MTT assay and relative ALP activity were carried out to investigate the cell proliferation and differentiation for both hPDLCs and hBMSCs on 5Li-β-TCP bioceramic disks.

The cementogenic/osteogenic differentiation of hPDLCS and hBMSCs on 5Li-β-TCP bioceramic disks

hPDLCs and hBMSCs were collected from 5Li-β-TCP and β-TCP bioceramic disks; qRT-PCR and western blotting were carried out for cementogenic/osteogenic markers (*ALP, OPN, OCN, CEMP1, CAP*) and canonical Wnt signalling markers (*WNT3a, LRP5, AXIN2, CTNNB*) after 7 days culturing in osteogenic medium.

Chapter 3: Literature Review

3.1 THE ROLE OF THE CANONICAL WNT SIGNALLING PATHWAY IN DEVELOPING TEETH

3.1.1 Brief review of tooth molar formation

Teeth have long been regarded as the most ideal model organs for use in research, because they can be easily observed, obtained to be manipulated, can be cultured *in vitro* and explanted to other ectopic positions to get the recognizable structure [2]. Tooth formation is typical of epithelial-mesenchymal interactions with a series of inductive signals transferred between the neural crest-derived mesenchyme and oral epithelium [3]. Depending on different species, teeth can be formed from ectoderm only or both endoderm and ectoderm [21].

The embryonic tooth molar formation

In mice, future dental mesenchyme is determined by the embryo midbrain and hindbrain-derived neural crest cells at embryonic day 8.5 (E8.5) [22-24]. Then the determination of tooth type starts at the first gill arch around E10.5, following by the appearance of the starting signs for tooth development on maxilla and mandible [25-26]. At E11.5, the first sign of tooth development can be seen as an obvious thickening of the oral epithelium with distinct morphological characteristics. During this period, the dental epithelial cells transform their morphology from cuboidal to high columnar, forming tooth primordia along the apical-basal axis. The thickened epithelium invaginates into neural-crest-derived mesenchyme to form the placodes with a U-shaped area known as the dental laminar. At the bud stage of E12.5 to E13.5, the budding dental epithelium is accompanied by the subsequently condensed dental mesenchyme cells around the bud to form dental papilla, while the dental epithelial cells maintain columnar shape [27]. The epithelium undergoes proliferation and extends further to the mesenchyme to form a cap at the E14.5, which is thought to be induced by enamel knot. The enamel knot, a centre of non-proliferating epithelial cells, expresses a set of unique signalling molecules involving all the pathway families, and is therefore considered to control the patterning of the tooth

cusp. These growth factors regulate the proliferation of neighbouring epithelial cells, leading to the uneven growth of dental epithelium to a cap shape. It is at this stage that the tooth crown development is initiated [28]. Following by this, mesenchymal cells differentiate into dentin-producing odontoblasts and the adjacent dental epithelium differentiates into enamel-producing ameloblasts at bell stage of E16.5. Then these two types of cells further elongate and the nuclei move to the other basal axis to begin secreting dentin and enamel. The dental follicle, which is mesenchymal part of the tooth germ, gives rise to periodontal structures including periodontal ligament (PDL), alveolar bone and cementum. In addition, the neural-crest-derived mesenchyme not only can form the dentin-producing odontoblasts, but also the future alveolar bone [29-30].

Human tooth development shares great similarity with that of early mouse development, including dental lamina, the bud and the cap stages shown in Figure 3-1 [1]. At the 5^{th} week of human gestation, tooth germ commences with a potential epithelium shape change from cubic to columnar, and subsequently form a U-shaped band around the upper and lower jaw, that is dental laminar. Following this, at the 7^{th} week of embryonic development, continuous but asymmetric proliferation in the laminar epithelium entrap into the below dental mesenchyme, leading to bud structure with 10 presumptive deciduous teeth at intervals. Thereafter, from the 9^{th} to 10^{th} week, human deciduous tooth bud invaginates into the mesenchyme, generating the primary enamel knot to control the tooth cusp formation. At the same time, the permanent tooth germ can be formed along the superficial side of the tooth bud. However, there is a major difference between the human tooth development and other mammals. A prominent epithelial branch can be produced by dental mesenchymal bud around the lingual or buccal side, which is the prospective permanent tooth bud epithelium. It can also interact with mesenchyme to become permanent tooth germs as well as the deciduous epithelium, but permanent tooth germ will pause at the late bell stage until the primary teeth are replaced.

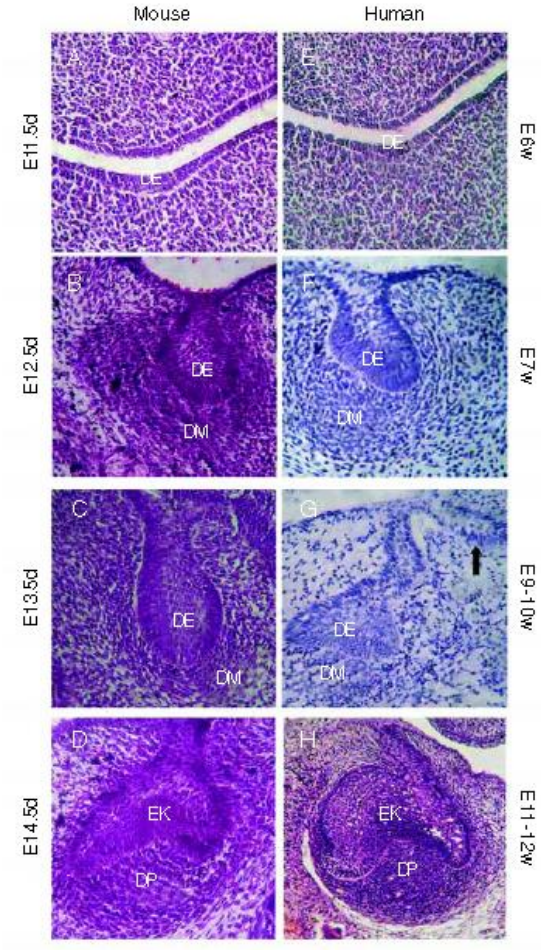

Figure 3-1. The early stages of molar tooth development in the mouse and human embryos [1]. (**A-D)** the mouse molar tooth germ at the lamina stage (**A**), the early bud stage (**B**), the late bud stage (**C**), and the cap stage (**D**). (**E-H**) The human molar tooth germ at the lamina stage (**E**), the early bud stage (**F**), the late bud stage (**G**), and the cap stage (**H**). The presence of the secondary tooth germ (STG) in (**G**). Abbreviation: DE, dental epithelium; DM, dental mesenchyme; DP, dental papilla; EK, enamel knot; STG, secondary tooth germ.

Tooth root development

During the late bell stage, it is known that the size and shape of the crown become permanent as the ameloblasts and odontoblasts secrete the mineralized matrix of enamel and dentin, respectively. The tooth development has demonstrated that the crown is always covered by the epithelium, but the epithelia stem cell niche is lost when the root formation starts among the non-continuously growing teeth including all human teeth [31].

Following the completion of crown formation, an epithelium called Hertwig's Epithelial Root Sheath (HERS) directs root growth and participates in the root development and the completion of tooth organ. HERS, known as a morphologically boundary structure of two dental ectomesenchymal tissues: dental papilla and dental follicle, continuously proliferate and are invaded by the epithelia cells covering the surface of the root termed as epithelia cell rests of Malassez. Both HERS and Malassez have the limited proliferative competence, but more findings indicate that

the interaction between them plays an important role during root formation including the bio-mineralization for cementogenesis and root dentin formation [32-35].

After the initiation of root formation, interaction between HERS and dental follicle cells leads to the development of periodontium cementoblasts, root osteoblasts and fibroblasts derived from the mesenchymal cells of dental follicle [23, 36]. Then the cementoblasts differentiate into cementum that covers the root surface and the fibroblasts form periodontal ligament fibres that are inserted in cementum adjacent to the root dentin, and alveolar bone will be produced by osteoblasts [37-38]. Then, supporting tooth root structure forms with the complete periodontal tissues including periodontal ligament, cementum and alveolar bone.

3.1.2 Molecular network in developing tooth

Classical studies have indicated that mammalian tooth development depends on the continuous molecular interactions between the dental epithelium and mesenchyme derived from the cranial neural crest in various mesoderms [3]. It is clear that embryonic cell and tissue interactions are mediated by diffusible protein signalling molecules known as growth factors. Soluble growth factors play a critical role in the process of interaction between dental epithelium and neural-crest-derived mesenchyme [28, 39]. The role of the growth factor is to activate a series of intracellular reactions to produce transcription factors in the neighbouring target cells or tissues. The transcription factors are intracellular proteins combined with the DNA specific region, thereby activating or inhibiting downstream related genes expression. It is temporal and spatial expression of growth factors and transcription factors that leading to a continuous and mutual induction during the organ development [2, 40].

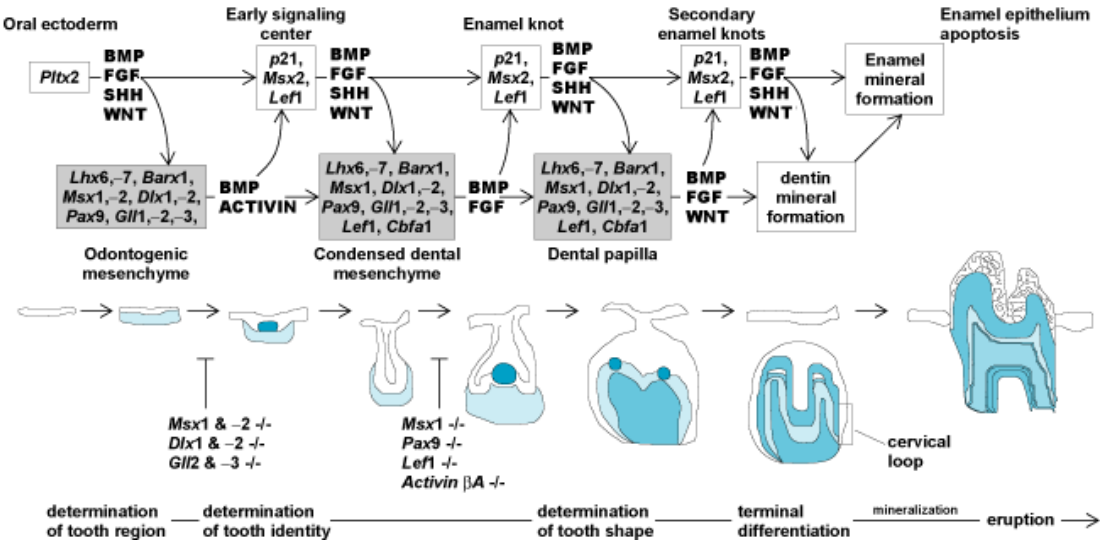

Figure 3-2. Gene and signalling pathways involved in tooth formation including early embryonic and root development [3]. Four classical signalling pathways have been found to have an imperative role in the tooth formation.

Growth factors regulate the interaction during odontogenesis

It has been clear that growth factors mediate the inductive interaction between cells and tissues during the tooth development. Prominent among those are hedgehog (Hh) families, bone morphogenetic proteins (BMPs), fibroblast growth factor (FGFs) and Wnt (wingless-type MMTV integration site family member) families [41-43]. These extracellular ligands function antagonistically/synergistically to pattern the organs development, through binding to their particular cell membrane-bound receptors. And then the complex can activate intracellular signals, leading to signal transduction into the nucleus where potential transcription factors can be activated, eventually lead to target genes expression [44]. Recent findings have revealed that all the ligands, their receptors and other key signalling molecules are involved with mediating the epithelial-mesenchymal interactions in the tooth development [45-46].

Wnts gene family has a large number of members that participate in cell proliferation and differentiation during many organs formation [47]. Hedgehog protein family (Hhs) consists of three members in vertebrates: sonic hedgehog (Shh), india hedgehog (Ihh) and desert hedgehog (Dhh). Among the three members, Shh is the only ligand expressed in the early embryonic tooth development and has been implicated in tooth patterning and growth through short-range and long-distance activation of downstream genes [48-49].

The role of transcription factors in tooth development

In the process of organ development, transcription factors can recognize and bind to the specific DNA region on the specific gene transcriptional regulatory region (promoter), which inhibits or activates relative gene expression. RNA *in situ hybridization* (ISH) is a type of hybridization that uses a labelled complementary RNA probe to localize a specific RNA sequence in a portion or section of tissue. RNA ISH results have demonstrated that many transcription factors and growth factors often have overlapped expression in several regions during tooth development, suggesting the existence of potential interaction between them [47]. A series of homeobox genes, have played an imperative role in the process of tooth development, and a homeobox gene code hypothesis for the tooth pattern formation was raised [26, 48, 50-51]. The overlapping expression areas of homeobox genes may subdivide the jaw into upper and lower regions in order to specify the tooth's position.

Signalling network involved in regulation of tooth formation

It is well known that BMP, FGF, Hhs and Wnt signalling pathways have always played an imperative role in cell differentiation and proliferation in epithelial and mesenchymal tissues during tooth morphogenesis.

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily. The findings demonstrated that many members, such as *BMP2, BMP3*, *BMP4, BMP5, BMP6, BMP7*, are involved in the occurrence of teeth, but many studies have shown that *Bmp4* is the most critical gene to mediate the interactions between epithelium and mesenchyme [27].

Several FGF family members have been detected with the expression in the early stages of tooth development and also can control all the steps during the teeth development from the teeth initiation to the last cusp formation [42].

Hedgehog gene was originally discovered in mutant fruit flies. Among the three members of Hh family in vertebrates, Shh is the only Hh ligand to be detected in early tooth development [48]. Odent *etc.* have found that *Shh* was expressed in human neural tube substrate, spinal cord, lungs, teeth and limbs development. In the

Shh knockout mice, the mineralization of enamel and dentin is still preserved, but the level and polarity of ameloblast and odontoblast would be severely damaged [52].

A number of Wnt genes are found to express in the developing tooth germ, however, most Wnt genes have limited expression in the dental epithelium. *Wnt10a* and *Wnt10b* expression can be detected in thickening epithelial of the molars and incisors at E11.5. However, expression of both *Wnt10a* and *Wnt10b* was only confined in the enamel knot of cap stage at E14.5 [53]. In contrast, expression of *Wnt5a*, *sFrp2*, and *sFrp3* are limited in the dental mesenchyme [54]. The absence of certain Wnt genes in knockout mice, such as *Wnt1*, *Wnt2*, *Wnt3*, *Wnt3a* or *Wnt5a*, can cause premature embryonic death [55].

3.1.3 The canonical Wnt signalling in developing tooth

The Wnt signalling pathway plays essential roles in cell maintenance, proliferation and differentiation during the entire tooth development. The Wnt pathway involves a large number of proteins known as extracellular Wnt signalling ligands and their interactions with receptors on target cells, which can trigger the cell physiological responses [31].

Brief overview of Wnt pathways

Wnt proteins are paracrine-signalling molecules and have at least 19 family members in mammals. They can be placed into 12 subfamilies and form a family of evolutionarily conserved secreted glycoproteins [56]. Traditionally, mammalian Wnts can be subdivided into two groups based on their abilities of whetter activate canonical Wnt signalling. Members of the Wnt1 class (Wnt1, Wnt3, WNT3A, Wnt7A, Wnt7B, Wnt8A) have been considered to effectively activate canonical β-catenin pathway, whereas members of the Wnt5a class (Wnt4, Wnt5A, and Wnt11) have been considered poor activators or even inhibitors of the canonical pathway as they act through the non-canonical (β-catenin independent) pathways [57]. Wnt ligands activate several known pathways: the Wnt/β-catenin or canonical signalling pathway, the Wnt/Ca^{2+} pathway involving Protein Kinase A and the non-canonical planar cell polarity (PCP) pathway [58].

The canonical Wnt pathway involved with the stabilization and accumulation of β-catenin in the nucleus. In the absence of Wnt ligands, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) and is associated with adenomatous polyposis coli (APC) and Axin proteins, leading to the destruction of β-catenin via the ubioquitination-proteosome degradation. When Wnt proteins bind to the cell-surface receptors of the Frizzled family and/or low-density lipoprotein (LDL) receptor known as LRP5/6, ligands-receptors complex could activate dishevelled protein homolog Dvl (Dsh in Drosophila) family proteins. Dvl protein is a key component of a membrane-associated Wnt receptor complex which inhibits the proteins complex including Axin, GSK-3, and APC. The inhibition of this "β-catenin destruction complex" can stabilize a pool of cytoplasmic β-catenin, and some accumulated β-catenin is able to enter the nucleus where it interacts with TCF/LEF (T cell factor/lymphoid enhancer factor) family transcription factors to promote specific gene expression (Fig. 3-3) [59].

During the past 20 years, increasing findings revealed that β-catenin-independent Wnt signalling pathways have been identified to control aspects of gastrulation movements. The non-canonical Wnt signalling pathway, also termed the atypical Wnt-Frizzled signalling pathway, has two intracellular signalling cascades that consist of the Wnt/Ca^{2+} signalling pathway and the Wnt/PCP signalling pathway. While the signalling events of the β-catenin-independent pathway are relatively poorly defined, partly because there are at least three mechanisms branches, which also overlap with other signalling pathways (Fig. 3-3) [60]. Potential mechanisms of non-canonical Wnt signal transduction are also quite diverse, including signalling through calcium flux, JNK, and both small and heterotrimeric G proteins. There are indications that vertebrate non-canonical Wnt signalling may also be involved in processes as diverse as cochlear hair cell morphology, heart induction, dorsoventral patterning, tissue separation, neuronal migration, and cancer [61].

The Wnt/Ca^{2+} pathway regulate cell fate determination, cell migration, and axon guidance and collaborate with the PCP pathway in regulating tissue polarity. In Wnt/Ca^{2+} signalling pathway, Wnt proteins (consisting Wnt1, Wnt5a and Wnt11) bind with frizzled homologs resulting in activating phospholipase C (PLC). Then PLC leads to increased intracellular Ca^{2+} and activation of calcium calmodulin kinase II (CaMKII), protein kinase C (PKC) and calcineurin. CaMKII and PKC inhibit the

canonical Wnt signalling. PKC also activates Cdc42 regulating cytoskeletal dynamics during convergent extension. Calcineurin activates NFAT which affects gene transcription controlling cell fate and cell migration [62].

The non-canonical planar cell polarity (PCP) pathway is best characterised in *Drosophila* where it controls the remodelling of the cytoskeleton, tissue polarity, coordinated cell migration and axon guidance via a β-catenin-independent manner [63]. For Wnt/PCP signalling, Wnts proteins bind to Frizzled receptors, followed by activating Rho/Rac small GTPase and Jun N-terminal Kinase (JNK) via Dvl and Diego (Dgo) to assist in the subsequent regulation of cytoskeletal organization and gene expression. Dvl is connected via Daam1 to downstream effectors of Rho and Rho-Associated Kinase (ROCK), resulting in actin stress fibre formation. Dvl also activated Rac, leading to the protrusion of actin-based lamellipodia and c-Jun-dependent transcription. Activation of Rac1, Cdc42 and JNK also leads to stabilisation of microtubule filaments. Thus, direct interaction of Dvl with both RhoA and Rac1 is fundamental to the regulation of cell polarity through the remodelling of the actin and microtubule cytoskeletons [54].

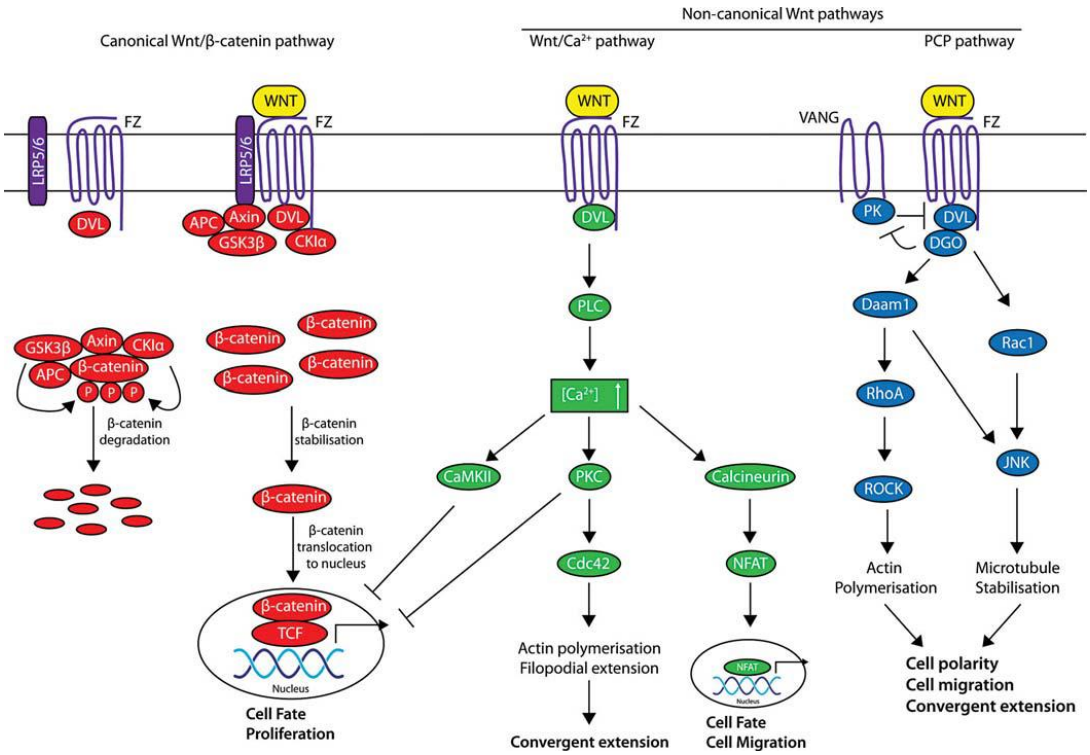

Figure 3-3. Wnt signalling pathways [60]. In the canonical Wnt signalling pathway, in the absence of Wnt signalling, β-catenin is in a complex with axin/APC/GSK3β, and gets

phosphorylated for degradation. In the presence of Wnt signalling, β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where it binds TCF/LEF transcription factors, thus activating target genes controlling cell fate and proliferation. The non-canonical Wnt/Ca^{2+} pathway involves intracellular Ca^{2+} increase which activates CaMKII, PKC and calcineur in controlling cell fate and cell migration. Non-canonical PCP signalling mediates cytoskeletal changes through activation of RhoA and ROCK via Daam1, which leads to actin polymerisation, and Rac1 and JNK, which leads to microtubule stabilisation.

Wnt pathway antagonists

In the canonical Wnt signalling pathway, Wnt receptors complex that activate intracellular cascade event contains two catalogues: a member of Frizzled (Fzd) family (10 family members of these seven-transmembrane-span proteins in human) and either of co-receptors LRP5/6 (two single-span transmembrane proteins). However, activation of non-canonical Wnt pathways is mediated by Fzd family receptors; it is not clear whether LRP5/6 are required in these non-canonical pathways [60].

The negative regulation of Wnt signalling pathway can occur at both extracellular and intracellular level. Secreted Wnt antagonists can block the Wnt signalling pathways through different mechanisms. Thus, they can be divided into two functional categories. Members of secreted frizzled related protein (sFRPs) class, which includes the sFRPs family, Wnt inhibitory factor (WIF-1) and Cerberus, bind with Wnts ligands directly, thereby preventing Wnts binding with receptors to block both canonical Wnt and non-canonical signalling pathway. Members of Dickkopf family (Dkks) class, which includes Dkks family, sclerostin and Krmen 1/2(Krm1/2), can inhibit co-receptor LRP5/6 complex to further block the canonical Wnt signalling pathway [64-65].

Recent finding revealed that sclerostin, a secreted protein in humans that encoded by the *SOST* gene, could interact with extracellular Wnts co-receptors LRP5/6 domains to competitively prevent the various Wnts binding with co-receptors. Sclerostin was initially characterized as a BMP antagonist, based primarily on its homology to the DAN family of cystine knot-containing proteins [4, 66]. Thus, in theory, sFRPs class antagonists could inhibit both canonical and non-canonical signalling pathways,

whereas those of DKKs and sclerostin class specially inhibit the canonical Wnt signalling pathway.

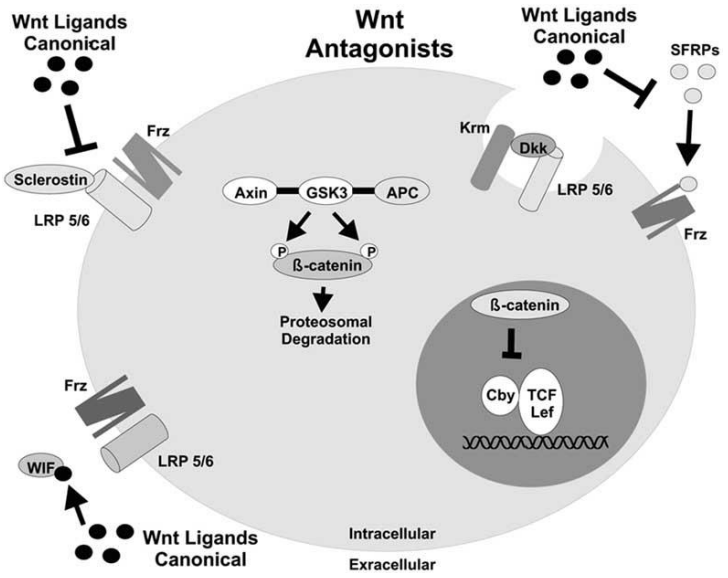

Figure 3-4. Functional extracellular and intracellular Wnt antagonists [67]. Extracellular antagonists include sclerostin that bind and inhibit LRP5/6, WIFs that bind Wnt ligands, and SFRPs that bind Frz and competively inhibit the LRP interaction with Fzd. Krm and Dkks facilitate LRP inactivation through internalization of the receptor. The intracellular inhibitors include GSK3β/Axin/APC complex that inhibit β-catenin translocation to the nucleus and Chibby that blocks β-catenin association with its transcription factors in the nucleus.

Intracellular inhibitors include the GSK3β/Axin/APC complex and Chibby (Fig. 3-4). GSK3β/Axin/APC complex inhibit β-catenin translocation into the nucleus. Chibby competes with Tcf/Lef transcription factors, leading to the blockage of canonical Wnt signalling [68]. The antagonist regulation of Wnt cascade is critical in a variety of disease processes, and therefore, has tremendous potential in therapeutic regimens.

Regulation of the canonical Wnt signalling pathway in tooth development

The canonical Wnt signalling is dynamically active in tooth-forming regions at multiple stages of tooth development, and plays multiple critical roles in these events [4, 69].

Multiple Wnt genes are expressed at the initiation of mouse tooth development. These include *Wnt10b*, which is expressed specifically in the molar and incisor

dental epithelial thickenings. *Wnt4, Wnt6* and *Fz6* are observed in dental epithelium. At the early cap stage, *Lef1, Wnt3, Wnt6, Wnt10b*, and *Fz6* are detected in the primary enamel knot, a transient signalling centre in the developing tooth. While *Wnt5a* and *Fz1* show strong expression in the dental papilla mesenchyme [70].

β-catenin is a critical member of canonical Wnt signalling pathway, through post translation modifications that inhibits its cytoplasmic degradation as the result of the upstream activation of the pathway [71-72]. β-catenin expression was found in developing tooth bud, enamel knot and secondary enamel knot. *Wnt 10a* was expressed in secretory odontablasts and is an upstream regulatory molecule for dentin sialophosphoprotein (*Dspp)* which is a tooth-specific non-collagenous matrix protein regulating dentin mineralization. Overexpression of *Wnt3* in dental epithelium resulted in the loss of ameloblasts and a reduction of enamel in post-natal incisors, suggesting that the canonical Wnt signalling might play a role in enamel formation [73]. Liu *etc*. demonstrated that constitutively mutation of β-catenin in dental epithelium caused formation of large, misshapen tooth buds and ectopic teeth. In addition, inducible Dkk1 expression after the bud stage caused formation of blunted molar cusps and loss of restricted ectodin expression. These data demonstrated that canonical Wnt signalling is essential both for patterning tooth development in the dental lamina, and for controlling the shape of individual teeth [4]. Together, these results indicate that activation of the mesenchymal odontogenic program during early tooth development requires concerted actions of Bmp, Fgf and Wnt signalling from the presumptive dental epithelium to the mesenchyme [74].

In postnatal stage, it is noted that HERS is important for tooth root formation. Enhanced canonical Wnt signalling is expressed in the HERS [62]. It is known that cementum formation is sensitive to intra/extracellular phosphate/pyrophosphate distribution, and treatment of inorganic phosphate in immortalized mouse cementoblasts alters the expression of several Wnt pathway genes. Expression of the secreted canonical Wnt signalling Sfrp4 inhibitor is enhanced, while expression of the Wif1 and Dkk3 inhibitors and the Wnt10b and Wnt4 ligands is diminished [75]. Sfrp4 has been identified as a potential phosphaton in a putative circulating regulator of phosphate concentration [66]. Analysis of these data suggests that Wnt signalling may be involved in cementum formation.

Recently, several research papers concern the role of canonical Wnt signalling in tooth root formation. An OC-Cre;Catnb(lox(ex3)/+) mouse model was created to obtain persistent stabilization of β-catenin in the dental mesenchyme; and the results demonstrated that malformed teeth were characterized by aberrantly formed dentin and short molars roots covered with the excessively formed cellular cementum [76]. Furthermore, more details were found in OC-Cre;Catnb(lox(ex3)/+) mouse model that dental papilla cells were prematurely differentiated and had a disorganized arrangement at the beginning of root formation in mutant molars. In addition, Dkk2 was upregulated in the cementum of mutant mice. These results suggest that temporospatial regulation of canonical Wnt signalling plays an important role in cell differentiation and matrix formation during root and cementum formation [77].

To further understand the role of canonical Wnt signalling in the dento-alveolar complex formation, Col1a1-cre mediated conditional activation of β-catenin was generated in osteoblasts and odontoblasts. Mutant mice showed that tooth formation and eruption was disturbed with hypoplastic cementum and periodontal ligament. While cementum matrix proteins were decreased, bone matrix proteins were increased in the cementum and alveolar bone of mutant mice [78]. Zhang *etc.* conditionally knocked out beta-catenin gene (Ctnnb1) within developing odontoblasts and cementoblasts during the development of tooth roots, and observed rootless molars as well as incomplete incisors. The formation of dentin and periodontal tissues were greatly hampered. This study provides strong *in vivo* evidence to confirm that canonical Wnt signalling is functionally significant to root odontogenesis and cementogenesis during the tooth root development [79]. All these recent findings indicate that local modulation of canonical Wnt signalling has therapeutic potential to improve the regeneration of dentin and periodontium.

Role of Axin2 in tooth development

Axin, body axis development suppressor gene (axis inhibitor), was first cloned from the mutant mice and encode two axis protein members of Axin1 and Axin2 in vertebrates [80]. Axin2, also known as Conductin or Axil, is a negative Wnt signalling pathway regulatory factor and the direct target gene of canonical Wnt signalling pathway [81]. Axin2 is also a homolog of Axin1, baring 45% amino acid sequence with Axin. Although they have similar biological characteristics, they

function completely differently in the organogenesis. Axin1 is expressed in every organ in the embryonic development, while Axin2 expression is concentrated in specific organs, and its expression must be activated by Wnt signalling [82-84].

Axin2 has a series of protein binding sites as a structure protein, and can combine with many members of the Wnt signalling pathway, such as APC, GSK-3β, β-catenin and Dvl protein. Then they can form a protein complex that promote the phosphorylation and degradation of intracellular β-catenin to maintain at a low level. Therefore, Axin2 plays two functions in the canonical Wnt signalling pathway. First, similar to Axin1, it is a negative regulation of Wnt signalling pathway through the formation of complex induce degradation of the cytoplasmic β-catenin. Secondly, Axin2 is a direct target gene of the canonical Wnt signalling pathway and its expression level directly reflects the Wnt signalling pathway [85]. Hence, this forms a negative feedback loop.

In humans, loss of Wnt10a or Axin2 can cause tooth agenesis [86-87]. The expression of canonical Wnt signalling is concentrated in dental mesenchyme in the early mouse tooth development, but Axin2, as the reporter gene of canonical Wnt signalling pathway, can be observed in both epithelium and mesenchyme in the developing tooth [88]. At the lamina and bud stage of molar, *Axin2* is observed in both the epithelium and underlying mesenchyme. At the cap and bell stage, *Axin2* was strongly expressed in the primary and second enamel knots respectively. Weak *Axin2* expression can be detected in the odontoblasts, which resembles the localization of nuclear β-catenin. High levels of *Axin2* expression were found in odontoblasts, dental pulp and surrounding the developing root in the postnatal teeth, while *Axin2* was lost in the crown odontoblasts. During the late stages of cementogenesis, *Axin2* continues to express in HERS and developing root in high levels. Between the roots, *Axin2* was also highly expressed forming a V-shape [89]. High level of canonical Wnt signalling pathway was also noted in the developing molar roots in accordance with *Axin2* expression during root formation (Fig. 3-5). All indicates that canonical Wnt signalling has a new role in controlling root development in late stages of postnatal tooth development in addition to an important role in embryonic tooth development.

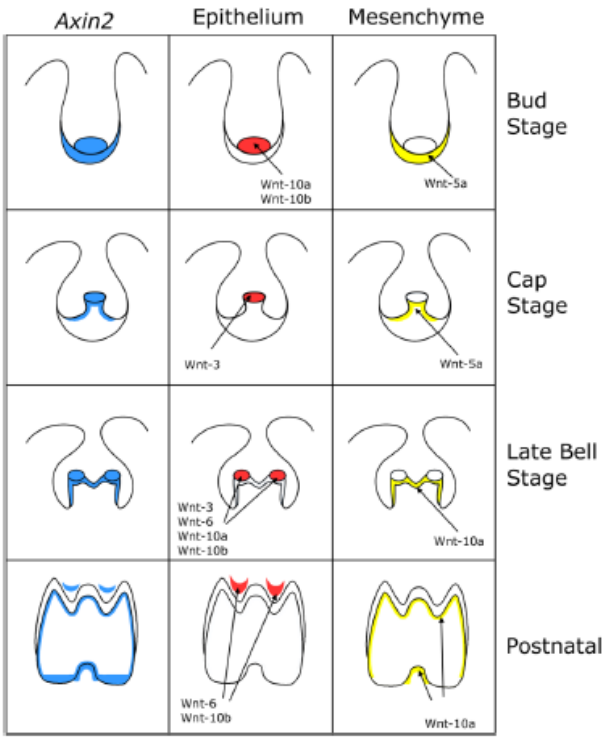

Figure 3-5. The expression pattern during tooth crown and root formation [89]. Diagrammatical representation of *Axin2* expression (blue) was identified both mesenchyme and epithelium at the different stages of tooth formation and postnatal teeth.

3.2 CURRENT PROGRESS OF PERIODONTAL REGENERATION

3.2.1 The structure of cementum in periodontium

Normal tooth cementum is a bone-like mineralized tissue with collagen fibrils, as it is a key organic component presented on the surface of root dentin. It also can provide the "bridge" to support the periodontal ligament between the root dentin and surrounding alveolar bone. The periodontium structures are cementum, periodontal ligament and alveolar bone as shown in Figure 3-6.

Cementum is secreted by the cementoblasts, which are the cementum-forming cells develop from undifferentiated dental follicle mesenchymal cells. The inorganic portion of cementum is hydroxylapatite (HAp) crystals ($Ca_{10}(PO_4)_6$). The organic part is composed primarily of collagen, protein polysaccharides and water. The periodontal ligament is made up of Sharpey's fibres inserted in the cementum and alveolar bone, which attaches the tooth to the alveolus. Cementum is avascular and therefore, cannot spontaneously be regenerated [90].

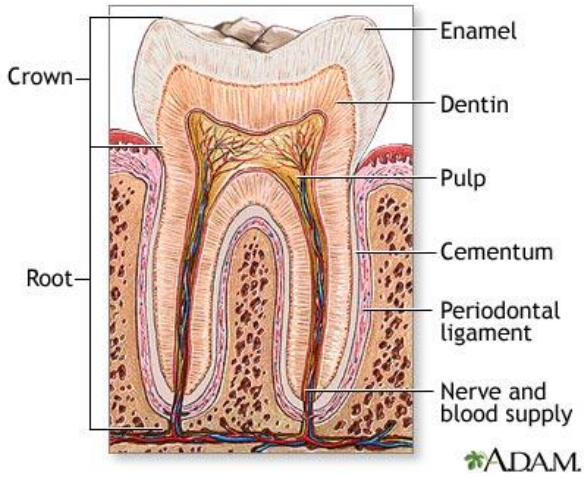

Figure 3-6. The natural structure of tooth with two anatomical parts: crown and tooth root [90]. The crown of a tooth is the part which is covered with enamel and this is usually visible in the mouth. The root includes periodontal ligament, alveolar bone and cementum and tooth root dentin.

Collagen fibres in the cementum can be divided into two different kinds: intrinsic fibres (fibres arranged in parallel with cementum surface), and extrinsic fibres (fibres embedded into the ends of the primary fibres). There are two kinds of cementum formed: acellular and cellular. Acellular cementum, also called acellular extrinsic fibre cementum or primary cementum, is the first formed cementum during tooth development and is located on the coronal half and mid portion of the tooth root. The acellular layer of cementum is the living tissue that does not have cells incorporated into its structure. Whereas, many of the cementoblasts become entrapped by the cellular cementum they produce, becoming cementocytes. Moreover, cellular cementum is found more frequently on the apical and interradicular half [91]. Additionally, there are many noncollagenous components in cementum, such as bone sialoprotein, osteocalcin and osteopontin; these glycoproteins impart the cohesion and structural integrity to the cementum [92-93]. Recently, cementum-derived attachment protein (CAP), and human cementoblastoma-derived protein cementum protein 1 (CEMP 1) have been regarded as specific factors to the cementoblast differentiation and cementum matrix [94].

To date, the cellular origin is still controversial for cementum regarding the cell source, which is from epithelial or mesenchymal. This disagreement is perhaps partly due to the poor understanding of the events during the initiation and development of tooth root. It is clear that bone tissues are secreted by osteoblasts that are derived

from mesenchymal progenitor cells, but it is still unclear with respect of the cell source for cementum and whether cellular and acellular cementum are from the same progenitor cells [90, 95]. One theory is that cementoblasts are derived from the mesenchymal progenitor cells/dental follicle cells and directly differentiate into both cellular and acellular cementum by invading the epithelial HERS [95]. Other speculation suggests that epithelia HERS cells formed cementoblasts through the epithelium-mesenchymal transformation process [96]. However, these proposals are not based on gain-of-function and loss-of-function research methodology. Recently, Feng's lab demonstrated that the cell source for cellular cementum is mesenchymal periodontal ligament progenitor cells in which osterix (OSX), plays a vital role during cementogenisis [97]. In their study, they carried out gain- and loss-of-function methodologies to study the cellular cementum origin targeting OSX. Using the OSX over-expression mice model with a Collagen 1A promoter, they found that in an increase in cementum mass. On the other hand, the deletion of OSX gene can reduce the cementocyte numbers and morphology with a significant reduction in mineralization rate. Taken together, these data support the idea that derivation of cellular cementum may originate from mesenchymal dental follicle cells [97].

3.2.2 Cell sheets techniques in periodontal tissue regeneration

The periodontal tissue regeneration is a complicated functional replacement of the tooth supporting structures, including the periodontal ligament, cementum and alveolar bone to maintain the teeth in the jaw. Due to periodontal diseases lead to the destruction of periodontal tissues, which affect 30-40% of the population. It is of great importance to reconstitute the periodontium complex including alveolar bone, PDL and root cementum [98]. In the process of periodontal tissue regeneration, one of the most critical and difficult components is cementum regeneration. Ideally, the newly formed periodontal fibres are inserted into the regenerated cementum to attach with the root surface and new alveolar bone. Tissue engineering provides a new method based on the molecular and cellular biology for regeneration of periodontal/cementum which is a complicated biological process in itself [99].

Tissue engineering was emerged in 1990s involving three elements: signalling molecules, scaffold and stem cells. In periodontal regenerative therapy, researchers are trying to establish new treatments to accelerate the regeneration of periodontal

tissues by combining typical human recombinant cytokines and human mesenchymal stem cells into hard-tissue forming cells. Along with the recent progress in tissue engineering, cell sheet engineering have been developed as the foundations for periodontal regeneration, including alveolar bone, cementum and periodontal ligament [7]. The cell sheet technique was invented by Okano *et al.* using a surface-grafted temperature-responsive polymer called poly N-isopropylacylamide (PIPAAm). PIPAAm is fully hydrated at temperatures lower than 32^{o}C with an extended-chain conformation; however, PIPPAAm is dehydrated and compacted when temperature exceed 32^{o}C. PIPPAAm was then engrafted into atemperature-responsive dish to allow intact cells to be harvested at lower temperature [7]. It has been reported that cell sheets can be fabricated from different types of cells including fibroblasts, hepatocytes, endothelial cells, macrophages and retinal pigmented epithelial cells. Some of these cell sheets have been applied *in vivo* and have demonstrated remarkable results [100-102]. Regarding periodontal regeneration, periodontal ligament cells have shown the potential ability to regenerate periodontium by application of the cell sheet technique in previous studies in beagle dog [20]. Dehiscence defects were healed with bone, periodontal ligament and cementum formation in the experimental group in which periodontal ligament cell sheets were engrafted [20]. Following the successful research in beagle dogs, newly-formed cementum-like hard tissues were regenerated on the dentin surfaces in the transplanted periodontal ligament cells-dentin block constructs in athymic rats [103-104]. Based on these studies above, three-layered osteogenic medium cultured periodontal ligament cell sheet were transplanted autologously onto dental root surface in canine periodontal defect model. The bone defect was filled with beta-tricalcium phosphate (β-TCP). The results showed that cell sheet transplantation regenerated more new bone and cementum connective tissues and well-organized collagen fibres when compared to the non-cell sheet control groups [105]. Another study investigated the regenerative potential of different cell sheets derived from periodontium (gingival connective tissue, alveolar bone and periodontal ligament) supported by a calcium phosphate coated melt electrospun polycaprolactone (CaP-PCL) scaffold and then were transplanted in an athymic rat periodontal defect model. It was noted that hPDLCs and alveolar osteoblasts cell sheet with a CaP-PCL scaffold resulted in significant periodontal attachment formation. While the gingival cell sheets did not promote periodontal regeneration on the root surface and inhibited

bone formation within the CaP-PCL scaffold [106-107]. Furthermore, PDL cell sheets were placed on the electrospun membrane and osteoblasts were cultured in the bone compartment. Then the cell-seeded biphasic scaffolds were placed onto a dentin block and implanted for 8 weeks in an athymic rat subcutaneous model. Data demonstrated that a thin mineralized cementum-like tissue was deposited on the dentin surface for the scaffolds incorporating the multiple PDL cell sheets, as observed by H&E and Azan staining [108]. These results suggest that periodontal ligament cells possess multiple differentiation properties, which can assist regeneration of injured periodontal tissues using cell sheet grafting technique.

3.2.3 Stem cells as powerful therapeutics for periodontal regeneration

Since stem cells, also known as precursor cells, have been considered as attractive candidates for tissue regeneration, due to they have the capability of self-renewal and multi-lineage differentiation. Based on the varying potentials, stem cells can be divided into two categories: embryonic stem cells and adult stem cells [109]. Due to the ethics considerations and regulations, adult crest-derived mesenchymal stem cells, such as periodontal ligament stem cells (PDLSCs) [110], dental follicle precursor cells (DFPCs) [111] and bone marrow mesenchyme stem cells (BMMSCs) [112], have been identified as more appropriate candidates for cementum regeneration. PDLSCs, which are isolated from the periodontal ligament of human third molars, are capable of differentiating into cementoblast-like cells *in vitro*, and show the ability to produce cementum-like tissues *in vivo* [113]. DFPCs have also been isolated from human third molars, but there is no cementum formation observed in transplants *in vivo*. The expression of cementum specific makers, however, such as CAP and CP-23, has been detected in the cultured DFPCs after incubation with human recombinant protein of rhBMP2 and rhBMP7 or enamel matrix derivatives (EMD) for 24 hours [114]. Subsequently, other dental mesenchymal stem cells were isolated from human exfoliated deciduous teeth (SHED) and noted to form bone and dentin *in vivo* [115]. Stem cells from apical papilla of human teeth (SCAP) have aslo been shown to form dentin tissues in immunocompromised mice and generate root-periodontal complex after transplantation with periodontal ligament cells in minipigs [116-117]. The dental follicle progenitor cells can be obtained from human third molar and have been proven to differentiate into osteoblasts/cementoblasts *in vitro*; they can generate PDL-like tissues during *in vivo* implantation [111]. These data

demonstrated that dental mesenchymal stem cells are appropriate cell sources for periodontal regeneration application.

Non-dental mesenchyme stem cells, BMMSCs also have the potential to produce the cementum-like tissue, when BMMSCs were loaded around the fixtures of the porous poly-DL-Lactide-co-Glycolide scaffolds with a hollow root-shape. Periodontal-like tissues including cementum, alveolar bone and periodontal ligament can be detected around the implants after one-month implantation in goats [118]. Apart from BMSCs, adipose-derived stem cells (ASCs) can generate new PDL-like and alveolar bone-like structures, when transplantation occurs in a rat and canine periodontal defect models. Although current evidence suggests that ASCs can be induced to periodontal tissues, the mechanism by which these stem cells can be directed towards strict periodontal cell differentiation remains uncertain [119]. These results, therefore, suggest that both dental and non-dental derived stem cells are able to be applied in cementum/periodontal regeneration.

3.3 THE CONCEPT OF PERIODONTAL TISSUE ENGINEERING

The current tissue engineering principles and strategies should be applied to accelerate restoration of periodontal complex. Recently, Chen etc. addressed the concept of periodontal tissue engineering with three key factors: biological cues, stem cells or progenitor cells and suitable biomaterials. The bioactive cues can be absorbed or encapsulated in the biomaterials scaffolds to implant at the periodontal defect, which can lead to cell homing/recruitment from healthy tissues to the defect. The homed cells will proliferate and differentiate into fibroblasts, cementoblasts and osteoblasts. Subsequently, differentiated tissues will be achieved in the periodontal defects including periodontal ligament, alveolar bone and cementum. In this regard, periodontal defects can likely be reconstructed by utilizing bioactive cues, biological active proteins, biopolymers/bioscaffolds, and/or patient-derived regenerative ‘tools’ (Fig. 3-7). Meanwhile, more efforts are still required to optimize the cell-delivery materials and strategies to accelerate the periodontal tissue regeneration process. Further development of periodontal bioengineering requires the incorporation of multiple areas covering biology, engineering, dentistry, biomaterials and the dentists who can access the fundamental knowledge into clinical application.

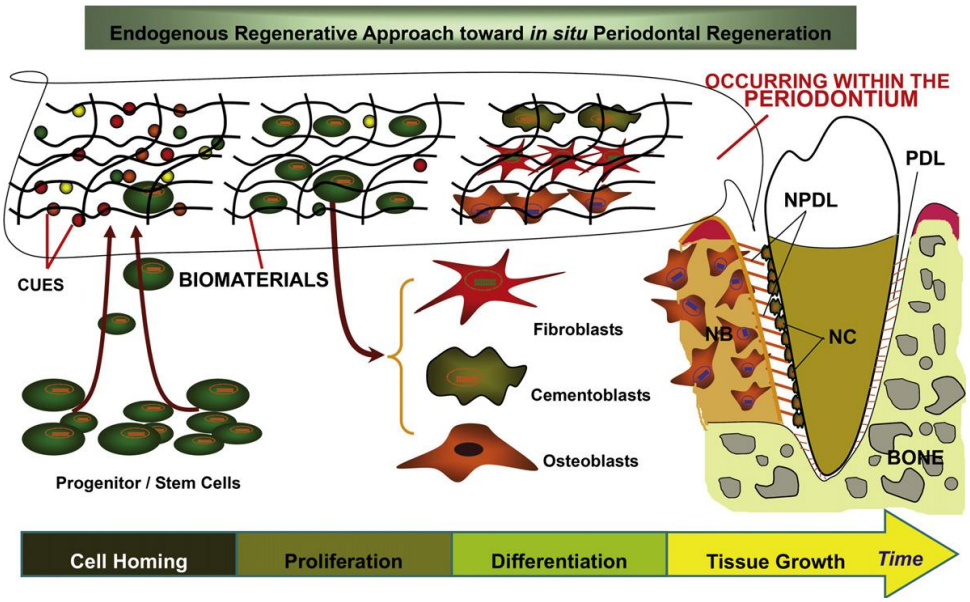

Figure 3-7. Schematic illustration of the periodontal regeneration using an endogenous regenerative approach [14]. This approach requires the partnership of bioactive cues, biomaterials and/or patient-derived progenitor cells and transplantation into the periodontal defect. The cells proliferate and differentiate into specific periodontal fibroblasts, osteoblasts and cementoblasts which can differentiate into periodontal ligament, alveolar bone and cementum finally over time. NB, new bone; NPDL, new periodontal ligament; PDL, periodontal ligament; NC, new cementum.

Clinically, several procedures have been attempted to regenerate periodontal tissues, including bone grafting (autograft, allograft and xenograft), conventional flap surgery, guided tissue/bone regeneration (GTR/GBR) and the use of various growth factors/host modulators (Emdogain and parathyroid hormone) [120-121]. GTR/GBR is a technique that involves the placement of a barrier membrane at the periodontal defect to aid the repair of periodontal tissues. As mentioned above, the proper growth factors (GF), a stem cell graft and scaffold for delivering and retaining cells are required for a promising alterative of periodontal regeneration [122-124]. The role of the scaffold is to provide an environment for guiding or assisting the regeneration *in vivo* and *in vitro*. Subsequently, the scaffolds need to be designed with suitable surfaces for cell adheshion and differentiation, and then be optimized to ensure adequate mechanical integrity and osteoconductive properties [15]. Therefore, it is the tissue engineering that could provide a variety of laboratory products (such as stem cells, material niches, tissue constructs or engineered tissues) for clinical therapies and transplantation.

3.3.1 Mesoporous bioactive glass

Bioactive glass, due to their versatile properties of osteoconductivity, composition, and good degradation rate have been very attractive materials for bone regeneration. The first generation of bioactive glass was synthesized by Hench named with 45S5® bioactive glass, belonging to SiO_2-Na_2O-CaO-P_2O_5 system using the traditional sintering melt method; it was found that 45S5 glass was able to bond with closely with bone tissues [125-126]. Since then, many other ions, such as silicate, borate and phosphate glasses, have been proposed by researchers for bone tissue engineering. However, 45S5 glass was only limited formed as powders, particles and dense bulk of large shapes and sizes without microporous structure. On the other hand, mesoporous materials possess significantly ordered mesoporous structure pore size within 2-50 nm and well-defined hydrophobic/hydrophilic surface; however, mesorporous materials have lower bioactivity [127].

Thus, to overcome the problems related to poor bioactivity of pure-silica mesoporous biomaterials and conventional bioactive glass without mesoporous structure, it is of great importance to synthesize mesoporous bioactive glass (MBG) that combine efficient mesoporous structure and excellent bioactivity. The first MBG was prepared by the combination of sol-gel method and it was found that MBG possess more optimal surface area, pore volume and excellent *in vitro* cytocompatibility compared to the conventional non-mesoporous bioactive glass (NBG) [128]. Currently, based on the different preparation methods, MBG materials can be synthesized in different forms: particles, powders, fibres and scaffolds [129-130]. Among them, MBG three-dimensional porous scaffolds have been attractive for bone tissue engineering, due to their highly interconnective pore structure and optimal porosity [129]. A significant advantage of MBG is that it possesses superior apatite-mineralization ability in biological solution.

MBG has been regarded as a potential bioactive bone regeneration material owing to its superior bioactive behaviour. Further *in vitro* and *in vivo* osteogenesis has been studied in the past several years. Wu *et al.* reported a new facile method to prepare hierarchical and multi-functional MBG scaffolds that demonstrated excellent pore size, mechanical strength and osteoinductive ability for bone regeneration application [131]. They further confirmed that the incorporation of MBG particles onto PLGA

enhanced the proliferation and relative ALP activity of human osteoblasts. In addition, they incorporated Fe, Sr, Zr and B ions into MBG scaffolds which also enhance cell proliferation and osteogenic differentiation [129]. The presence of Sr significantly enhanced oesteogenesis/cementogenesis-related gene expression (such as *ALP*, *CEMP1* and *CAP*) of human periodontal ligament cells [132]. It was also reported that the ionic product from nanoscale bioactive glass can not only increase osteogenic markers, thereby enhancing proliferation, cell attachment and mineralization, but also enhance cementoblast viability, proliferation and mitochondrial activity [133]. Furthermore, Wu *et al.* also prepared MBG/silk scaffolds and found that MBG/silk scaffolds could enhance expression of Collagen I and new bone formation after transplantation into a rat calvarial defect [134]. Recently, three-dimensional printed MBG scaffolds were placed into the defects of rat femur. After four weeks of implantation, MBG scaffolds induced a great amount of new bone ingrowths in the defects. The results further indicate that MBG has excellent *in vivo* osteoinduction for potential bone repair application [135]. These results demonstrated that MBG is a new bioactive material, which has significant potential to improve bioactivity and osteoconduncivity *in vitro* and *in vivo* for bone tissue regeneration application.

It is reasonable to suggest that bioactive glass could have a similar effect on periodontal ligament cells osteogenic/cementogenic differentiation capability. It is also reasonable to hypothesize that an increase in osteogenic proliferation of hPDLCs *in vitro* could response by the presence of MBG. In this study, it has been investigated involving the potential application for MBG scaffolds during osteogenic/cementogenic differentiation for hPDLCs.

3.3.2 Bioceramics: beta-tricalcium phosphate (β-TCP)

In natural bone, the three main components are calcium phosphate, water and collagen (predominantly type I collagen), together with other organic materials in smaller quantities, such as non-collagenous proteins, polysaccharides, and lipids [136]. Calcium phosphate in bone is in the form of crystallized hydroxyapatite (HA) and amorphous calcium phosphate (ACP). Bioceramics or mineral-based scaffolds have long been recognized as potent osteoconductive materials for regenerative hard tissues, such as bone and dentin as they can possess a crystalline structure [137].

There are several variables influencing the efficacy of bioceramics, such as chemical composition, surface roughness, hydrophilicity and topography of surface of ceramics, which would directly influence their cells response, osteoconductivity, vascularisation, osseointegration and material resorption [138]. The most popular material is β-TCP ceramics which possess high biocompatibility, osteoinduction and biodegradability [139]. β-TCP biodegrades more readily, thereby gradually being resorbed and replaced by host tissues in the body [140].

While β-TCP has been well established as a drug carrier for bone regeneration, recent data suggested that β-TCP alone or combining with other materials, have vital role as a cell carrier for periodontal regeneration [139]. It was reported that a dentin-pulp complex was successfully constructed, when porcine dental papilla cells were seeded on a β-TCP scaffold and transplanted into nude mice [141]. Consequently, when human PDLCs were seeded into β-TCP porous scaffold, vascular tissue ingrowth, differentiation of hPDLCS into osteoblasts and cementoblasts were reconstructed *in vivo* [142]. It was also reported that akernanite ceramics can stimulate cell attachment, proliferation and osteogenic differentiation of hPDLCs compared to that of β-TCP ceramics [143]. Similarly, when seeding dog PDLCs on a nano-HA/collagen/PLA scaffold, it showed significant enhancement of cell attachment, proliferation and osteogenic differentiation compared to conventional β-TCP [144]. Although β-TCP bioceramics are generally biodegradable and biocompatible, they are still far from optimal for stimulating the osteogenic/periodontal differentiation of stem cells to achieve further shorten healing time of tissue defects. In the past several years, strontium modified β-TCP bioceramics enhanced cell viability and differentiation for osteoblast-like cells with improved bioactivity have been prepared for bone regeneration application [145]. This inspired us that bioactive ions modification to β-TCP bioceramics may be a viable method to stimulate differentiation of mesenchyme stem cells for better tissue regeneration. In this research, whether β-TCP is another class of suitable biomaterial for cementum regeneration applications was investigated.

3.3.3 Cell and growth factors delivery through biomaterials

For periodontal tissue engineering, solid materials should be designed to posess pore size that allows the cellular ingrowth inside of scaffolds as periodontium is not only

made up of bone tissues. The cell-scaffold constructs stimulate the formation of reparative tissue while maintaining adequate integrity following the implantation [119]. Another clinical challenge is the neogenesis of osseous and ligamentous interfacial structures where the three-dimension shape of the natural tissues have to be achieved. Interconnecting porosity facilitates nutrient supply into central regions of engineered transplants, thereby preventing adverse effects such as tissue necrosis. Consequently, it is still under investigation for the optimal geometries and dimensions of tissue-engineered solid scaffolds for periodontal applications [15].

Recently, it was shown that the fabrication of composite hybrid polymeric scaffolds (Poly-ε-caprolactone/poly (glycolic acid)-PCL/PGA) for gene modified human cells transplantation can form dentin-ligament-bone complexes [146]. Furthermore, a fibre-guiding scaffold was also developed that fit complex anatomical defects to guide the new oriented ligament fibres *in vivo*, due to periodontal bioengineering requiring functional restoration of complex topologies and tissue integration. The results showed that cell delivery with hybrid scaffolds lead to a predicted fibre orientation and better control of tissue infiltration compared to the conventional transplantation complex [147]. More recently, biophasic scaffolds were developed made of a fused deposition modelling bone components (β-TCP) and a micro-fibrous periodontal component (PCL) to provide simultaneous delivery for PDLCs and osteoblasts to form an alveolar bone/PDL complex. The *in vitro* results showed β-TCP bone component supported the cell growth and PCL periodontal component allowed harvesting multiple cell sheets. It is an ectopic assay but it would fulfil some of the principles of GTR allowing enough space and stabilization for successful periodontal regeneration [148]. Thus, it is noted that cell-based research in periodontal tissue engineering has started to focus on designing and developing various advanced physical and geometric biomaterials. In addition, the increasing degree of sophistication will require a better understanding of how cells interconnect with the biomaterials and how the cell signalling pathways process this procedure to initiate the biological responses.

To date, the use of bone grafting materials, exogenous growth factors and cell-based approaches have been employed easily in clinical practise to induce promotion/augmentation of self-healing capacity for the treatment of periodontitis. It is well known that the application of growth factors of platelet-rich growth factor

(PDGF), BMPs, fibroblast growth factors (FGFs) and transforming growth factors β (TGF-β) for stimulating robust and self-regenerative localized mechanisms have been of increasing interest in periodontal regeneration [149-150]. These growth factors (GFs) have been proven to stimulate the systematic cellular signalling responses to regulate critical activities, such as cell survival, proliferation and mineralization that may lead to functional tissue regeneration. Growth factors also can degrade the extracellular matrix (ECM) proteins to orchestrate intracellular signalling transduction by binding with the extracellular domain of a targeted growth factor receptor [150-151]. However, there are still a few known issues associated with GF-based therapeutics that should be considered, such as the short half-lives *in vivo*, their relatively large size, slow penetration, and potential denaturation during manipulation. Ideally, the delivery of GFs to the periodontium should be localized, following specific and distinct kinetics in order to mimic the healing process in the injured periodontal tissues [15, 119]. To address this problem, the GFs require the carriers and suitable delivery systems derived from diverse biomaterials to not only act as a cell vehicle but also as a carrier controlling the release of GFs during tissue regeneration process. Many of them, by means of combination of growth factors with biomaterials, can provide a well-controlled release into a microenvironment to yield desirable concentrations over time. Most frequently used biomaterials for tissue engineering are polylactic acid (PLA), β-TCP and natural polymers such as collagen, gelatine and dextran [14, 18, 151]. Given all these facts, previous findings and efforts using growth factors presents only the beginning the development of an ideal system for periodontal regeneration. In this study, I am trying to investigate the cellular signalling activation in the biomaterials delivery system without growth factors for periodontal regeneration.

3.3.4 The canonical Wnt signalling in osteogenesis and bone regeneration

In recent years, the role of canonical Wnt signalling in bone biology has gained considerable attention. This part of review will underline the significance of therapeutic potential canonical Wnt signalling in bone regeneration, thus inspired us to investigate the role of canonical Wnt pathway during periodontal regeneration application in this study. Given the numerous findings demonstrated that canonical Wnt signalling regulates osteogenesis as shown in animal studies and genetic disorders in human. Besides, there are multiple levels of regulators, which modulate

the osteogenesis, such as Wnt receptors, secreted antagonists and intracellular mediators. Thus inducing this pathway has arisen as an attractive therapeutic target to promote bone formation for treating osteogenic disorders.

The canonical Wnt signalling stimulates bone formation

Homozygous mutations of Sost is associated with a progressive increase in bone mass, hyperostotic skeleton and increased bone mineral density in autosomal recessive diseases Sclerostosis and Van Buchen disease[152]. Sclerostin is directly involved in the regulation of osteoblastic differentiation through the antagonism of the Wnt signalling pathway *Sost* knockout mice have a high bone mass phenotype characterized by marked increases in bone mineral density, bone volume, bone formation, and bone strength [153]. Overexpression of *Lrp5* leads to increased bone mass in mice, while knockout mutations reduce bone mineral density from a defect in osteoblast proliferation [154]. Furthermore, conditional deletion of β-catenin results in increased bone mass in mice, leading to impaired mineralization secondary to a deficiency of terminally differentiated osteoblasts in mice [155].

The Axin/GSK3β/APC complex has been shown to play a role in osteogenesis. Axin 2 knockout mice present with increased osteoblast proliferation, craniosynostosis, increased chondrocyte differentiation, reduced limb length, and increased bone mass phenotypes [156]. GSK3β also regulates osteogenesis. Heterozygous Gsk3β alleles mice exhibit increased bone mass and lithium, as GSK3β inhibitors is able to increase bone density and enhance osteoblast differentiation [157] .

Target extracellular Wnt signalling antagonist of sclerostin for bone regeneration

Inhibition of extracellular Wnt antagonists with monoclonal antibodies has been a popular approach to modify the Wnt signalling pathway and promote bone regeneration. One highly promising endeavour is to target sclerostin using humanized neutralized sclerostin antibodies. Sclerostin is a glycoprotein with a molecular mass of 24 kDa, which is the product of the *SOST* gene expressed almost exclusively in osteocytes. It is produced by osteocytes to locally regulate bone formation and is vital for enabling bone responding to mechanical loading [158]. Sclerostin binds to the extracellular domain of Wnt receptors LRP5/6, preventing the binding of various Wnt ligands to these co-receptors and inhibiting canonical Wnt signalling. Sclerostin can, therefore, negatively regulate osteoblastic bone formation

[10, 159]. Thus, neutralized sclerostin antibody (Scl-Ab) could activate the canonical Wnt signalling to promote bone regeneration (Fig. 3-8 and Table 3-1).

The most recent study has investigated effects of sclerostin antibody on fracture healing in femur defects models [11]. In a rat closed femur fracture model and a nonhuman primate fibular osteotomy model, sclerostin antibody was administrated subcutaneously at 25 mg/kg twice per week for 7 weeks or 30 mg/kg every 2 weeks for 10 weeks respectively, to investigate the effects of sclerostin antibody on fracture healing. The results showed that sclerostin antibody could significantly increase bone mass and strength at the cortical and trabecular fracture sites [11, 160]. In a rat closed femur fracture model, 4 weeks of treatment with sclerostin antibody twice weekly at 25 mg/kg resulted in improved callus density, increased maximum load and stiffness and increased bone strength parameters [161]. In a 6 mm femoral defect model for Lewis rats, 12 weeks of continuous administration and treatment also resulted in bone healing with pronounced radiographic healing and bone volume compared to non-treatment group [11].

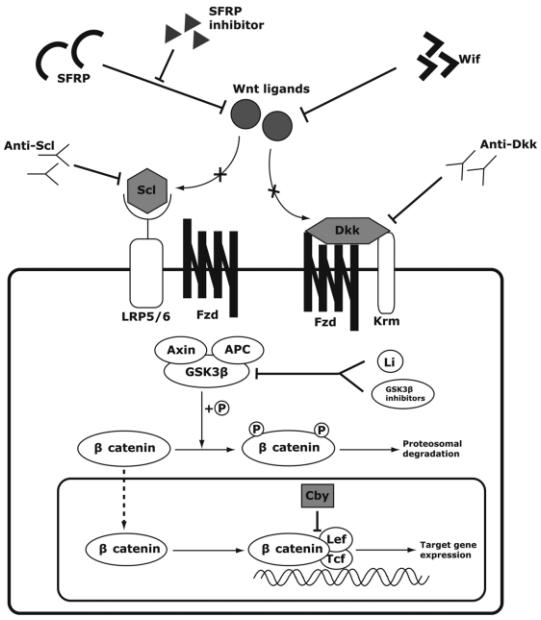

Figure 3-8. Mechanism of Scl-Ab and lithium in Wnt signalling pathway [162]. In the presence of sclerostin, the ligands cannot bind their receptors to activate the Wnt signalling. The inhibitory effects of sclerostin proteins can be neutralized by Scl-Ab, thus activating the Wnt signalling pathway downstream. GSK3β normally targets β-catenin for degradation by phosphorylation. However, lithium can inhibit the GSK3β activity, thus allowing β-catenin to accumulate in the cytoplasm and the nucleus expressing the target genes. Scl-Ab, sclerostin antibody; GSK3β, glycogen synthase kinase 3β.

Studies have showed that subcutaneous injection of neutralized sclerostin antibodies markedly increased bone formation in ovariectomised rats after 5 weeks of treatment [163-164]. In a 3-mm circular femoral defect model in Sprague-Dawley ovariectomised rats, sclerostin antibody was administered twice weekly for 3 weeks, sclerostin antibody significantly improved both intramembranous and endochondral bone formation and enhancing bone formation and bone volume [165-166]. Recently, in a phase I clinical trial, a single dose injection of anti-sclerostin antibody given to post-menopausal women increased the bone mineral density of the lumbar spine and increased production of collagen type I over 85-day study period [167].

Ligature-induced experimental periodontitis (EP) generated in alveolar bone defects. Following 4 weeks of disease induction, Scl-Ab were systemically delivered twice weekly for up to 6 wks to determine the ability of Scl-Ab to regenerate bone around tooth-supporting osseous defects. 6 weeks of Scl-Ab significantly improved maxillary bone healing, indicating Scl-Ab treatment restored alveolar bone mass loss in a preclinical animal model of periodontitis [168]. The current knowledge presented by different research groups already identified an important role of sclerostin antibody in improving bone formation in fracture healing models; however, not few studies investigate the effect of sclerostin antibody in periodontal defect model.

Target intracellular GSK3β inhibitor of lithium for bone regeneration

The intracellular molecules involved in the Wnt pathway also show promise in osteogenic therapeutic interventions. For instance, inhibiting GSK3β from phosphorylating β-catenin could stabilize the cytoplasmic β-catenin, allowing further progression through the Wnt downstream cascade. Lithium, a well characterized as a GSK3β inhibitor (Fig. 3-8 and Table 3-1), has been widely used as a long-term mood stabilizer in the treatment of bipolar and depressive disorders in the past 50 years. Its mode of action is enhancing the remyelination of peripheral nerves [169-171].

Li^+ has been reported to activate the canonical Wnt signalling pathway by inhibiting phosphorylating β-catenin in the cytoplasm, thereby targeting it for ubiquitination and degradation [172-174]. The effect of lithium on GSK3β was first investigated from the observation that lithium's effects on *Xenopus* development were very similar to those caused by reductions of GSK3β activity. Furthermore, it revealed

that lithium directly binds and inhibits the phosphorylation of substrates of GSK3β, such as the microtubule associated protein tau [175]. Previous studies have shown that lithium chloride can stimulate the proliferation of retinoblastoma cells via elevated canonical Wnt signalling [176]; and the *in vitro* proliferation and neuronal differentiation of neural progenitor cells was enhanced by long-term treatment by lithium [177]. A study that is of particular interest for the application of Li^+ ions in bone tissue engineering applications, is the finding that Li^+ is capable of enhancing *in vivo* bone mass, and also activate the canonical Wnt signalling pathway in cultured calvarial osteoblasts from *Lrp5*$^{-/-}$ mice [12]. Additionally, mice treated with lithium after bone injury exhibited enhanced fracture healing [178].

Table 3-1. The mode action of Scl-Ab and lithium in activation of canonical Wnt signalling

Molecule of Wnt	Physiological role	Target agent	Mode of action	Effects on osteogenesis
Sclerostin	Inhibits canonical Wnt signalling via binding LRP5/6	Sclerostin antibody	Neutralizes sclerostin	Increased bone mass, density, and strength in OVX rats, aged male rats, and cynomolgus monkeys Phase I clinical trial: Dose-dependent increase in bone formation without adverse events
GSK3β	Inhibits canonical Wnt signalling via targeting β-catenin for proteosomal degradation	Lithium	inhibits GSK3β	Restores bone mass in LRP5 KO mice and increases bone mass in WT mice Enhances fracture healing in mice Increased bone mass and density with increasing accumulated dose of clinical use of lithium

GSK3β, glycogen synthase kinase 3β; KO, knockout; LRP, low-density lipoprotein receptor-related protein; OVX, ovariectomized; WT, wild type

Increasing evidences demonstrated that Li may be associated with hyperparathyroidism, a risk factor for osteoporosis. The association between Li and enhanced bone formation in mouse models is consistent with epidemiological data in humans. Li can enhance fracture healing in mice, while clinically it reduces the incidence of fractures with increased bone mass and density with increasing accumulated dose of clinical use of lithium [178-180]. Its role in fracture healing is further demonstrated by increased β-catenin expression in fracture tissue in patients taking Li [181]. Yet, there are some reports that argue against the pharmacologic benefit of Li reducing fracture risks [182]. O. Cohan's findings demonstrated although the clinical data does suggest an increase in bone turnover associated with lithium carbonate therapy treatment; however, no distinct effect was detected on bone density after short-or long-term lithium carbonate therapy, indicating that short-or long-term treatment with Li is not associated with increased risk for osteoporosis

[183]. These data demonstrated that GSK3β inhibitor of lithium possesses the promising osteogenic benefits. However, caution needs to be taken in over inhibiting GSK3β due to oncogenic risks.

3.4 SUMMARY

Periodontal regeneration especially cementum repair is a highly precise and programmed biological process that must be robustly controlled using a proper time frame and sequence to enhance the wound healing process. Ultimately, it is now necessary to unveil the distinct spatial and temporal patterns of cellular and molecular events, especially canonical Wnt signalling involving cementogenesis and cementum regeneration with *de novo* generated Sharpey's fibres tightly attached with the newly-formed cementoid. Tissue engineered cementum/periodontal tissues aim to mimic the natural structure of periodontium to guide periodontal regeneration in a physiological manner. This literature review summarizes natural anatomical structure and physiological functions of cementum in periodontal tissue development and regeneration. Also summarized is the potential applicable technology and scaffold materials for periodontal tissue engineering, and cells for cell-scaffold complex construction and strategies to engineer functional periodontium discussed based on current publications.

This chapter highlighted on important role of canonical Wnt signalling in bone regeneration. It inspired me to investigate whether canonical Wnt signalling can induce cementum regeneration *in vivo* and whether suitable biomaterials could stimulate canonical Wnt signalling pathway and further induce cementum tissue regeneration. Therefore, the purpose of this study was to discuss in general terms canonical Wnt signalling pathway that potentially control cementum regeneration *in vivo* and *in vitro*. It can provide a new perspective to better understand the potential mechanism of canonical Wnt signalling activation that mediate periodontal repair and help us in designing new biomaterials that would coordinate the process of cementum/periodontal regeneration clinically.

Chapter 4: Activation of canonical Wnt signalling induces cementum regeneration

Activation of the canonical Wnt signalling pathway induces cementum regeneration

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Statement of Contribution of Co-Authors for Thesis by Published Paper

Contributor	Statement of contribution*
Pingping Han Signature Date	Involved with experimental design, performing the laboratory experiments and data analysis. Wrote the manuscript.
Saso Ivanovski	Involved in the design of the project, data analysis and reviewing the manuscript.
Ross Crawford	Involved in the design of the project, data analysis and reviewing the manuscript.
Yin Xiao	Involved in the conception and design of the project. Assisted in sample collection, technical guidance and reviewing the manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name Signature Date

Abstract

The restoration of large periodontal defects due to periodontal diseases is a major challenge for regenerative therapy. It is well known that the canonical Wnt signalling plays a vital role in cell differentiation during cementogenesis. However, it is unclear whether canonical Wnt signalling pathway can effectively induce significant cementum repair *in vivo* for the regeneration of periodontal tissues and stimulate cementoinductive differentiation for human periodontal ligament cells (hPDLCs) *in vitro*. Therefore, the aim of this study was to investigate the interaction between canonical Wnt signalling activation and cementum repair using rat periodontal defect model and further explore hPDLCs cementogenic differentiation through activation of canonical Wnt signalling pathway. The results showed that the local activation of canonical Wnt signalling in rat periodontal defect area had significant new cellular cementum and well-organized periodontal ligament fibres formation, which was absent in the control group. *In vitro* experiments, using hPDLCs, showed that Wnt signalling pathway activator significantly increased mineralization, alkaline phospatase (ALP) activity, and gene and protein expression of bone and cementum markers of osteocalcin (OCN), osteopontin (OPN), cementum protein 1 (CEMP1), and cementum attachment protein (CAP). Our results suggest that activation of canonical Wnt signalling pathway can induce *in vivo* cementum regeneration and *in vitro* cementogenic differentiaiton of hPDLCs.

Key words: The canonical Wnt signalling pathway; regenerative medicine; cementum regeneration**;** tissue engineering

4.1 INTRODUCTION

The tissues forming the tooth-supporting apparatus of periodontium include alveolar bone, periodontal ligament (PDL), cementum, and gingival. Once the integrity of the peridontium has broken down, it is a major challenge for dental clinicians to restore its original structure and function by tissue regeneration [98]. By definition, regeneration of the lost periodontium involves the formation of all tooth-supporting structures including new cementum, PDL, alveolar bone and gingival tissue. In addition, the appropriate PDL tissue orientation, fibre directionality and integration to both cementum and alveolar bone are required for the function. Furthermore, appropriate mechanical loading is essential for the development of highly organized functional PDL fibres [184-185]. In periodontal tissue engineering and regeneration, cementum regeneration is a critical and challenging phase for the functional PDL formation, however, the molecular mechanisms underlying the regeneration process of cementum is poorly understood [185-186]. There are few, if any, reports describing the interaction between cell signalling 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 to anchorage on tooth [90]. Increasing evidences demonstrated that cementogenesis is from dental mesenchymal cells in PDL differentiate to cementoblasts to form cementum [96-97]. Currently, PDLCs have been reported to be able to regulate the osteogenic differentiation during the process of periodontal regeneration; cell sheet of PDLCs have been confirmed to form new periodontal-cementum complex without/with growth factors indifferent animal models [19-20, 105]. However, the capacity of PDLCs to become cementoblast-like cells is not clear yet, let alone the molecular mechanisms behind such a process.

Understanding the critical regulators associated with cementogenesis is of great importance for developing the molecular therapies for cementum regeneration. It is well known that canonical Wnt signalling is involved in multiple stages of tooth morphogenesis by mediating the transcription of the target genes [74, 89]. Recently, it has been demonstrated that the activation of the canonical Wnt signalling in osteoblasts and odontoblasts leads to aberrant dento-alveolar complex formation with

hypoplastic cementum and periodontal ligament in mice [77-78]. Furthermore, constitutive β-catenin stabilization in the dental mesenchyme leads to the excessive formation of dentin and cellular cementum in mice [76]. These data prompted us to ask whether activation of canonical Wnt signalling might assist in regeneration of cementum/periodontal tissues *in vivo*. Although its essential role in tooth morphologies is well documented, little is known about the involvement of canonical Wnt signalling pathway in the differentiation of PDL cells into cementum regeneration *in vivo* and *in vitro*.

Experiments using neutralizing antibodies have shown that inhibition of sclerostin (Scl-Ab) could promote bone formation via activation of canonical Wnt signalling [10-11]. A similar effect is also seen with the administration of lithium chloride (LiCl), which by inhibiting GSK3β enhances bone healing by activating canonical Wnt signalling [175, 187]. In the present study, we tested the hypothesis that activation of the canonical Wnt signalling using either neutralizing Scl-Ab, lithium or overexpression of β-catenin can have favourable 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: (i) local injection of LiCl; (ii) local injection of a lentiviral construct overexpressing of β-catenin (LV-Ctnnb); and (iii) local injection of neutralizing Scl-Ab. Furthermore, the *in vitro* activation of the canonical Wnt pathway (by either LiCl or LV-Ctnnb) 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 signalling pathway can enhance cementum regeneration *in vivo* and *in vitro*.

4.2 METHODS AND MATERIALS

4.2.1 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). 12-week-old adult male Lewis rats (Rattus norvegicus, Strain-LEW/CrlArc, Animal Resources Centre, WA, Australia) were anaesthetized by isoflurane (AttaneTM, Bomac Animal Health Pty Ltd, Australia) inhalation. A rat

periodontal defect model was created according to a previously published protocol [188].

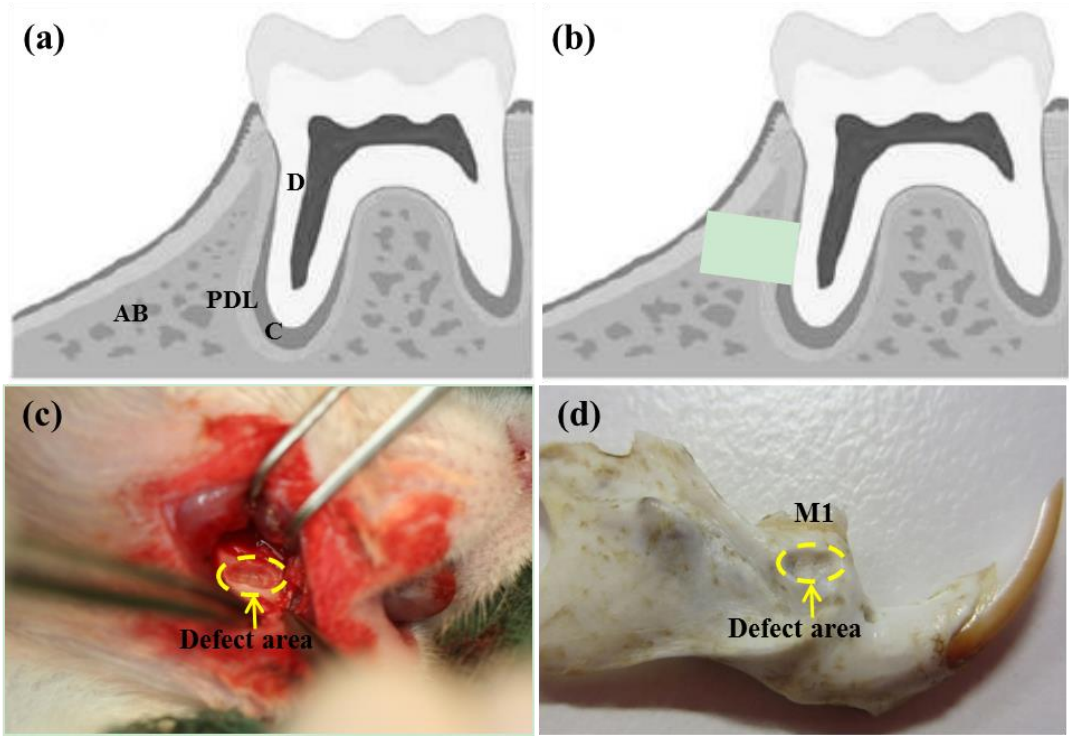

Figure 4-1. Rat periodontal defect model. (a) Schematic illustration of rat mandibular first molar. (b) Schematic illustration of periodontal fenestration defect. (c) Macroscopic observation of the periodontal defect during surgery. (d) The periodontal defect was successfully established. D: dentin; PDL: periodontal ligament; C: cementum; M1: mandibular first molar.

Briefly, a vertical incision was made along the mesiopalatal side of the right buccal mandible. The mucoperiosteal flaps were subsequently elevated by dissection of the underlying masseter muscle and the periosteum covering the surface of first and second mandible molars to expose the mesial alveolar bone. The alveolar bone covering the root between first and second molar was removed with a dental bur with 0.9% saline irrigation. The created periodontal window defect was approximately 1.5 mm in width, 3 mm in length and 2mm in depth, in which all the periodontal ligament tissues and cementum on the tooth root surface were removed. The flap was re-positioned and pressed against the root surface and sutured (Fig. 4-1).The rats were sacrificed 7, 14, and 28 days after the surgery and all the mandibles (n=3 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).

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

To investigate the regenerative potential of the canonical Wnt signalling during periodontal regeneration, rats with periodontal defects were treated on a daily basis for 2 weeks with either vehicle (n=4) or Scl-Ab (humanized monoclonal Scl-Ab III, Amgen Inc., Thousand Oaks, CA, USA; n=4) or LiCl (Sigma-Aldrich, 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 in terproximal gingival papillae (at the dose of 25 mg/kg and 149 mg/kg respectively). Local injection of phosphate-buffered saline (1x PBS, Life Technologies, Australia) served as the vehicle control in the study.

4.2.3 Local injection of LV-Ctnnb in rat periodontal defect

To further confirm the effect of activation of the canonical Wnt signalling on periodontal regeneration, lentivirus particles containing rat gene of β-catenin (Ctnnb) were produced by 293T cell line which can be efficiently transduced by calcium phosphate DNA precipitation [189]. The HIV-1 lentivirus was produced by co-transfection of 293T cells with 10 μg plenti-CTNNB (Thermo Scientific, Australia) gene expression vector, 5 μg of envelope vector pCMV-VSVG and 5 μg of packaging plasmid pCMV-dR8.2. The medium was changed 12 hours after the transfection and then the lentivirus-containing medium was collected. LV-GFP without insert gene was used as control in this study. Virus concentration was determinate by transduction of 293T cells and quantification of transducted cells by flow cytometry for GFP expression. Lentivirus particles ($1x10^9$) 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 1xPBS (n=3) and LV-GFP (n=4) were served as the vehicle control.

4.2.4 Micro-computed tomography (μCT) scanning analysis

Mandible 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 1xPBS for Micro-CT scanning. Tissues were scanned by micro-computed tomography scanning (μCT40, SCANCO Medical AG, Brüttisellen, Switzerland) with high resolution of 12 μm and a voltage of 45 kVp and a current of 177 mA. The Three-dimensional (3D) images were reconstructed from the scans by the micro-CT system software.

4.2.5 Histomorphometric analysis

For histological analyses, jaws were dissected and fixed in 4% paraformaldehyde at 4^{o}C overnight. After rinsing with 1xPBS buffer, the specimens were decalcified in 10% EDTA/PBS solution over a period of 4–8 weeks, and then embedded in paraffin. The samples were sectioned to a thickness of 5μm. Serial sections were performed by Hematoxylin & 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 different colours, with cell nuclei (dark red) by azocarmine, collagen (blue) usinganiline blue. Histological and immunohistochemical images were captured with a Zeiss Axio Scope A1 Microscope (Carl Zeiss Pty, Ltd, NSW, Australia).

Newly-formed cementum was defined as mineralized tissue covering the root surface with cementocytes. The percentage of newly-formed cementum was calculated by dividing the length of the defect on the root surface. Percentage of new alveolar bone formation was calculated by dividing the area of new bone with the alveolar bone defect area.

4.2.6 In situ hybridization and immunohistochemistry (IHC) analysis

RNA-RNA *in situ* hybridization was assessed in this study to measure and localize mRNAs and transcripts within tissue sections. All samples were fixed overnight in 4% formaldehyde solution at 4^{o}C, decalcified in 1% EDTA and then embedded in paraffin. Antisense riboprobes labelled with digoxin were used for *in situ* hybridization. Probes of Axin2 were used for *in situ* hybridization for the canonical Wnt signalling.

Immunohistochemical staining was carried out following de-waxing, hydration and endogenous peroxidase activity. Then the sections were incubated with Axin2

(1:500, rabbit anti-rat; Cell Signalling Technology, Inc., USA) primary antibody overnight at 4^{o}C, followed by incubation, at room temperature with a biotinylated universal swine-anti-mouse, rabbit, goat secondary antibody (DAKO, CA, USA) for 15 min, and then with horseradish perioxidase-conjugated avidin-biotin complex (DAKO, CA, USA) for another 15 minutes. The antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 minutes. Mayer's haematoxylin (HD Scientific Pty Ltd.) was used for counter staining.

4.2.7 Cell culture, proliferation and differentiation assay

The isolation and culture of human periodontal ligament cells (hPDLCs) and human alveolar bone-derived osteoblasts (hOBs) were performed according to previously published protocols [190]. Informed consent was given by all patients involved and the research protocol had been approved by the Human Ethics Committees of Queensland University of Technology, Brisbane, Australia. hPDLCs and hOBs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS; Thermo Scientific) and 50 U/mL penicillin and 50 mg/mL streptomycin (P/S; Gibco-Invitrogen) at 37^{o}C in a humidified CO_2 incubator. For osteogenic differentiation, cells were cultured in osteogenic DMEM medium containing 10% FBS, 50μg/ml ascorbic acid, 3mM β-glycerophosphate and 10nM dexamethasone in the absence or presence of LiCl (Sigma-Aldrich, Australia) and LV-Ctnnb.

Cell proliferation was assessed by MTT assay using the methods as described in our previous protocols [191]. Briefly, 0.5 mg/mL of MTT solution (Sigma-Aldrich, Australia) was added to each well and incubated at 37^{o}C for 4 h to form formazan crystals. The formazan was solubilized with dimethyl sulfoxide and the absorbance read at 495 nm on a 96 well plate reader.

Alizarin red staining was carried out as described before [192]. The medium was discarded and cells were fixed with ice-cold 100% methanol for 20 min, 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 day 7 and 14 after culture in osteogenic differentiation medium. Cells were lysed in a lysis buffer containing 0.2% Triton X-100 and 50mM Tris-HCl. The whole cell lysis was measured using

QuantiChromTM Alkaline Phosphatase Kit (BioAssay System) by adding pNPP as a substrate assay buffer containing $MgCl_2$, and then the kinetic of absorbance was read at 405nm. The enzyme activity was expressed as OD value per min per μg of protein. The total protein in the cell lysis was measured using the BCATM Protein Assay Kit (PIERCE).

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

4.2.8 qRT-PCR and western blotting analysis

Total cellular RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions and reverse transcription was performed using a DyNAmo cDNA Synthesis Kit (FINNZYMES). The qRT-PCR primers for each gene are shown in Table 4-1. The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an ABI 7300 Real-Time PCR System and the relative gene expression was calculated using the following formula: $2^{-\Delta\Delta Ct}*10^4$.

Western Blotting analysis was performed for the detection of the protein expression. The protein concentration was determined by the BCA Protein Assay Kit (Thermo Fisher Scientific, Australia) and then 10 μg proteins from each sample were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred into a nitrocellulose membrane (Pall Corporation, East Hills, NY, USA). After blocking for 1 h with Odyssey® blocking buffer (Millennium Science, Australia), the membranes were incubated with the 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 Signalling Technology), Axin2 (1:2000 rabbit anti-human; Abcam) and GAPDH (1:1000, mouse anti-human; Abcam) overnight at 4^oC and then with fluorescent secondary antibodies (1:4000, Cell Signalling Technology) and then targeted proteins were visualized using the Odyssey® infrared imaging system.

4.2.9 Statistical analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) version 21.0 software. 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 of less than 0.05 were considered to be statistically significant.

4.3 RESULTS

4.3.1 No cementum formation takes place during periodontal wound healing

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 micro-CT images were reconstructed to evaluate the primary healing of the defect at different time points. As shown in Fig. 4-2a, mineralized tissue covering the defect could be seen to increase with time.

Periodontal tissue biopsies including gingival, alveolar bone and tooth were obtained at different time points. The representative images of H&E and AZAN staining showed that the new osteoblasts were formed along the surface of the old bone fragments at days 7 and 14 after surgery. At day 14, a high density of fibroblast-like cells had formed along the root surfaces of the surgical wounds. Newly deposited connective tissue fibres were formed along the root dentin surface, and the fibres were not well-oriented. At day 28, new bone formation could be seen mainly at the edge of old bone fragment. However, there was no new cementum found during this process (Fig. 4-2b, c).

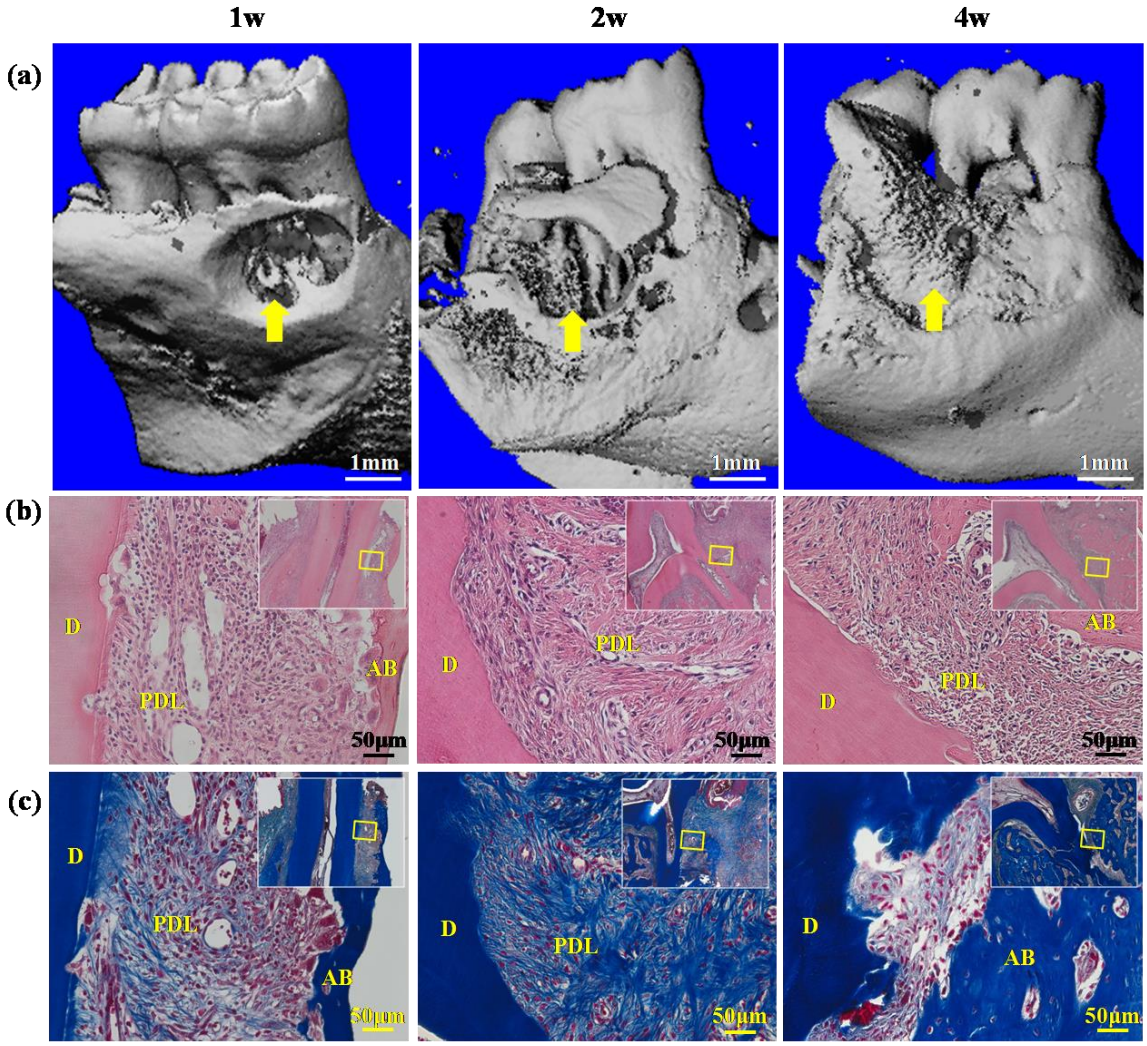

Figure 4-2. Primary periodontal wound healing in control samples. (a) Representative μCT reconstruction images showing increased new bone formation (yellow arrow) with the time. (b) The representative H&E and AZAN staining images showed the periodontal remodelling process at 1, 2 and 4 weeks after the surgery. M1: mandibular first molar; D, dentin; AB, alveolar bone; PDL, periodontal ligament.

4.3.2 Local activation of the canonical Wnt signalling with LiCl and Scl-Ab in established periodontal defect

To elucidate the role of canonical Wnt signalling in alveolar bone repair, the Wnt signalling activators LiCl and anti-sclerostin primary antibody were injected locally to the periodontal defect area for two weeks. After 2 weeks, μCT analysis of samples from rats with LiCl and Scl-Ab injections showed a slight increase in alveolar bone compared with PBS injection control group (Fig. 4-3a and 4-4a).

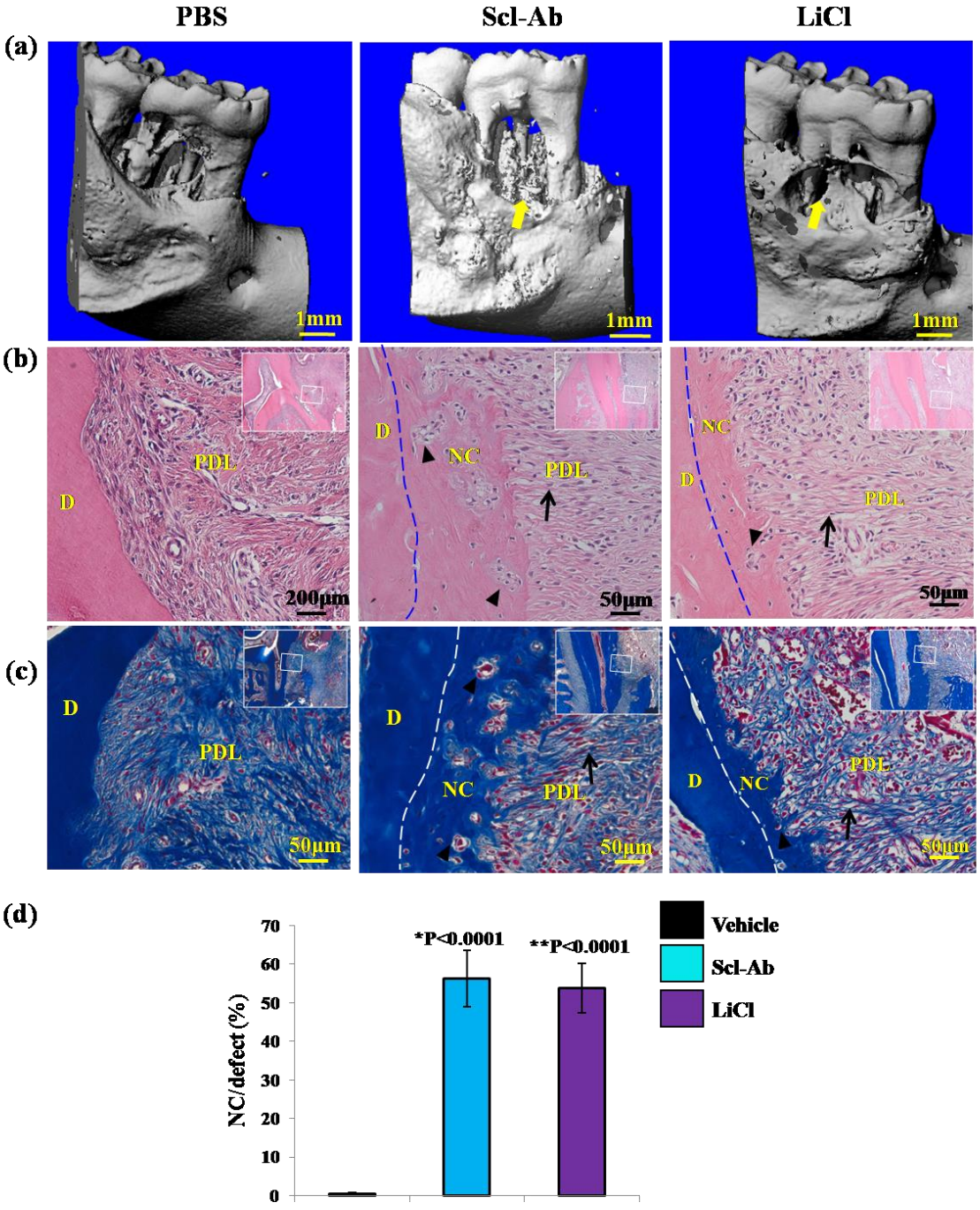

Figure 4-3. Activation of canonical Wnt signalling inducing periodontal regeneration. (a) Representative μCT images of the periodontal healing process via activation of canonical Wnt signalling. (b, c) Representative images of H&E and AZAN staining for histological sections of periodontal defect 2 wks after the local injection at the surgical area. Scl-Ab and LiCl injection had new cemtum formation with well-orientated PDL fibres (black arrow) inserted, while vehicle group had no cementum formation. Black triangles indicate new cementocytes formed in newly-formed cementum. (d) Scl-Ab and LiCl significantly enhanced new cementum formation compared to control group. M1: mandibular first molar; D, dentin; AB, alveolar bone; PDL, periodontal ligament; NC, newly-formed cementum.

After analysis of H&E and AZAN histological staining of sections, the LiCl and Scl-Ab injection groups had a significant increase of new cementum formation, whereas control groups showed no cementum formation along the root surface (Fig. 4-3 b, c). After 2 week of Scl-Ab injection, newly formed cementum was seen on the denuded root surface with well-orientated PDL collagen fibres inserted. The LiCl injection group, after 2 weeks of local injections, also showed obvious cementum formation with cementocytes entrapped and well-orientated Sharpey's collagen fibres inserted.

4.3.3 LiCl and Scl-Ab injections enhanced alveolar bone formation

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; while the LiCl and Scl-Ab groups had enhanced new bone formation in the defect site along the old bone (Fig. 4-4). There was a significantly higher percentage of bone and cementum coverage for LiCl and Scl-Ab groups compared to PBS injection groups (Fig. 4-3d and 4-4d) ($p < 0.05$).

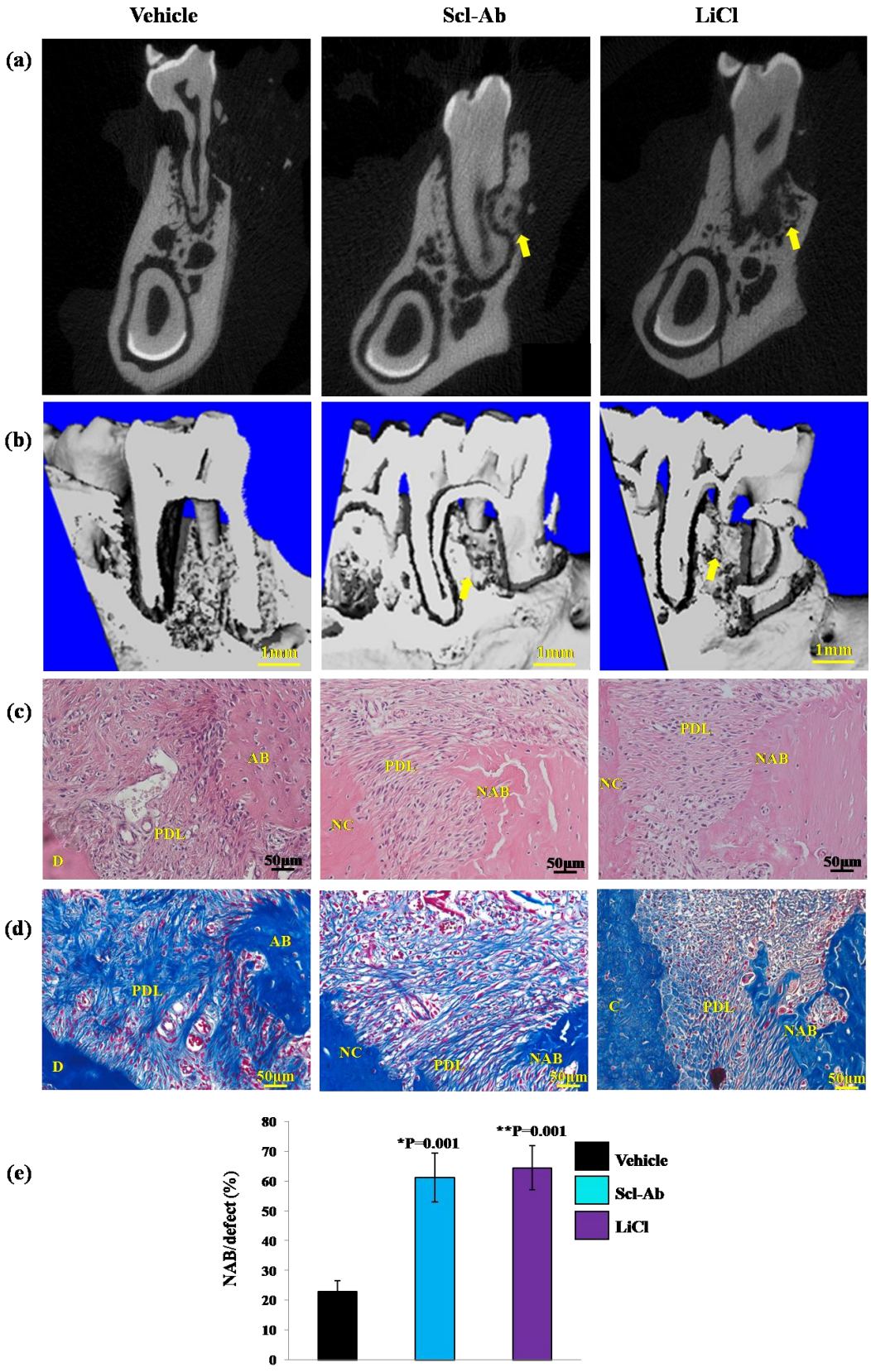

Figure 4-4. Enhanced alveolar bone formation via LiCl and Scl-Ab local injections. (a) After 2 week's injections, LiCl and Scl-Ab groups had induced bone formation between first molar cups (yellow arrow) compared to the PBS injection group. (b, c) Representative H&E and AZAN stained sections of the defects after 2 weeks injections. At 2 week, new bone was

observed in the LiCl and Scl-Ab injections groups, with PDL fibres inserted. D: Dentin; NAB: newly-formed Alveolar Bone; PDL: Periodontal Ligament; NC, newly-formed cementum.

4.3.4 Regeneration mode: detection of Axin2 in periodontal regeneration tissues

In situ hybridization and immunohistological analysis of AXIN2 expression were carried out to investigate whether the canonical Wnt signalling was activated in the defect area.

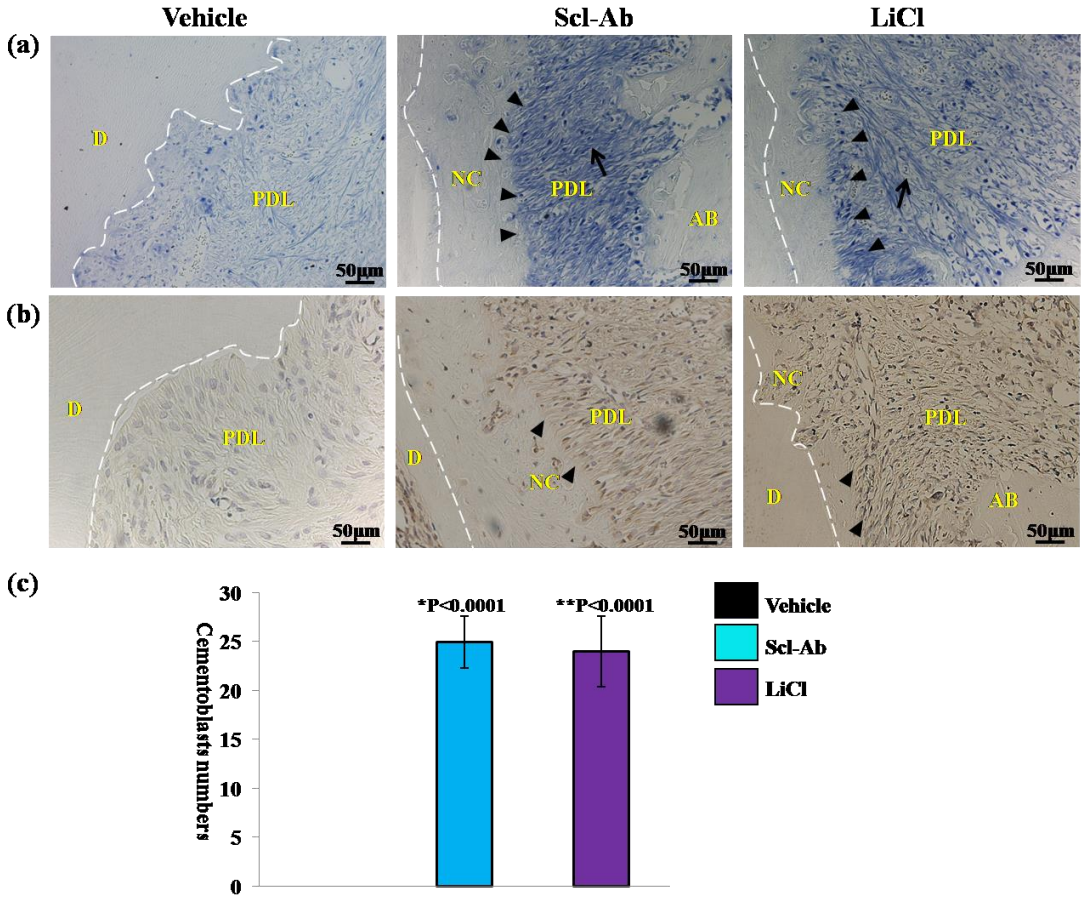

Figure 4-5. *In situ* hybridization and IHC detection of AXIN2 expression in regeneration study. Representative sections showed that RNA (a) and protein (b) expression of AXIN2 was increased in newly-formed PDL fibres (black triangle) and cementocytes (black arrow) via local activation of canonical Wnt signalling with LiCl and Scl-Ab for 2 weeks. Figure c demonstrated that LiCl and Scl-Ab not only enhanced newly-formed cementum, but also increased alveolar bone formation significantly, when compared to that of vehicle group. D: Dentin; PDL: Periodontal Ligament; NC, newly-formed cementum.

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 and newly-formed periodontal fibres in LiCl and Scl-Ab groups (Fig. 4-5a), indicating the activation of canonical Wnt signalling in local periodontal defect area. IHC data confirmed that protein expression pattern of Axin2 was observed locally in periodontal defect area. Activation of canonical Wnt signalling with LiCl and Scl-Ab not only increased new cementum formation but also led to the increased new alveolar bone formation (Fig. 4-4c).

4.3.5 Overexpression of β-catenin facilitates periodontal regeneration

To further confirm whether activation of canonical Wnt signalling has enhanced effect of cementum regeneration, lentivirus particles for overexpression of Ctnnb were injected locally in periodontal defect area for two weeks. Confocal laser scanning microscopy (CLSM) images provided evidence of newly-formed cementocytes and well-oriented PDL fibres with strong GFP expression in LV-Ctnnb injected animals compared to controls (Fig. 4-6a). The representative H&E and AZAN staining images showed that LV-Ctnnb had enhanced cementum formation with well-ordered PDL fibres (Fig. 4-6 b, c).

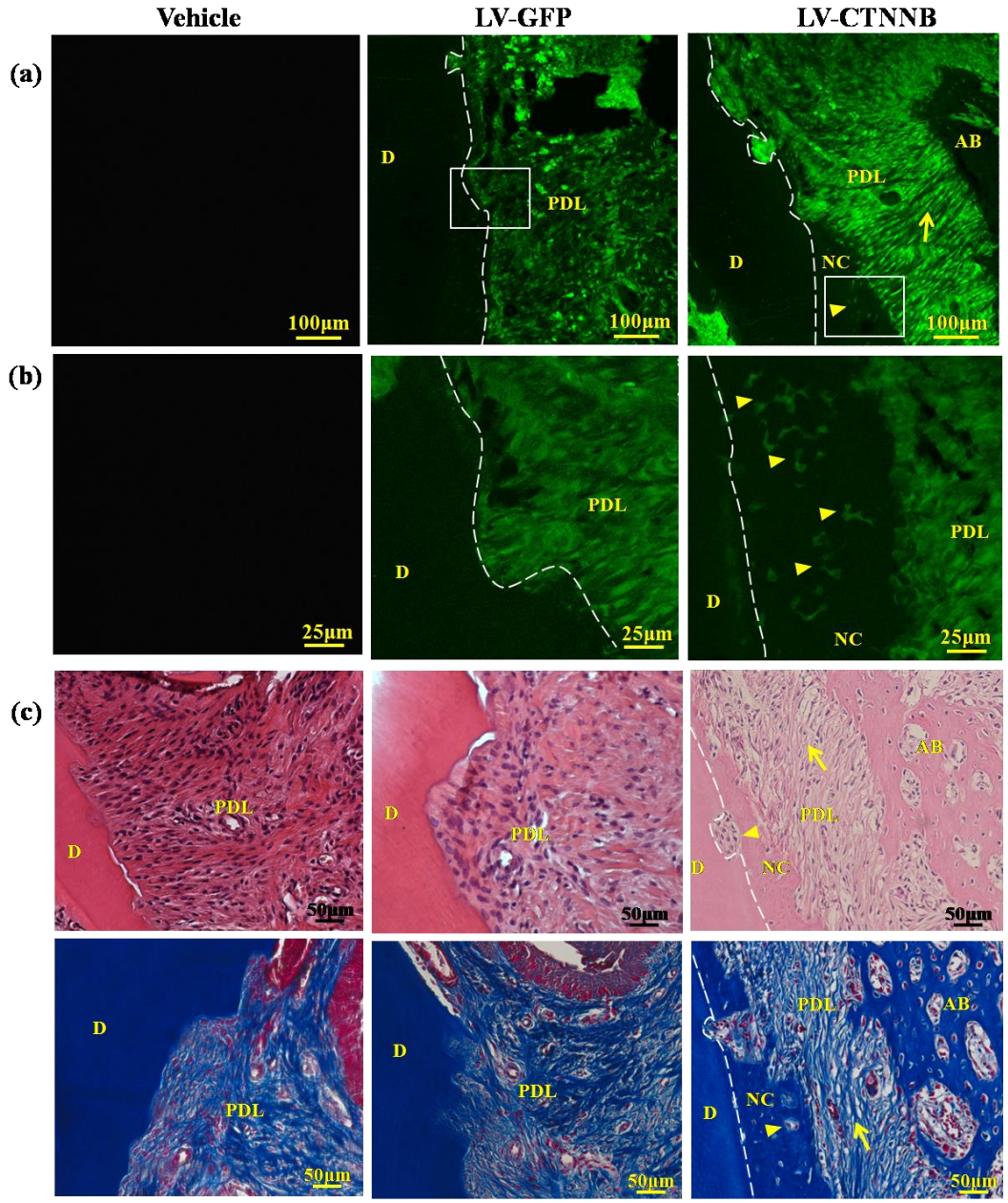

Figure 4-6. Overexpression of Ctnnb facilitates periodontal regeneration after 2 weeks. (a) After injection of LV-Ctnnb for two weeks, newly-formed cementocytes (yellow triangles) formed with well-oriented PDL fibres (yellow arrows) inserted compared to LV-GFP group. (b) Higher magnification of GFP-positive cementocytes (yellow triangle) in different groups. (c) The representative H&E and AZAN staining images show LV-Ctnnb increased cementum and PDL fibres formation. D: Dentin; PDL: Periodontal Ligament; NC, newly-formed cementum, AB, alveolar bone.

4.3.6 Activation of canonical Wnt signalling pathway during hPDLCs osteogenic differentiation

LiCl induced activation of canonical Wnt signalling was carried out to investigate whether it can regulate osteogenic differentiation of hPDLCs. The effect of different concentrations of LiCl on proliferation of hPDLCs was assessed by MTT test. The MTT assay results showed that LiCl significantly stimulated proliferation of hPDLCs compared to control group in 1, 3 and 7 days culture; 5mM of LiCl was the optimum concentration for proliferation (Fig.4-7c). The mineralization was examined using Alizarin Red staining, relative ALP activity and intracellular calcium assay. As shown in Supplemental Fig. 1a, 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 amount as revealed by calcium assay (Fig.4-7c).

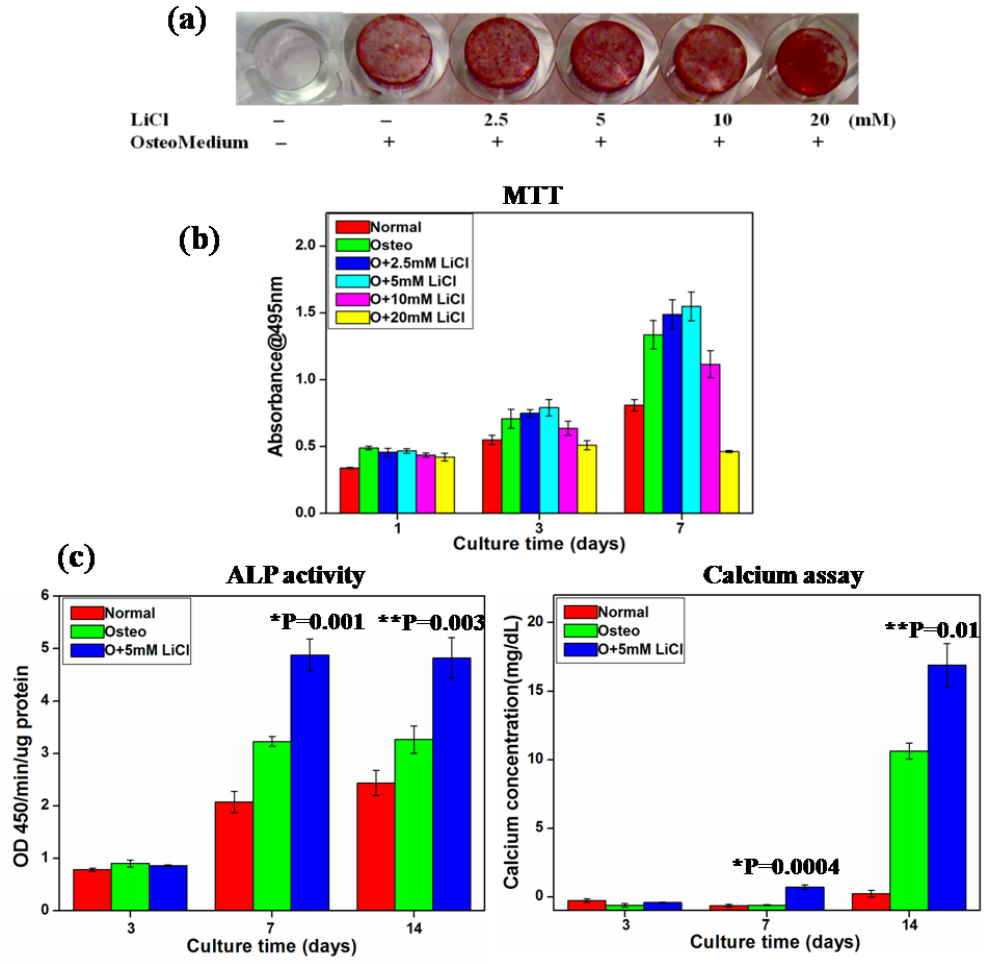

Figure 4-7. Activation of canonical Wnt signalling enhanced proliferation and differentiation for hPDLCs. (a, b) The Alizarin red staining and cell proliferation assay for hPDLCs in the presence of different concentrations of LiCl. (c) LiCl increased relative ALP activity and calcium concentration for hPDLCs with different time points.

4.3.7 Cementogenic/osteogenic gene/protein expression and signalling pathway for hPDLCs

Quantitative RT-PCR and western blot analysis were carried out to investigate the effect of LiCl treatment on cementoblastic/osteogenic differentiation markers in hPDLCs, (Fig. 4-8). The results showed that the gene expressions of the osteogenic markers *OCN* and *OPN* increased in the presence of LiCl over time compared to control group (Fig. 4-8) as did the expression of the cementogenic markers *CEMP1* and *CAP* (Fig. 4-8b).

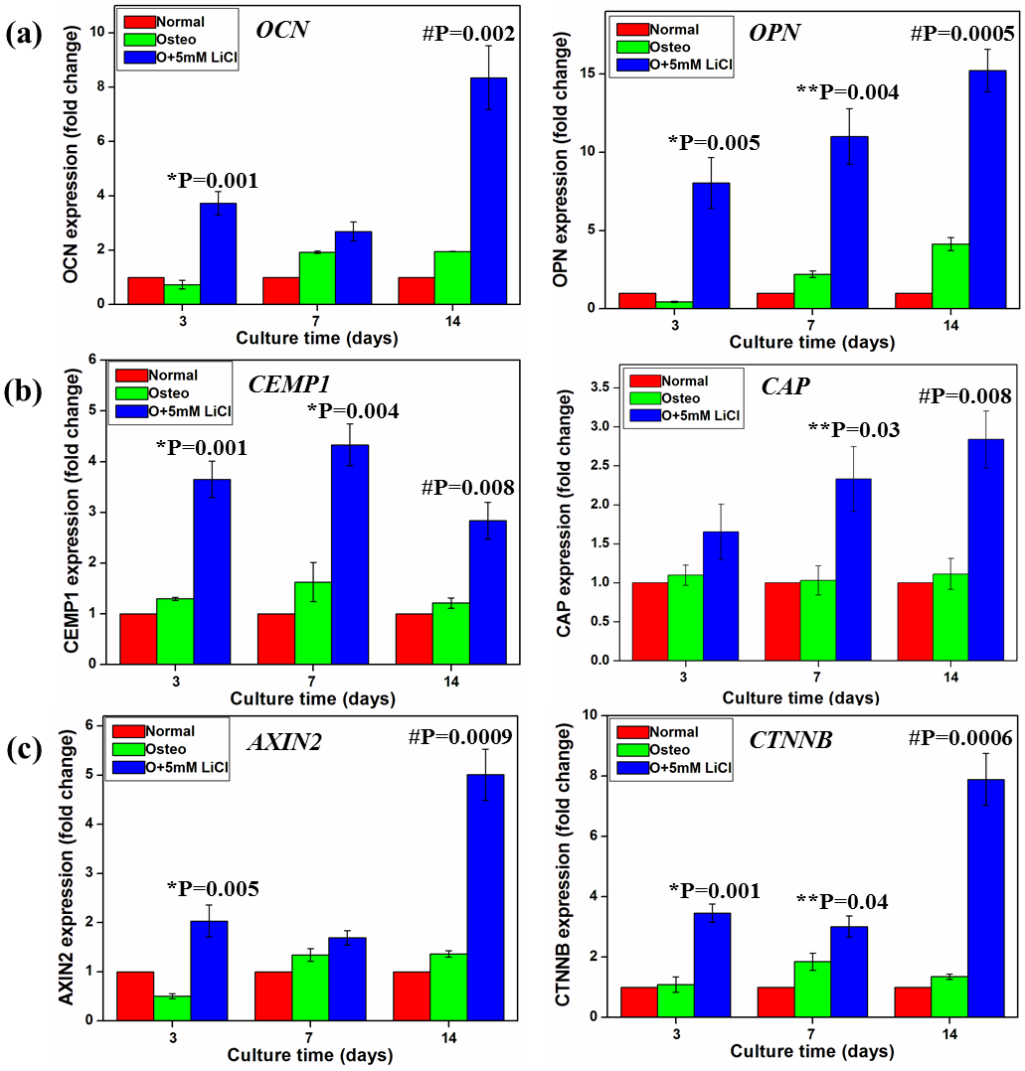

Figure 4-8. Activation of canonical Wnt with LiCl increased bone and cementum-related gene/protein expression for hPDLCs. The relative gene expression of osteogenic markers of OPN, OCN (a), cementum-related markers of CEMP1, CAP (b) and Wnt-related genes of AXIN2, CTNNB (c) for hPDLCs in the presence of LiCl. O/OM: osteogenic differentiation medium.

The analysis of the expression of canonical Wnt-related genes such as *AXIN2* and *CTNNB* was performed by qRT-PCR after culturing hPDLCs with LiCl (5mM) for 3, 7 and 14 days. The results showed that the expression of *AXIN2* and *CTNNB* of hPDLCs in the presence of LiCl was significantly higher than control group at day 3, 7 and 14 (Fig. 4-8c).

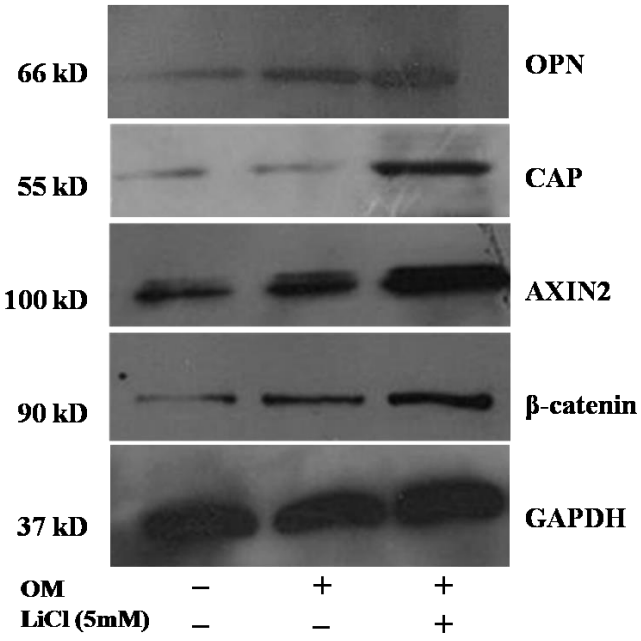

Figure 4-9. LiCl had enhanced protein expression for cementogenic/osteogenic and Wnt-related genes after 7 days culture. O/OM: osteogenic differentiation medium.

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 as the result of LiCl compared to two other groups (Fig. 4-9).

4.3.8 The effect of CTNNB overexpression on hPDLCs differentiation

hPDLCs were infected 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. 4-10a). CTNNB gene/protein expression assays further confirmed that LV-Ctnnb infected hPDLCs activated canonical Wnt signalling, resulting in enhanced CAP and β-catenin protein expression (Fig. 4-10 b, c).

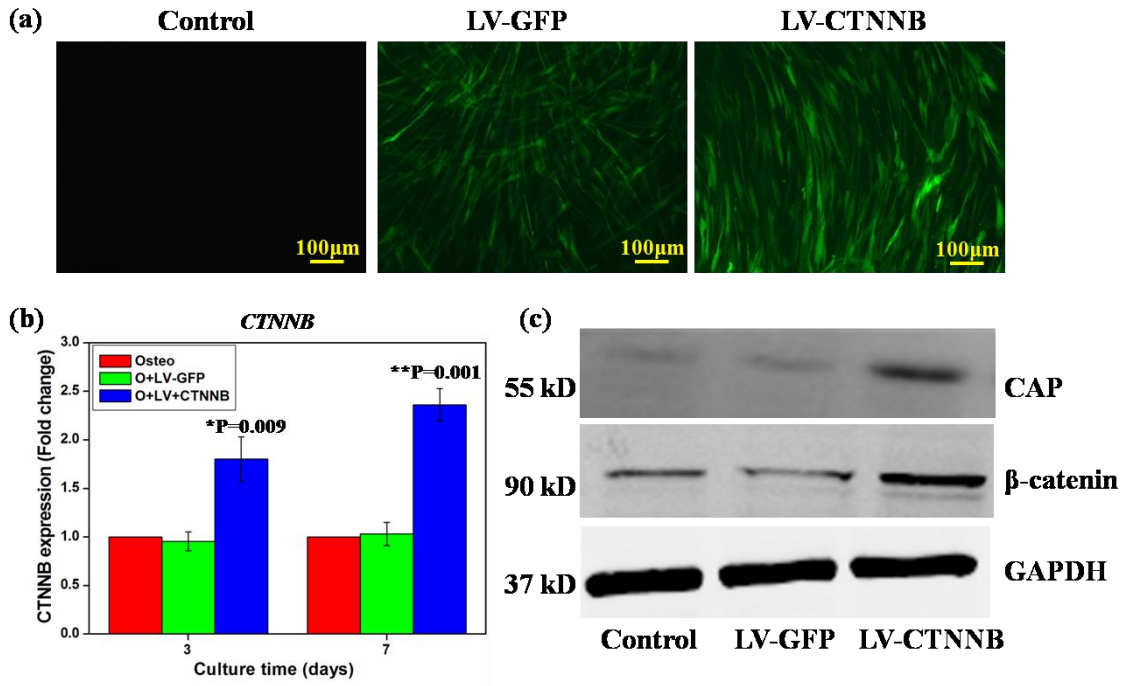

Figure 4-10. Overexpression of Ctnnb for hPDLCs. (a) GFP expression was indicated after transduction with lentivirus containing Ctnnb gene. (b) To confirm LV-Ctnnb transduction with enhanced expression of Ctnnb. (c) LV-Ctnnb increased CAP and Ctnnb protein expression. Cells without lentivirus transduction and LV-GFP were used as control.

4.3.9 Canonical Wnt signalling in differentiation of osteoblasts

To investigate the role of canonical Wnt signalling in alveolar bone derived osteoblasts cementogenic differentiation, LiCl were applied in the culture medium. qRT-PCR results showed a failure of osteoblasts to express CAP gene and protein in response to LiCl treatment (Fig. 4-11). However, LiCl treatment did result in enhanced AXIN2 and β-catenin protein expression osteoblasts (Fig. 4-11).

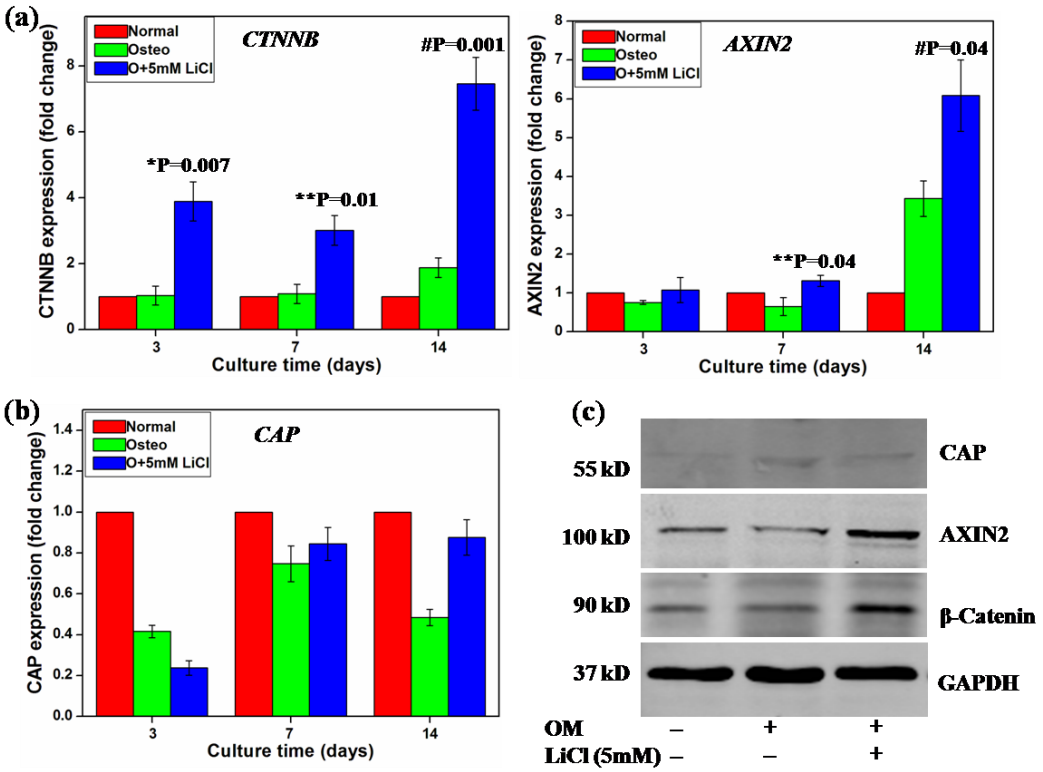

Figure 4-11. Activation of canonical Wnt signalling for hOBs. (a) The gene expression of canonical Wnt signalling of AXIN2 and CTNNB for hOBs in the presence of LiCl. (b) CAP expression for hOBs under culture with LiCl. (c) hOBs had no CAP protein expression under activation of Wnt signalling with enhanced AXIN2 and CTNNB protein expression.

4.4 DISCUSSIONS

This study was conducted to investigate the interaction between activation of canonical Wnt signalling pathway and cementum repair/regeneration *in vivo* and *in vitro*. To the best of our knowledge, this is the first study of its kind, which activates canonical Wnt signalling *in vivo* by local injection of LiCl, Scl-Ab and LV-Ctnnb into a periodontal defect. The results showed that the activation of canonical Wnt signalling stimulates *de novo* alveolar bone formation, with well-orientated PDL fibres and cementum formation. In addition, the regulation of canonical Wnt signalling was also investigated *in vitro* for hPDLCs proliferation and osteogenic/cementogenic differentiation. These data indicate that canonical Wnt signalling pathway is an important regulator for cementum regeneration and suggests this signalling 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 the 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 [193-194]. We can report that in this study, therat model of periodontal defect was successfully established, with the exposed tooth root surface documented by macroscopic and micro-CT observations. Although some periodontal tissues were formed 4 weeks after the surgery, including periodontal ligament fibres and alveolar bone, the cementum still lags behind in terms of repair during the natural healing progress following the periodontal defect surgery.

There is much evidence to support the essential role of canonical Wnt signalling 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 [74]. However, the molecular mechanism of canonical Wnt signalling in cementum regeneration is largely unknown. Many studies suggested that LiCl and anti-sclerostin neutralizing antibody could enhance the *in vitro* cell proliferation and improve bone mass in mice *in vivo* by activating canonical Wnt signalling pathway [12, 174, 195]. For this reason, it is important to elucidate the involvement of canonical Wnt signalling pathway with cementum repair to establish its role during periodontal tissue engineering.

The pharmacological inhibition of sclerostin using neutralizing monoclonal antibodies have been studied in clinical trials for postmenopausal osteoporosis, and in preclinical models of osteoporosis, bone repair, and fracture healing [164-165, 167]. Moreover, oral use of lithium has been used to treat humans with bipolar disease for over a half-century, with substantial benefit [169-171]. 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 with increasing accumulated dose use but also restore bone mass in LRP5 knockout mice and increase fracture healing in mice [175, 187]. 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 signalling; however, effects within the

periodontal complex have never been studied. Experiments were, therefore, designed to compare the transcriptional events induced by LiCl and Scl-Ab. Importantly, similar results were obtained in terms of newly-formed cementum, alveolar bone and periodontal fibres, suggesting that both LiCl and Scl-Ab act in a similar fashion to induce new cementum/periodontal regeneration.

Our study provides early preclinical evidence that Scl-Ab and LiCl have an ongoing 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 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 fibres inserted compared to the control group.

Axin2 is recognised as the most accurate reporter gene in canonical Wnt pathway since it is a direct target gene of Wnt ligand binding and activation of the Wnt signalling pathway [83] and has been found to be expressed during the tooth formation including the crown and root developments [83, 89]. In this study, *in situ* hybridization and IHC results demonstrated that Axin2 transcript/protein expression was increased in response to Scl-Ab and LiCl treatments compared to the controls, whereas there was only weak Axin2 expression of in PBS injection group. The Axin2 expression coincided with newly-formed Sharpey's fibres and cementocytes in the LiCl and Scl-Ab treatment groups, indicating that activation of canonical Wnt signalling is correlated with periodontal regeneration. It is anticipated that the role of the canonical Wnt signalling will share a similar pattern between early cementogenesis and cementum regeneration, which may provide evidence to promote an entirely new approach for cementum regeneration.

β-catenin is a critical member of Wnt/β-catenin signalling pathway, and although its expression is not directly linked to the activation of the Wnt pathway, it is the inhibition of its cytoplasmic degradation that is the direct consequence of the upstream activation of the pathway [71-72]. Overexpression of β-catenin in this study enhanced cementum formation with well-oriented PDL fibres inserted compared to control groups.

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 which is mediated by BMP-like molecules [19-20, 105]. Recent findings demonstrated that the Wnt/β-catenin signalling pathway stimulates cell proliferation and osteoblastic differentiation in hPDLCs [196-197]. However, it is unknown whether canonical Wnt signalling is involved with hPDLCs osteogenic/cementogenic differentiation. In this study, LiCl, a GSK-3β inhibitor, was used to examine the possible involvement of the canonical Wnt signalling in regulating hPDLCs behaviour. In agreement with many recent reports, our results showed that activation of canonical Wnt signalling could enhance the expression of late osteoblasts makers OPN and OCN, and the cementobolast-related markers CAP. As a control group, human alveolar bone-derived osteoblasts showed no expression of CAP in the presence of activated canonical Wnt signalling (S. Fig. 2). These findings indicate that hPDLCs are the responsible cell type to differentiate into osteoblast/cementoblast-like cells compared to hOBs.

Several issues must be considered before the use of lithium and Scl-Ab as potential therapeutic targets to regenerate cementum and periodontal tissues becomes a viable option. For instance, over-activation of canonical Wnt signalling may be tumorigenic. Short-term lithium treatment (60 days) in mice with the cancer predisposing *Apc* mutations caused a modest increase in tumor size and, more importantly, it has been reported that rates of cancer increased in patients receiving chronic lithium therapy [198-199]. Another consideration is whether local administration of lithium or Scl-Ab in the oral environment may in fact have detrimental effects on the periodontal regeneration. More in depth studies would need to be conducted with gradient concentrations of LiCl and Scl-Ab on periodontal regeneration, especially in cementum regeneration. Although Scl-Ab showed increased cementum formation during periodontal healing, this is a costly reagent with a short half-life, whereas lithium is inexpensive and its ion release can be carefully controlled to activate canonical Wnt signalling. For this reason, lithium was the focus of the *in vitro* experiments in this study. There is also the issue of what constitute cementum-specific markers. Although cementum attachment protein (CAP) has been reported as a cementum-specific marker, PDL tissues also express CAP strongly [200]. This makes it challenging to distinguish between what is

osteogenic differentiation and cementogenic differentiation. This problem can only be solved by identifying yet unknown cementum-specific markers.

We put forward the hypothesis that activation of canonical Wnt signalling 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 signalling pathway. Additionally, future evaluation of cementum forming capacity is required in large animal models to demonstrate that canonical Wnt signalling can initiate bone/cementum regeneration as well as its crosstalk with other signalling pathways in the relation to differentiation of PDLCs to cementoblasts.

4.5 CONCLUSIONS

In summary, local injections of LiCl, Scl-Ab and LV-Ctnnb in a rat periodontal defect model resulted in *de novo* cementum formation with well-orientated PDL fibres, *via* the regulation of canonical Wnt signalling pathway. The canonical Wnt signalling activation by LiCl and LV-Ctnnb significantly enhanced cell proliferation, and bone/cementum-related gene/protein expression in hPDLCs. Targeting canonical Wnt signalling pathway may provide a new potential application in periodontal tissue regeneration.

4.6 ACKNOWLEDGEMENTS

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Chapter 5: Design of lithium-containing MBG for cementogenic differentiation

The cementogenic differentiation of hPDLCs via the activation of the canonical Wnt signalling by Li^+ ions released from bioactive scaffold

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Statement of Contribution of Co-Authors for Thesis by Published Paper

Contributor	Statement of contribution*
Pingping Han Signature Date	Involved with experimental design, performing the laboratory experiments and data analysis. Wrote the manuscript.
Chengtie Wu	Involved in the design of the project, data analysis and reviewing the manuscript.
Jiang Chang	Involved in the design of the project, data analysis and reviewing the manuscript.
Yin Xiao	Involved in the conception and design of the project. Assisted in sample collection, technical guidance and reviewing the manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name Signature Date

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Abstract

Lithium (Li) has been widely used as a long-term mood stabilizer in the treatment of bipolar and depressive disorders. Li^+ ions are thought to enhance the remyelination of peripheral nerves and also stimulate the proliferation of neural progenitor cells and retinoblastoma cells via activation of the canonical Wnt signalling pathway. Until now there have been no studies reporting the biological effects of released Li^+ in bioactive scaffolds on cemetogenesis in periodontal tissue engineering applications. In this study, parts of Li^+ ions were incorporated into the mesoporous bioactive glass (MBG) scaffolds and showed that this approach yielded scaffolds with a favourable composition, microstructure and mesopore properties for cell attachment, proliferation, and cementogenic differentiation of human periodontal ligament-derived cells (hPDLCs). This study went on to investigate the biological effects of Li^+ ions themselves on cell proliferation and cementogenic differentiation. The results showed that 5% Li^+ ions incorporated into MBG scaffolds enhanced the proliferation and cementogenic differentiation of hPDLCs on scaffolds, most likely via activation of the canonical Wnt signalling pathway. Further study demonstrated that Li^+ ions by themselves significantly enhanced the proliferation, differentiation and cementogenic gene expression of hPDLCs. These results indicate that incorporation of Li^+ ions into bioactive scaffolds is a viable means of enhancing the canonical Wnt signalling pathway to stimulate cementogenic differentiation of hPDLCs.

Keywords*:* Lithium; Bioactive scaffolds; Cementum/periodontal tissue engineering; Gene expression; Signalling pathway

5.1 INTRODUCTION

For the past 50 years, Lithium (Li^+) has been widely used as a long-term mood stabilizer in the treatment of bipolar and depressive disorders and its mode of action is thought to be by enhancing the remyelination of peripheral nerves [169-171]. Li^+ has been also been reported to activate the canonical Wnt signalling pathway by inhibiting glycogen synthase kinase-3β (GSK-3β), an enzyme that phosphorylates β-catenin in the cytoplasm, thereby targeting it for ubiquitination and degradation [172-174]. Previous studies have shown that lithium chloride can stimulate the proliferation of retinoblastoma cells via elevated the canonical Wnt signalling [176]; and the *in vitro* proliferation and neuronal differentiation of neural progenitor cells was enhanced by long-term treatment by lithium [177]. A study that is of particular interest for the application of Li^+ ions in bone tissue engineering applications is the finding that Li^+ is capable of enhancing *in vivo* bone mass, and also activating the canonical Wnt signalling pathway in cultured calvarial osteoblasts from *Lrp5*$^{-/-}$ mice [12].

The treatment of periodontital diseases is a major challenge due to difficulties in periodontal tissue regeneration, especially the cementum. Periodontal tissue engineering has come into focus as an alternative approach for the regeneration of alveolar bone, root cementum, and periodontal ligament [98, 201-203]. Human periodontal ligament cells (hPDLCs) have the capacity *in vitro* to differentiate into osteoblasts and cementoblasts [204] due to the multilineage differentiation potential [110]. The cementogenic differentiation of hPDLCs is spatially and temporally regulated by the cell signalling proteins and genes and influenced by the ions presented in the culture media[205]. In the previous study, it has demonstrated that the stem cell transplantation can lead to the formation of new cementum, however stem cell transplantation to periodontal site is still a clinically challenged technique to deliver. It is known that bioactive scaffolds play an important role to influence the biological response of tissue cells and the subsequent tissue formation [206-207]. It is, therefore, of great clinical relevance to design bioactive scaffolds with properties that stimulate the cementogenic differentiation of PDLCs for better periodontal regeneration application. As such, mesoporous bioactive glass (MBG) has attracted significant attention in recent years [128, 191, 208-211]. The significant feature of MBG, compared with non-mesopore bioactive glass (NBG), is that it possesses

significantly improved specific surface area and nanopore volume which is evidenced by greatly enhanced apatite-mineralization ability and degradation [208, 212-213]. It has been demonstrated that MBG has improved *in vitro* and *in vivo* bioactivity, degradation and drug delivery properties, compared to NBG [214-215] and, as a bioactive material; MBG has great potential for cementum/periodontal regeneration applications. In an earlier study Li^+ was incorporated into 45S5 bioglass disks (not scaffolds) and was found that Li^+ could influence the apatite-mineralization of 45S5 bioglass with a dose-dependent way [216]. However, there is no study about how Li-containing biomaterials and Li^+ ions influence the cementogenic differentiation of cells.

With these earlier studies in mind, it is reasonable to hypothesise that the incorporation of Li^+ ions into MBG scaffold system may improve the cementogenic differentiation of PDLCs for periodontal tissue engineering application. Therefore, the aim of this study, therefore, was to prepare Li^+-containing bioactive scaffolds and then investigate the effect of Li^+ ions released from the scaffolds on the proliferation and cementogenic differentiation of hPDLCs.

5.2 MATERIALS AND METHODS

5.2.1 Preparation and characterization of porous Li-MBG scaffolds

Porous lithium-containing mesopore-bioglass (Li-MBG) scaffolds were prepared by incorporating Li (molar: 0, 2 and 5%) into MBG using co-templates of non-ionic block polymer P123 (EO20-PO70-EO20) and polyurethane sponges. P123 is used to produce mesoporous structures (mesopore size: around 5 nanometers) and polyurethane sponges are used to create large pores (large pore size: 300-500 hundred micrometers) as described in previous publications [191, 211]. To prepare MBG scaffolds containing of 5% Li, 6 g of P123 (Mw=5800, Sigma-Aldrich), 10.1 g of tetraethyl orthosilicate (TEOS, 98%), 1.4 g of $Ca(NO_3)_2{\cdot}4H_2O$, 0.125 g of LiCl (Sigma-Aldrich), 1.10 g of triethyl phosphate (TEP, 99.8%) and 1.5 g of 0.5 M HCl were dissolved in 90 g of ethanol (Li/Ca/P/Si/ = 5/10/5/80, molar ratio, named 5Li-MBG) and stirred at room temperature for 1 day. The polyurethane sponges (25ppi) were cleaned and completely immersed into this solution for 10 min, then transferred to a Petri dish to allow evaporating at room temperature for 12 h. This procedure was

repeated three times. Once the samples were completely dry, they were calcined at 700^{o}C for 5 h yielding the 5Li-MBG scaffolds. MBG scaffolds without Li (Li/Ca/P/Si/ = 0/15/5/80, molar ratio, named: MBG) and with 2% Li (Li/Ca/P/Si/ = 2/13/5/80, molar ratio named: 2Li-MBG) were prepared by the same method except for their Li and Ca contents.

The large-pore structure and inner microstructure of the calcined Li-MBG scaffolds were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

5.2.2 Isolation and culture of human periodontal ligament cells (hPDLCs)

Isolation and culture of human PDLCs were performed according to previously published protocols [20]. Teeth were obtained from healthy patients (n=3, 18–25 years old) undergoing third molar extraction surgery. Informed consent was given by all patients involved and the research protocol had been approved by the Human Ethics Committees of Queensland University of Technology, Brisbane, Australia. Periodontal ligament tissues were separated from the middle third of the root surface using a scalpel and were cultured in a T25 flask in Dulbecco's Modified Eagle Medium (DMEM; Gibco-Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS; Thermo Scientific) and 50 U/mL penicillin and 50 mg/mL streptomycin (P/S; Gibco-Invitrogen) at 37^{o}C in a humidified CO_2 incubator. The medium was changed after five days and the outgrown cells growing around the PDL tissues were passaged at approximately 80% confluence and expanded through two passages to obtain a sufficient number of cells for the *in vitro* assays. Cells at passages P2–P5 were used for the study.

5.2.3 Morphology and proliferation of hPDLCs on Li-MBG scaffolds

At an initial density of 1 x 10^{5}cells/scaffold, PDLCs were cultured on 5×5×5 mm Li-MBG scaffolds placed in 48-well culture plates. After culture for 7 days in DMEM culture medium with 10% FBS, the scaffolds were removed from the culture wells, rinsed in PBS, and then fixed with 2.5% glutaraldehyde in PBS for 1 h. After washing with buffer containing 4% (w/v) sucrose in PBS and post fixed in 1% osmium tetroxide in PBS, the scaffolds were dehydrated in ethanol series (50, 70, 90, 95 and 100%) and hexamethyldisilizane (HMDS) and then all the specimens were

coated with gold. Using SEM, the morphological characteristics of the attached cells was performed.

Cell proliferation was assessed by MTT assay using the methods as described in previous protocols [191]. Briefly, an 0.5 mg/mL of MTT solution (Sigma-Aldrich) was added to each scaffold and incubated 37^{o}C to form formazan crystals. The culture media was removed and the formazan was solubilized with dimethyl sulfoxide (DMSO) after 4 h. The absorbance of the formazan-DMSO solution was read at 495 nm using a plate reader. All the results were demonstrated as the optical density values minus the absorbance of blank wells.

5.2.4 Relative alkaline phosphatase (ALP) activity and calcium concentration test of hPDLCs on Li-MBG scaffolds

The relative ALP activity was assessed at day 7 and 14 after hPDLCs cultured in Li-MBG scaffolds with a seeding density of 1x10^{5} per scaffold. Briefly, the cells were washed with PBS three times to remove the media, and then lysed in 200 μL of 0.2% Triton® X-100. Lysates were centrifuged at 14,000 rpm for 5 min at 4^{o}C and 50 μL of supernatants mixed with 150 μL ALP assay working solution according to the manufacturer's protocol (QuantiChrom™ Alkaline Phosphatase Assay Kit, BioAssay Systems, USA). The total protein content was measured by the bicinchoninic acid protein assay kit (Thermo Scientific, Australia). The optical density (OD) was measured at 405 nm on a plate reader. The relative ALP activity was obtained as the changed OD values divided by the reaction time and total protein content.

The calcium concentration of hPDLCs was measured at day 7 and 14 in culture on the Li-MBG scaffolds. The cells were washed with ddH_2O three times to remove the medium, then lysed in 100 μL of 0.6N HCl. The lysates were centrifuged at 14,000 rpm for 15 min at 4^{o}C and 5 μL of supernatant mixed with 200 μL of the assay working solution according to the manufacturer's protocol (QuantiChrom™ Calcium Assay Kit, BioAssay Systems, USA).

5.2.5 Cementogenic-related gene expression of hPDLCs on Li-MBG scaffolds

The effect of Li-MBG scaffolds on hPDLCs cementogenic differentiation was assessed by real-time quantitative PCR (RT-qPCR) to measure the mRNA

expression of *alkaline phosphatase (ALP),osteopontin (OPN), osteocalcin (OCN), cementum protein 1 (CEMP1)* and *cemtum attached protein (CAP)* in all treatment groups (Table 5-1). The media was changed after 24 h to osteogenic differentiation medium after 1 x 10^6 hPDLCs cultured in each scaffold. Cells were harvested on day 3 and 7 and then total RNA was isolated using Trizol Reagent® (Invitrogen) according to the manufacturer's instructions. From 1 µg total RNA, complementary DNA was synthesized using DyNAmo™ cDNA Sythesis Kit (Finnzymes, Genesearch, Australia) following the manufacturer's instructions. RT-qPCR primers were designed based on cDNA sequences from the NCBI Sequence database and the primer specificity was confirmed by BLASTN searches. RT-qPCR was performed on an ABI Prism 7300 Thermal Cycler (Applied Biosystems, Australia) with SYBR Green detection reagent. Briefly, 2.5 µL of cDNA template, 2.5 µL of gene-specific reverse and forward primers (GeneWorks Pty Ltd, Australia) and 12.5 µL of 2×SYBR Green qPCR Master Mix (Invitrogen) was mixed with 5 µL RNAse free water for a 25 µL final reaction volume in a 96-well PCR plate. The relative mRNA expressions of *ALP, OPN, OCN, CEMP1* and *CAP* were assayed and normalized against the house keeping gene *GAPDH*. Each sample was performed in triplicate. The mean cycle threshold (Ct) value of each target gene was normalized against Ct value of GAPDH and the relative expression calculated using the following formula: $2^{-(\text{normalized average Cts})} \times 10^4$.

Table 5-1. Primer pairs used in qRT-PCR analysis

Gene	Forward primer	Reverse primer
ALP	5' TCAGAAGCTAACACCAACG 3'	5' TTGTACGTCTTGGAGAGGGC 3'
OPN	5' TCACCTGTGCCATACCAGTTAA 3'	5' TGAGATGGGTCAGGGTTTAGC 3'
OCN	5' GCAAAGGTGCAGCCTTTGTG 3'	5' GGCTCCCAGCCATTGATACAG 3'
CEMP1	5' GGGCACATCAAGCACTGACAG 3'	5' CCCTTAGGAAGTGGCTGTCCAG3'
CAP	5' CTGCGCGCTGCACATGG 3'	5' GCGATGTCGTAGAAGGTGAGCC 3'
AXIN2	5' CCCCAAAGCAGCGGTGC 3'	5' GCGTGGACACCTGCCAG 3'
CTNNB	5'GCTACTGTTGGATTGATTCGAAATC 3'	5'CCCTGCTCACGCAAAGGT 3'
GAPDH	5' TCAGCAATGCCTCCTGCAC 3'	5' TCTGGGTGGCAGTGATGGC 3'
18s	5' TTCGGAACTGAGGCCATGAT 3'	5' CGAACCTCCGACTTCGTTC 3'

5.*2.6 The canonical Wnt signalling related gene expression of hPDLCs on Li-MBG scaffolds*

To investigate the effect of Li-MBG scaffolds on canonical Wnt signalling related the gene expression for hPDLCs and Wnt/β-catenin related genes (*AXIN2, β-CATENIN*) were analysed by RT-qPCR as described above.

5.2.7 Ion release from Li-MBG scaffolds

To investigate the ion release of Li-MBG scaffolds, the cell culture medium was collected after cell culturing for set periods. The ionic concentrations of Li, Ca, P, and Si ions in the medium were determined by inductive coupled plasma atomic emission spectrometry (ICP-AES) (Perkin-Elmer Optima 7000DV).

5.2.8 The effect of Li ions on cementogenic-relative gene expression of hPDLCs

To further investigate the effect of Li ions on the cementum-related genes expression of hPDLCs, *ALP*, *OPN*, *OCN*, *CEMP1* and *CAP* were carried out by RT-qPCR.

5.2.9 Statistical analysis

All the analysis was performed using SPSS software (SPSS Inc., Chicago, Il, USA). All the data were showed as means ± standard deviation (SD) and analyzed using one-way ANOVA with a Student's T-test; p-values < 0.05 were considered statistically significant.

5.3 RESULTS

5.3.1 Characterization of porous Li-MBG scaffolds

TEM analysis reveals that both MBG and 5Li-MBG scaffolds had a well-ordered and uniform mesoporous channel structure with the pore size of 50 nm in the inner of matrix (Fig. 5-1).

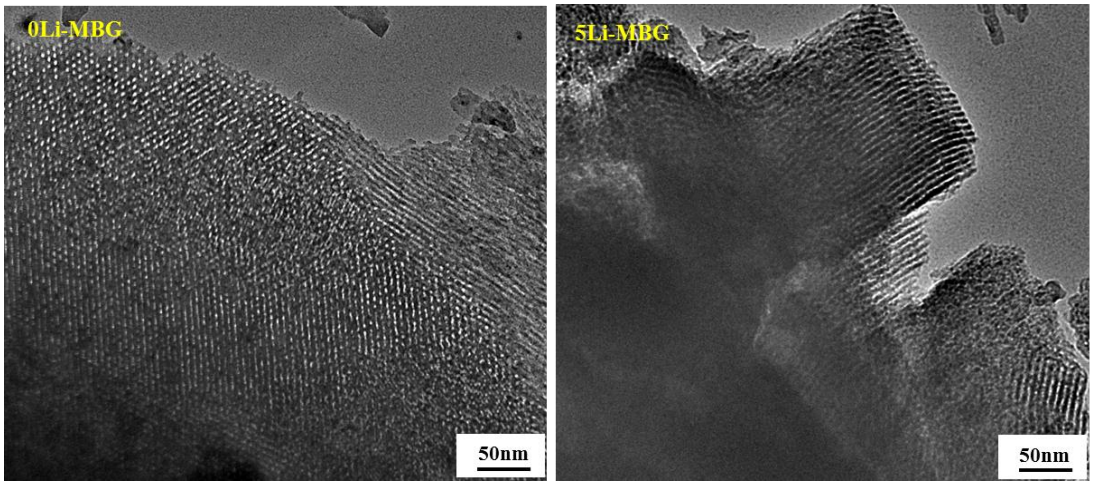

Figure 5-2. TEM analysis for 0Li-MBG (a) and 5Li-MBG (b) scaffolds.

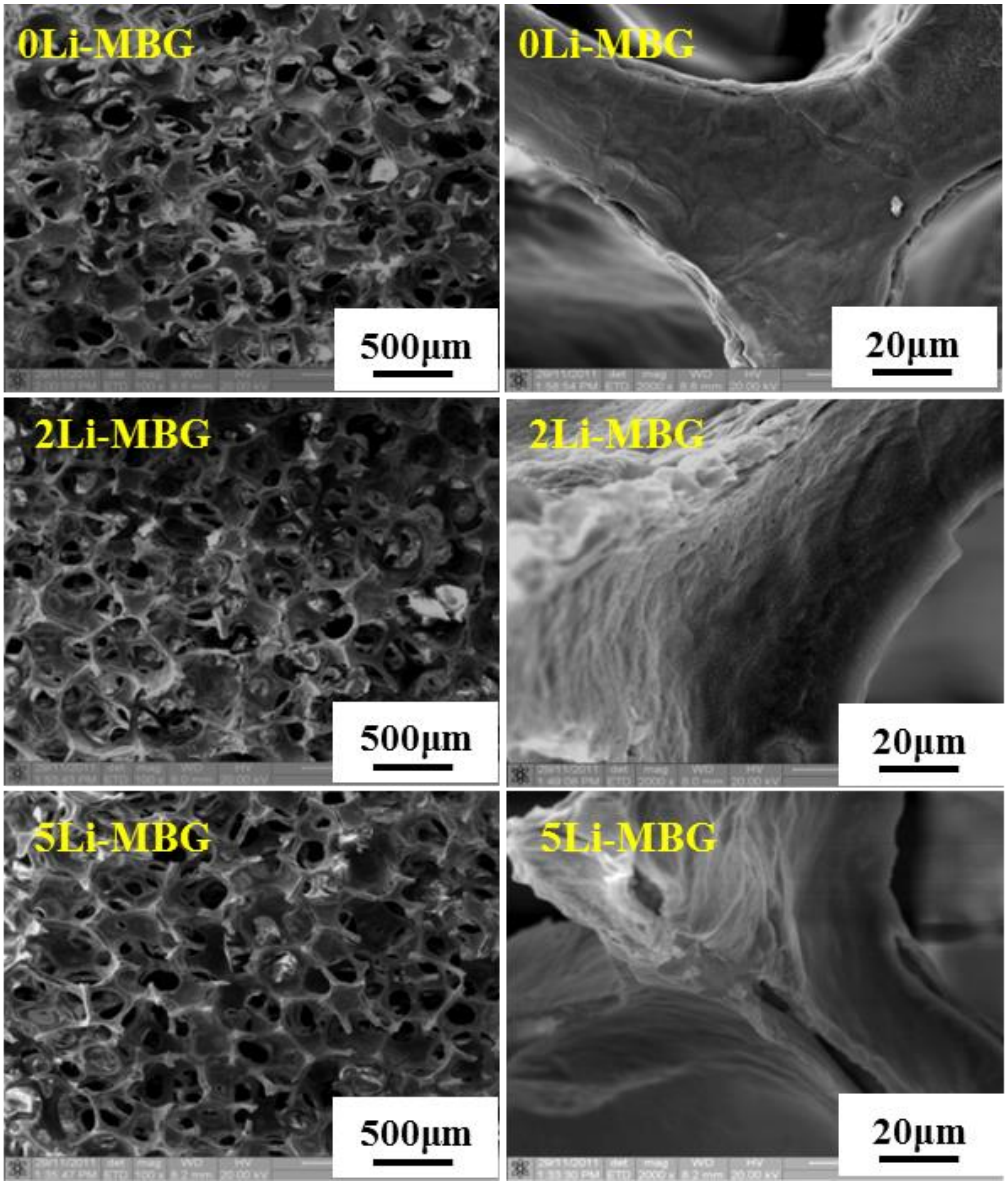

Figure 5-2. SEM analysis for 0Li-MBG (a, b), 2Li-MBG (c, d) and 5Li-MBG (e, f) scaffolds. (b), (d) and (f) are high magnification images.

SEM analysis revealed that the Li-MBG scaffolds (0, 2 and 5% Li^+) all had a highly porous structure with similar large-pore sizes ranging from 300 to 500 µm (Fig. 5-2a, c, and e). Higher magnification images showed smooth surfaced pore walls and no significant difference between the three scaffolds types (Fig. 5-2 b, d and f).

5.3.2 Attachment, morphology, proliferation of hPDLCs on Li-MBG scaffolds

Human PDLCs attachment and morphology on the Li-MBG scaffolds was examined by SEM (Fig. 5-3). After 7 days in culture, hPDLCs were attached to the surface of

the pore walls in all three scaffolds types. Cells were in close contact with the scaffold surfaces and had numerous filopodia.

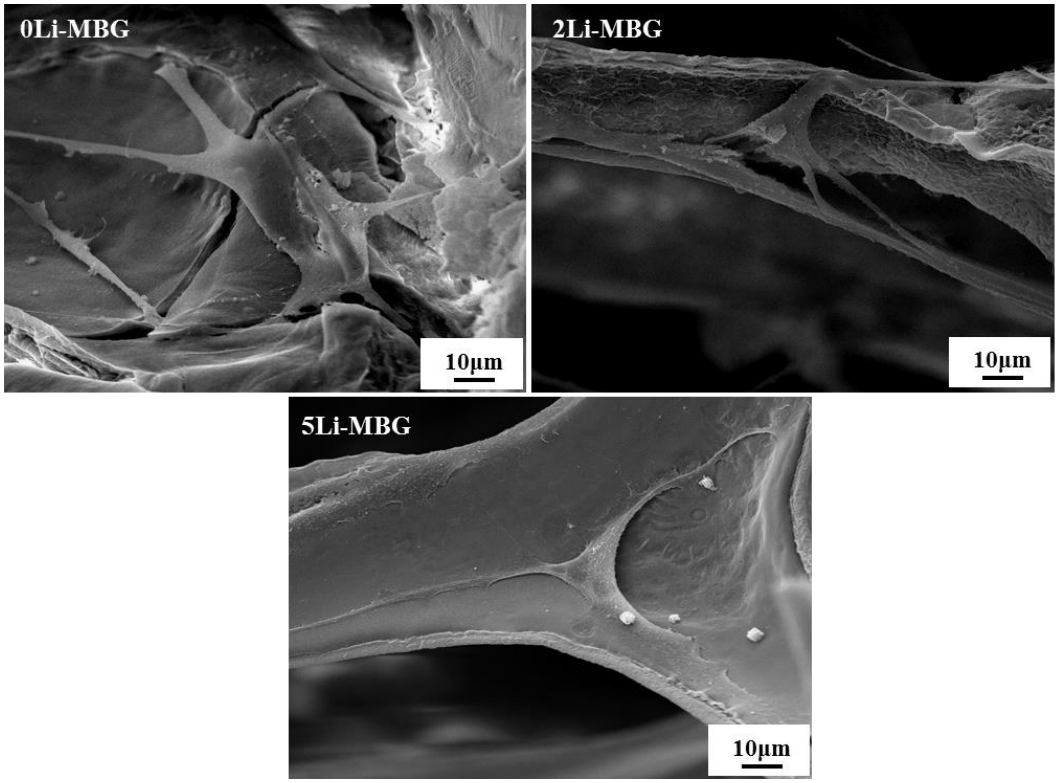

Figure 5-3. The attachment of hPDLCs on 0Li-MBG (a), 2Li-MBG (b) and 5Li-MBG scaffolds after culturing for 7 day.

MTT analysis showed that the number of cell increased with length of time in culture (Fig. 5-4a). The cell number on 5Li-MBG scaffolds was greater than that of the 2Li-MBG and MBG scaffolds at 7 days. Overall, the rate of cell proliferation on 5Li-MBG scaffolds was significantly higher than that on pure MBG or 2Li-MBG scaffolds with increased time in culture.

5.3.3 ALP activity and calcium content of hPDLCs on Li-MBG scaffolds

The relative ALP activity of hPDLCs on the 5Li-MBG scaffolds showed a significant increase compared to other two groups ($p<0.05$) at both 7 and 14 days (Fig. 5-4b). There was no significant difference for the calcium deposition across the three scaffold types (Fig. 5-4c).

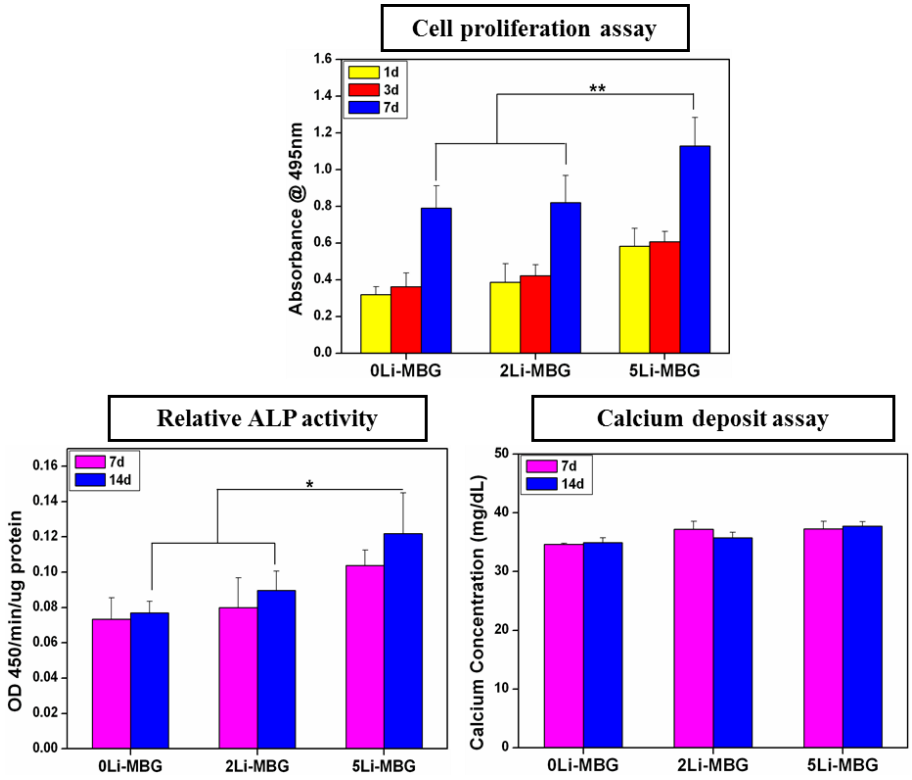

Figure 5-4. The cell proliferation (a), relative ALP activity (b) and calcium mineralization concentration (c) of hPDLCs on three scaffolds. *: significant difference ($p<0.05$) for 5Li-MBG group compared to other two groups (5Li: 0.1219±0.020076; 2Li:0.0897±0.010978; 0Li:0.077±0.006481). **: 5Li had an improved cell proliferation compared to other two groups ($P < 0.05$) (5Li:1.12825±0.156438; 2Li: 0.82125±0.113953; 0Li:0.7905±0.120876).

5.4.4 Cementum-related and specific genes expression of hPDLCs on Li-MBG scaffolds

5Li-MBG scaffolds showed significantly upregulated expression of the mineralization-related genes, such as *ALP*, *OPN* and *OCN* (Fig. 5-5 a, b and c) at both day 3 and 7 compared to other two groups (MBG and 2Li-MBG). 5Li-MBG scaffolds also had a significant increased expression of the cementum-related CEMP1 and CAP genes compared to two other groups at both day 3 and 7 (Fig. 5-5 d and e).

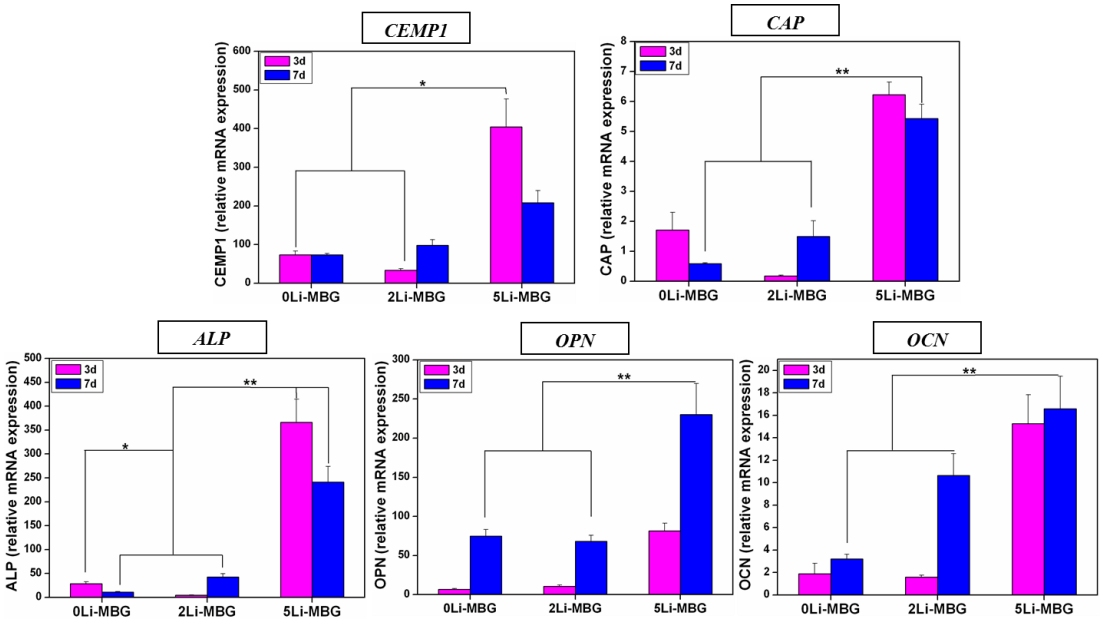

Figure 5-5. The effect of Li contents in MBG scaffolds on bone-related gene expression of ALP (a), OPN (b), OCN (c) and cementum-specific markers of CEMP1 (d) and CAP (e) for hPDLCs. *: significant difference ($p<0.05$) for 5Li- MBG group compared to other two groups at day 3. **: significant difference ($p<0.05$) for 5Li- MBG group compared to other two groups at day 7.

5.4.5 Wnt signalling pathway-related gene expression of hPDLCs on Li-MBG scaffolds

RT-qPCR results showed a significantly increased expression of Wnt related genes (*AXIN2*, *β-catenin*) in the 5Li-MBG scaffold samples compared with the other two groups at both day 3 and 7 (Fig. 5-6).

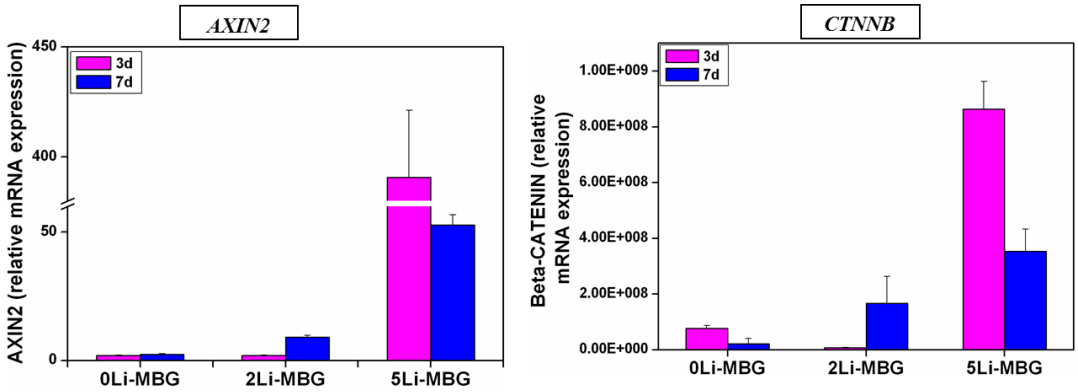

Figure 5-6.The relative AXIN2 (a) and β-CATENIN (b) gene expression for hPDLCs cultured in three scaffolds. **: significant difference ($p<0.05$) for 5mM Li group compared to blank control group at day 7.

5.4.6 Ion in cell culture medium after culture PDLCs with of Li-MBG scaffolds

Generally, there are almost no Li ions in MBG scaffolds medium. Li ion concentrations increased in the medium with the increase of Li contents in Li-MBG scaffolds at all time-points (Table 5-2). 5Li-MBG scaffolds released more Li ions than 2Li-MBG and MBG scaffolds. It was also noted that the incorporation of Li into MBG scaffolds increased Si ion release.

Table 5-2. Ions release of Li-MBG scaffolds in DMEM

Soaking time	Materials	Li^- (mg/L)	PO_4^{3-}(mg/L)	SiO_4^{4-}(mg/L)	Ca^{2+}(mg/L)
1d	0Li-MBG	0	421.44±5.487	68.56±1.098	122.90±4.215
	2Li-MBG	3.27±0.021	370.22±6.029	112.50±3.458	172.00±4.526
	5Li-MBG	8.52±0.214	364.26±5.031	109.99±4.921	167.00±3.856
7d	0Li-MBG	0	407.85±4.938	72.01±3.140	75.42±2.842
	2Li-MBG	5.33±0.359	276.68±3.526	84.08±4.529	302.70±5.693
	5Li-MBG	17.28±1.061	308.09±5.148	82.51±2.632	249.40±4.315
14d	0Li-MBG	0	383.48±5.804	68.50±1.093	50.25±1.578
	2Li-MBG	8.63±0.642	281.49±4.153	75.68±2.012	111.60±2.103
	5Li-MBG	16.01±1.073	293.02±4.965	79.38±3.201	66.76±2.065

5.4.7 The effect of Li^+ ions on cementum-related and cementum-specific genes expression for hPDLCs

ALP, OPN and *OCN* gene expression of hPDLCs in 5mM Li^+ ions was significantly upregulated at both day 3 and 7 compared to the control group without Li^+ ions (Fig. 5-7a, b and c). Similarly, the relative expression of CEMP1 and CAP was also upregulated by 5mM Li ions at day 3 and 7 compared to the control (Fig. 5-7d and e).

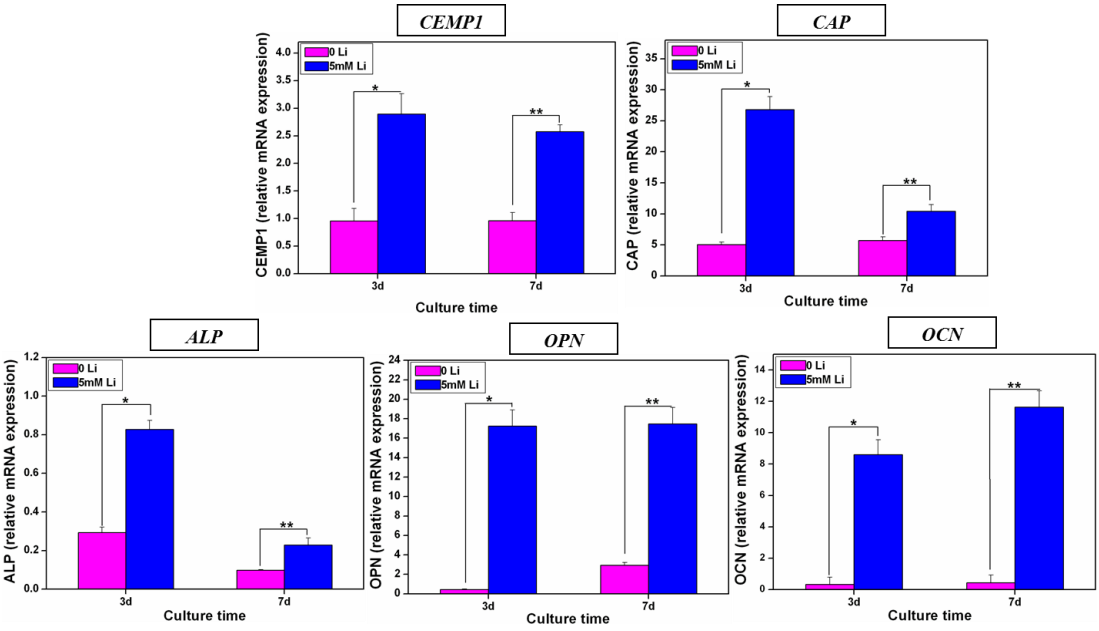

Figure 5-7. The effect of Li ions on gene expression of bone-related genes of ALP (a), OPN (b), OCN (c) and cementum-specific genes of CEMP1(d), CAP (e) for hPDLCs after culture for 3 and 7 days. *: significant difference ($p<0.05$) for 5mM Li group compared to blank control group at day 3. **: significant difference ($p<0.05$) for 5mM Li group compared to blank control group at day 7.

5.4 DISCUSSION

In this study, Li-MBG scaffolds have been successfully prepared with hierarchically large pores (300-500 μm) and well-ordered mesopores (5 nm) by incorporating Li^+ ions into the scaffolds. It was further investigated regarding the cell proliferation and cementogenic differentiation, including Wnt-related gene expression of hPDLCs cultured with Li-MBG scaffolds and Li^+ ions-containing medium. It was found that the incorporation of 5% Li^+ into MBG scaffolds significantly enhanced cell proliferation and cementogenic differentiation, as well as activation of Wnt signalling pathway in hPDLCs. It was found that Li^+ by itself was sufficient to promote the cell proliferation, differentiation and cementogenic related gene expression in hPDLCs. These results suggest that Li^+ ions released from biomaterials play an important role in enhancing cementogenesis of PDLCs on bioactive scaffolds and this biological reaction may be via activation of Wnt signalling pathway.

Earlier studies have shown that Li^+ ions could enhance the bone mass *in vivo* and stimulate neuronal differentiation of neural progenitor cells *in vitro*, but there are no

reports in which Li^+ ions have been incorporated into bioactive scaffolds and their effect on cell proliferation and differentiation for cementogenesis in periodontal tissue engineering applications. In the present study, Li-MBG scaffolds were firstly prepared with the polymer sponge method in which some of the Ca in the scaffolds was substituted with Li. The Li-MBG scaffolds thus prepared had a steady release of Li ions into the surrounding growth medium, which stimulated the cementogenic differentiation of PDLCs. The amount of Li^+ ions released depended on the amount of Li^+ incorporated into the scaffolds in the first place. Previous studies showed that low amount of Li (3%) could inhibit apatite mineralization of biomaterials [216]. In addition, if Li+ contents are too high, it will result in potential cytoxicity. Therefore, in this study, two low contents of Li (2 and 5%) were selected in scaffolds. Li ions release from Li-MBG scaffolds supported the cell attachment and proliferation of hPDLCs and 5% Li^+ incorporated into the MBG scaffolds significantly enhanced the cell growth and ALP activity. Most importantly, it was found that the incorporation of 5% Li into MBG scaffolds had the effect of significantly enhancing the mineralization related gene expression of *ALP*, *OPN* and *OCN*. Previous studies have shown that cementum-derived attachment protein (CAP) and cementum protein 1 (CEMP1) have limited expression in mature cementum matrix of adult periodontium and cementoblasts [217-218]. These results showed that the gene expression of these cementum-specific makers was enhanced by 5Li-MBG scaffolds. The results suggest that Li^+ ions released from the bioactive MBG scaffolds may play an important role in enhancing cementogenic differentiation of PDLCs. To further clarify the biological role of Li^+ ions in the scaffolds, additional studies were conducted to investigate what effect Li^+ ions by themselves could induce the similar biological reaction in PDLCs. The results of these experiments further confirmed the stimulatory effect of Li^+ ions on cell proliferation and cementogenic differentiation of PDLCs. The incorporation of Li^+ into bioactive MBG scaffolds therefore appear to be a viable means of enhancing the biological response of hPDLCs towards cementogenesis in periodontal tissues engineering applications.

The effect on the Wnt signalling pathway of Li^+ incorporation into MBG scaffolds was investigated in an effort to find possible molecular mechanisms for lithium's ability to stimulate cementogenic differentiation of hPDLCs. Axin2, also known as Conductin or Axil, is recognised as the most accurate reporter gene in the canonical

Wnt pathway since it is a direct target gene of Wnt ligand binding and activation of the Wnt signalling pathway [83, 219]. β-catenin is also a critical member of Wntβ-catenin signalling pathway, although its expression is not directly linked to the activation of the Wnt pathway *per se*, but rather from post translation modifications that inhibits its cytoplasmic degradation as the result of the upstream activation of the pathway [71-72]. In this study, 5Li-MBG scaffolds had the effect of significantly upregulation of WNT-related gene expression (*AXIN2* and *β-catenin*) of PDLCs compared to pure MBG and 2Li-MBG scaffolds. The results strongly suggest that Li-MBG scaffolds upregulated the canonical Wnt signalling of hPDLCs compared to the controls. It seems fair to speculate that the mechanism behind the enhanced cementogenic differentiation of hPDLCs in the presence of Li-MBG scaffolds may be directly linked to the stimulation of Wnt signalling pathway. This study does indicate Li-MBG scaffolds have the possibility of stimulating PDLCs differentiation into cementoblast-like cells. However, this concept needs to be tested *in vivo* to further demonstrate that an intact functional cementum can be initiated by the application Li^+ ions released from MBG scaffolds. Future studies will focus on the modification of the Li-MBG scaffolds for the functional cenmentum regeneration.

5.5 CONCLUSIONS

Li-MBG scaffolds with hierarchically large pores (300-500 mm) and well-ordered mesopores (5 nm) were prepared by substituting part of the Ca^{2+} ions with Li^+ ions in manufacture of the scaffolds. Li^+ ions released from the Li-MBG scaffolds significantly enhanced the proliferation and cementogenic differentiation of hPDLCs via the activation of Wnt signalling pathway. This study indicates that Li-MBG scaffolds have potential for the application of periodontal tissue engineering.

5.6 ACKNOWLEDGEMENTS

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Chapter 6: Design of lithium-containing TCP for cementogenic differentiation

Lithium release from β-tricalcium phosphate inducing cementogenic and osteogenic differentiation for both hPDLCs and hBMSCs

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(Manuscript published in Journal of **Biomaterial Science**)

Statement of Contribution of Co-Authors for Thesis by Published Paper

Contributor	Statement of contribution*
Pingping Han Signature Date	Involved in experimental design, performing the laboratory experiments and data analysis. Wrote the manuscript.
Mengchi Xu	Involved in the design of materials and reviewing the manuscript.
Jiang Chang	Involved in the design of the project, data analysis and reviewing the manuscript.
Nishant Chakravorty	Involved in data analysis and reviewing the manuscript.
Chengtie Wu	Involved in the design of the project, data analysis and reviewing the manuscript.
Yin Xiao	Involved in the conception and design of the project. Assisted in sample collection, technical guidance and reviewing the manuscript

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

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Name Signature Date

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Abstract

It is generally accepted that the accelerated differentiation of tissue cells on bioactive materials is of great importance to regenerate the lost tissues. Lithium (Li) ions could enhance the *in vitro* proliferation and differentiation of different progenitor cells by activating the canonical Wnt signalling pathway. However, it is unclear whether Li-containing bioactive ceramics, such as β-tricalcium phosphate (Li-β-TCP), can effectively induce the differentiation for different stem cells and present the significant osteo/cementostimulation for the regeneration of bone/periodontal tissues. Therefore, the aim of this study was to investigate the interactions of human periodontal ligament cells (hPDLCs) and human bone marrow stromal cells (hBMSCs) with Li-β-TCP bioceramic bulks and their ionic extracts, and further explore the osteogenic and cementogenic stimulation of Li-β-TCP bioceramics and possible molecular mechanisms. The results showed that Li-β-TCP bioceramic disks supported the cell attachment and proliferation, and in general significantly enhanced bone/cementum-related gene expression, canonical Wnt signalling pathway activation in both hPDLCs and hBMSCs, compared to conventional β-TCP bioceramic disks and blank controls. The Li-containing ionic products from 5Li-β-TCP powders could significantly promote the proliferation and bone/cementum-related gene expression for both hPDLCs and hBMSCs compared to pure β-TCP extracts. Our results suggest that Li-β-TCP bioceramics might be another promising biomaterial for bone/cementum regeneration, as they possess excellent *in vitro* osteogenic and cementogenic stimulation property by inducing bone/cementum-related gene/protein expression of both hPDLCs and hBMSCs.

Key words: *Bioceramics; tissue engineering; bone regeneration; β-tricalcium phosphate*

6.1 INTRODUCTION

For bone/periodontal tissues regeneration application, it is generally accepted that the quick differentiation of tissue cells on bioactive materials is of great importance to regenerate the deficient and injured tissues [16, 220-221]. To achieve this target, the strategy for applying growth factor to accelerate the differentiation of tissue cells has been widely studied [222]; however, the usage of growth factors is highly costly and may have potential health risks [223-224]. Hench *et al*. suggested that the third-generation of biomaterials should possess the ability to stimulate the differentiation of stem cells for tissue regeneration [225-226]. More and more studies have shown that bioactive materials with specific bioactive compositions and microstructures can induce the differentiation of stem cells and promote tissue regeneration [191, 207, 215, 227]. For this reason, it is of great importance to prepare proper bioceramics to release inorganic stimulus to stimulate the response of stem cells for bone and periodontal tissue regeneration without the usage of additional growth factors. It is well known that beta-tricalcium phosphate (β-TCP) is the most conventional used bioceramics for the regeneration of bone/periodontal tissues due to its high osteoconductivity and degradable ability [228-229]; however, there are few reports that β-TCP bioceramics can significantly induce the differentiation of stem cells and present the significant osteostimulation for the regeneration of bone/periodontal tissues. Although β-TCP bioceramics are generally biodegradable and biocompatible, they are still far from optimal for stimulating the osteogenic/periodontal differentiation of stem cells to achieve further shorten healing time of tissue defects [230-233]. In the past several years, bioactive ions (e.g. Sr and Si) modified β-TCP bioceramics with improved bioactivity have been prepared for bone regeneration application [234-235], which inspired us that bioactive ions modification to β-TCP bioceramics may be a viable method to stimulate differentiation of mesenchymal stem cells for better tissue regeneration.

Lithium chloride (LiCl) has been used as a mood stabiliser, which can stimulate the proliferation of retinoblastoma cells and endometrium epithelia cells via elevated canonical Wnt signalling [236-239]. Another study demonstrated that LiCl greatly enhances induced pluripotent stem cells (iPSCs) generation from both mouse embryonic fibroblast and human umbilical vein endothelial cells [240]. Previous studies showed that Li ions could be incorporated to bioactive glasses to regulate the

apatite mineralization and enhance the cementogenic differentiation of human periodontal ligament cells (hPDLCs) [216, 241]. However, there is no report about the preparation of Li-containing bioceramic, such as β-TCP and whether Li-containing bioceramics can induce the differentiation of different tissue cells, such as human bone marrow stromal cells (hBMSCs) and human periodontal ligament cells (hPDLCs) for both bone and periodontal regeneration applications. Furthermore, it is still unclear for the related molecular mechanisms in which whether canonical Want signalling pathway in the hPDLCs and hBMSC differentiation can be activated by Li-containing biomaterials. The effect of Li ions released from biomaterials on the bone/cementum-related protein expression of hPDLCs and hBMSCs is also unknown. It is believed that multipotent hBMSCs are involved in bone and periodontal defect healing [242]. As an attractive cell source for tissue engineering, BMSCs are easily available, highly proliferative and multipotent to differentiate into osteoblasts, chondrocytes, adipose cells, ligament cells, and neural cells [243-247]. hPDLCs are considered to be able to regenerate alveolar bone, periodontal ligament and cementum. Activation of osteogenic potential of both hPDLCs and hBMSCs by bioactive materials would greatly accelerate the new bone and cementum formation in the bone/periondontal defect site [248].

Therefore, the main aim of this study is to prepare Li-containing β-TCP bioceramics in an effort to improve their osteo/cementogenic capabilities compared to conventionally used β-TCP bioceramics, and to further elucidate the involved mechanism about the activation of the pro-osteo/cementogenic canonical Wnt signalling pathway in hPDLCs and hBMSCs by incorporation of Li ions. To achieve this aim, the effect of both Li-β-TCP bioceramic disks and their ionic products on the differentiation of hPDLCs and hBMSCs has been systematically explored in comparison to conventional β-TCP bioceramics.

6.2 MATERIALS AND METHODS

6.2.1 Preparation and characterization of 5Li-β-TCP bioceramic powders and disks

The Li-containing β-TCP (5Li-β-TCP) powders with the chemical composition of $(Li_{0.10}Ca_{0.95})_3(PO_4)_2$ were synthesized by a chemistry precipitation method using

$Ca(NO_3)_2 \cdot 4H_2O$, $(NH_4)_2HPO_4$ and lithium chloride (LiCl) as starting materials, in which 5% molar Ca was substituted by Li. The rational of to substitute 5% molar Ca was according to previous publication [190]. The solution containing 0.5 M of $Ca(NO_3)_2$ and LiCl in the designed molar ratio was added dropwise into 0.5 M of $(NH_4)_2HPO_4$ solution while stirring to produce the target (Ca+Li)/P ratio of 1.5 (stoichiometric for β-TCP). The pH of the solution was maintained between 7.5 and 8 during precipitation by the aid of ammonia. After the precipitation, the solution was aged overnight and then washed sequentially in distilled water and anhydrous ethanol, dried at 60 ^oC for 24 h and finally calcined at 800 ^oC for 3 h. Pure β-TCP powders were synthesized by similar method without adding LiCl and used for the controls.

To prepare ceramic discs, the synthesized 5Li-β-TCP powders were uniaxially pressed at 10 MPa in a mould of Φ10, using 6% polyvinyl alcohol as a binder. The green compacts were subsequently sintered in air at 1100 °C for 3 h with a heating rate of 2°C/min. Pure β-TCP ceramic discs were prepared by similar method and used for the controls. The crystal phase and surface morphology of the sintered ceramic discs were characterized by X-ray diffraction (XRD, Geigerflex, Rigaku, Japan) and scanning electron microscopy (SEM, FEI Company, USA).

6.2.2 The cell morphology of hPDLCS and hBMSCs on 5Li-β-TCP bioceramic disks

Human periodontal ligament cells (hPDLCs) and human bone marrow stromal cells (hBMSCs) were isolated and cultured according to previously published protocols [190, 227]. The ethics approval of hPDLCs and hBMSCs in this study was granted by the Human Ethics Committee of Queensland University of Technology and The Prince Charles Hospital, following informed consent taken from all the participants. While, hPDLCs were isolated from the teeth from healthy patients (n=3, 18–25 years old) undergoing third molar extraction surgery; hBMSCs were isolated from patients (n=3, mean age at 65 years) undergoing elective knee and hip replacement surgery. hPDLCs and hBMSCs at the 2-5th passage were seeded on 5Li-β-TCP ceramic disks at the initial seeding density of 10^5 cells per disk in 48-well plates and cultured under osteogenic induction medium with the supplement of 0.1μM dexamethasone (Sigma-Aldrich, Australia), 10mM β-glycerophosphate (Sigma-Aldrich, Australia) and

50μg/mL ascorbate-2-phosphate (Sigma-Aldrich, Australia). The cells cultured in β-TCP ceramics and blank wells were as control in this study.

The cell morphology on the disks was observed by SEM. The cellular samples were fixed in 2.5% glutaraldehyde solution and then dehydrated by graded ethanol series (50, 70, 90, and 100%) and dried by hexamethyldisilizane (HMDS). Then, the disks were sputter-coated by gold and observed by SEM (Quanta™ 200) for the morphological characteristics of hPDLCs and hBMSCs on two ceramics.

The confocal laser scanning microscopy (CLSM) was carried out to further determine the morphology of actin fibres and nuclei of hPDLCs and hBMSCs on the two ceremics disks through high-resolution optical images. After 1 and 7 days culture, cell-ceramics complex were fixed with 4% paraformaldehyde (PFA) solution for 30 mins at room temperature and then was permeabilized with 0.2% Triton X-100/PBS solution for 5 mins and then rinsed by PBS once. Samples were then incubated for 1 hr with 0.5% BSA/PBS containing 0.8 U/ml TRITC conjugated phalloidin (Sigma-Aldrich, Australia) and 5 μg/ml DAPI (Sigma-Aldrich, Australia). The cells were visualized with Leica SP5 Confocal microscope (Leica Microsystems, Germany).

6.2.3 Cell proliferation and relative alkaline phosphatase (ALP) activity assay for hPDLCs and hBMSCs on 5Li-β-TCP bioceramic disks

For the cell proliferation assay, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed in triplicate as described in the previous study protocol [190, 249]. The formazan crystals were formed by adding 0.5 mg/mL of MTT solution (Sigma-Aldrich) for 4 hours and then solubilised with dimethyl sulfoxide (DMSO, Sigma-Aldrich, Australia). The absorbance of formazan-DMSO solution was measured at λ=495 nm by SpectraMax Microplate Reader (Molecular Devices, Inc., USA).

After 7 and 14 days, the relative ALP activity was determined for both cell lines cultured on 5Li-β-TCP ceramic disks using previous protocols [191]. At each time point, the cells were lysed in 200 μL of 0.2% Triton X-100 and then mixed with ALP working solution according to the manufacturer's protocol (QuantiChrom^{TM} Alkaline Phosphatase Assay Kit, BioAssay Systems, USA). The optical density (OD) value

was measured at 405 nm on a plate reader. The relative ALP activity was calculated as the changed OD values divided by the reaction time and the total protein content measured by the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Australia).

6.2.4 Real-time quantitative (RT-qPCR) analysis of cementogenic/osteogenic gene expression and signalling pathway for hPDLCs and hBMSCs

Total RNA was extracted from the cells on 5Li-β-TCP, β-TCP ceramic disks and blank wells using TRIzol® Reagent (Ambion®, Life Technologies Pty Ltd., Australia). RT-qPCR was performed on osteogenic-related markers of *alkaline phosphatase* (*ALP*), *osteopontin* (*OPN*) and *osteocalcin* (*OCN*); cementum-related markers of *cementum protein 1* (*CEMP1*) and *cementum attachment protein* (*CAP*); canonical Wnt-related genes of *Wingless-3A* (*WNT3A*), *axis inhibition protein 2* (*AXIN2*), *beta-catenin* (*CTNNB*) as shown in Table 6-1. All reactions were conducted in triplicate for three independent experiments. The mean cycle threshold (Ct) value of target genes was normalized against Ct value of house-keeping gene *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) and *18S ribosomal RNA* (*18S rRNA*) and then the relative expression was calculated by the following formula: $2^{-(\text{normalized average Cts})} \times 10^4$.

Table 6-1. Primer pairs used in qRT-PCR analysis

Gene	Forward primer	Reverse primer
ALP	5' TCAGAAGCTAACACCAACG 3'	5' TTGTACGTCTTGGAGAGGGC 3'
OCN	5'GCAAAGGTGCAGCCTTTGTG 3'	5' GGCTCCCAGCCATTGATACAG 3'
CEMP1	5' GGGCACATCAAGCACTGACAG 3'	5' CCCTTAGGAAGTGGCTGTCCAG3'
CAP	5' CTGCGCGCTGCACATGG 3'	5' GCGATGTCGTAGAAGGTGAGCC 3'
WNT3A	5' TGGACAAAGCTACCAGGGAGT 3'	5' CCCACCAAACTCGATGTCCTC 3'
AXIN2	5' CCCCAAAGCAGCGGTGC 3'	5' GCGTGGACACCTGCCAG 3'
CTNNB	5' GCTACTGTTGGATTGATTCGAAATC 3'	5'CCCTGCTCACGCAAAGGT 3'
GAPDH	5' TCAGCAATGCCTCCTGCAC 3'	5' TCTGGGTGGCAGTGATGGC 3'
18s	5’ TTCGGAACTGAGGCCATGAT 3'	5’ CGAACCTCCGACTTCGTTC 3'

6.2.5 Ionic concentration analysis for the cell culture medium after culture 5Li-β-TCP bioceramic disks

To investigate the ion release of 5Li-β-TCP ceramics disks, the cell culture medium (n=3) was collected after cell culturing of hPDLCs and hBMSCs after the fixed culture time; then the ionic concentrations of Li, Ca and P ions in the medium were

measured by inductive coupled plasma atomic emission spectrometry (ICP-AES, Perkin-Elmer Optima 7000DV).

6.2.6 The effects of ionic extracts from 5Li-β-TCP powders on cementum/bone-related protein expression for hPDLCs and hBMSCs

The effect of ionic extracts from 5Li-β-TCP powders was further investigated on osteogenic/cementogenic-related proteins expression of hPDLCs and hBMSCs. According to International Standard Organization (ISO/EN) 10993-5 [250], the dissolution extracts were prepared by soaking 5Li-β-TCP powders in serum-free DMEM at the concentration of 200 mg/mL. Then serial dilutions of extracts (100, 50, 25, 12.5 and 6.25 mg/mL) were prepared after sterilization using a 0.2 μm filter in the following studies. The ionic concentrations of Ca, P and Li in the graded extracts were measured by ICP-AES. All experiments were conducted in triplicate.

After hPDLCs and hBMSCs were cultured with the β-TCP and 5Li-β-TCP extracts, respectively, cell proliferation and ALP activity assay was performed as described in Section 6.2.3. The RT-qPCR analysis for the cementogenic/osteogenic gene expression was performed as described in the Section of 6.2.4. All experiments were conducted in triplicate.

6.2.7 Statistical analysis

The statistical analysis was performed using one-way ANOVA followed by a Student's T-test. A p-value < 0.05 was considered statistically significant.

6.3 RESULTS

6.3.1 Characterization of 5Li-β-TCP bioceramic disks

SEM analysis showed that the prepared both 5Li-β-TCP and β-TCP bioceramics were fully sintered with dense surface microstructure and clear crystal boundaries (Fig. 6-1). The crystal size in 5Li-β-TCP bioceramics is generally smaller than that of β-TCP bioceramics. XRD analysis showed that 5Li-β-TCP ceramics had same characteristic peaks as β-TCP ceramics with pure β-TCP crystal phase (JCPD 09-0169) (Fig. 6-1).

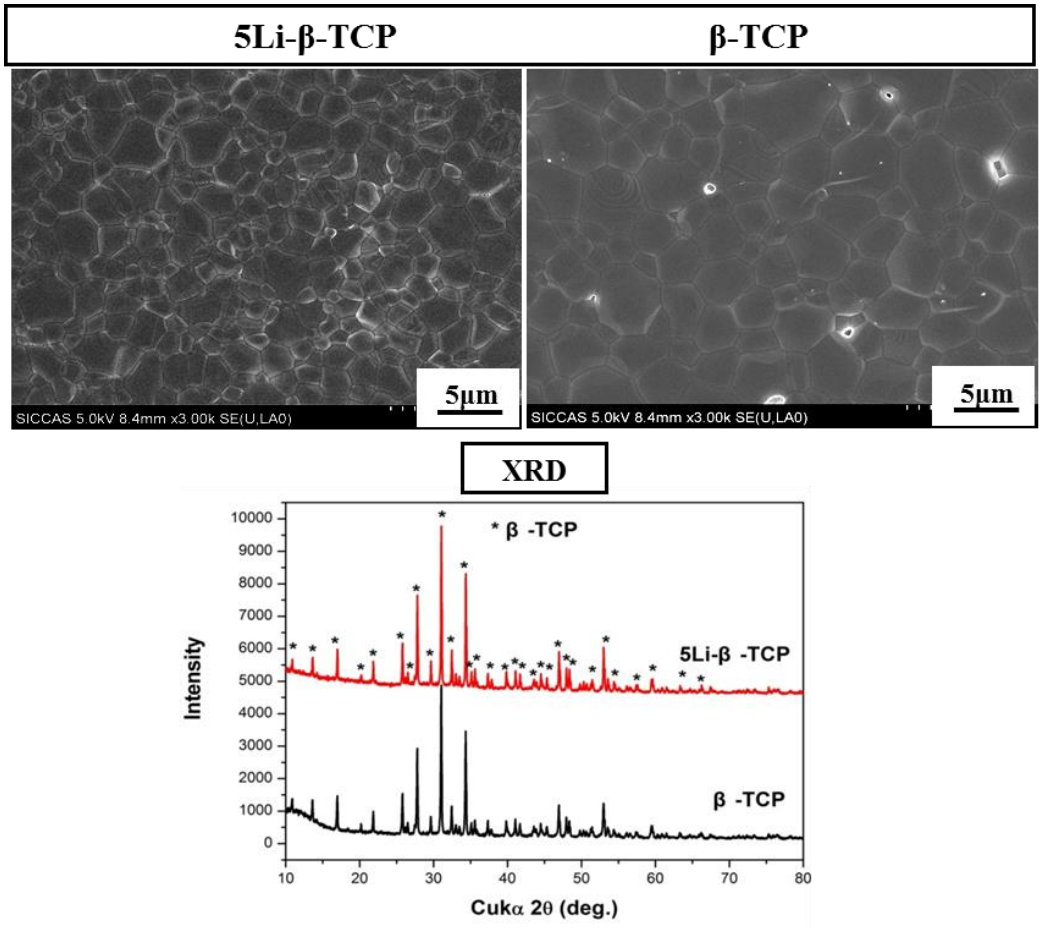

Figure 6-1. SEM and XRD analysis analysis for 5Li-β-TCP and β-TCP bioceramics. The crystal size in 5Li-β-TCP bioceramics is generally smaller than that of β-TCP bioceramics through SEM analysis.

6.3.2 The cell morphology, proliferation and ALP activity of hPDLCS and hBMSCs on 5Li-β-TCP bioceramic disks

The cell morphology of hPDLCS and hBMSCs was performed by SEM after 7 days culturing on 5Li-β-TCP and β-TCP bioceramics. Both hPDLCs and hBMSCs attached and spread well on the surface of two ceramic disks with close contact with ceramics at day 7 (Fig. 6-2).

After culture on two bioceramics disks for 7 days, CLSM was carried out to further confirm the morphology for both hPDLCs and hBMSCs were healthy and attached on the surface of disks very well (Fig. 6-3).

The MTT assay showed that the proliferation of both hPDLCs and hBMSCs increased obviously in a time dependent manner in each group. The proliferation rate

of hPDLCs on 5Li-β-TCP bioceramics disks was significantly higher than that on β-TCP ceramics at both day 1 and 7 (Fig. 6-4). 5Li-β-TCP than that on β-TCP ceramics groups at day 1, 3 and 7 (Fig. 6-4).

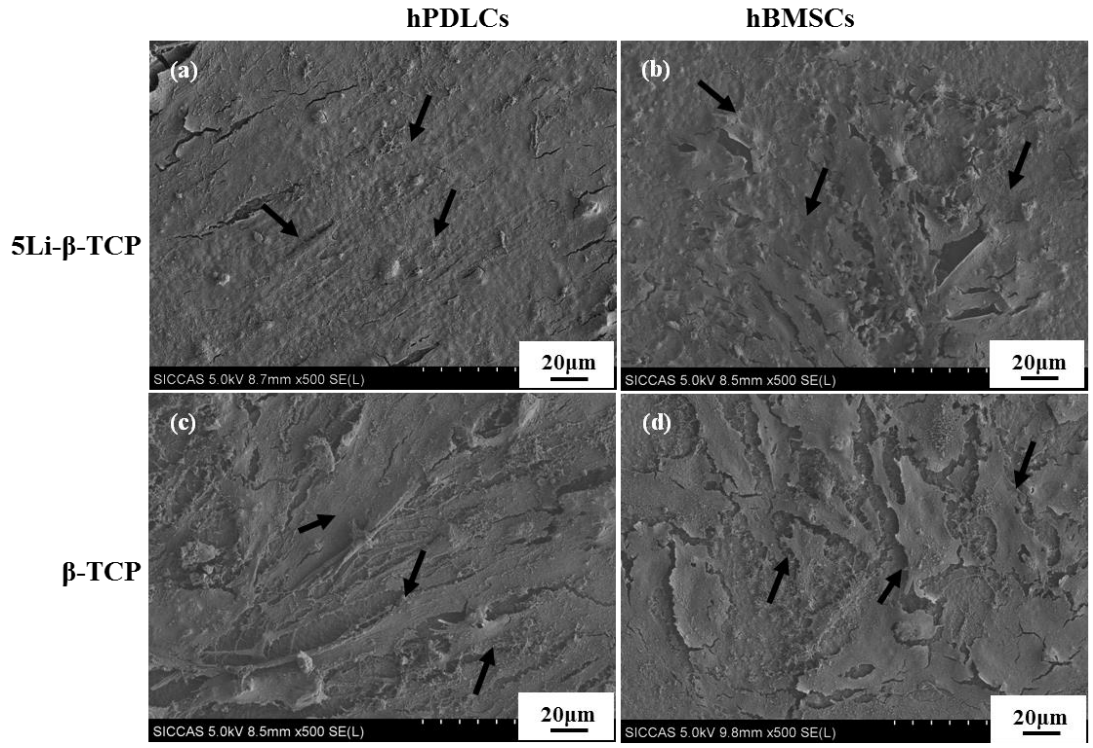

Figure 6-2. The cell morphology for (a) hPDLCs on 5Li-β-TCP, (b) hBMSCs on 5Li-β-TCP, (c) hPDLCs on β-TCP and (d) hBMSCs on β-TCP bioceramics after culturing for 7 days.

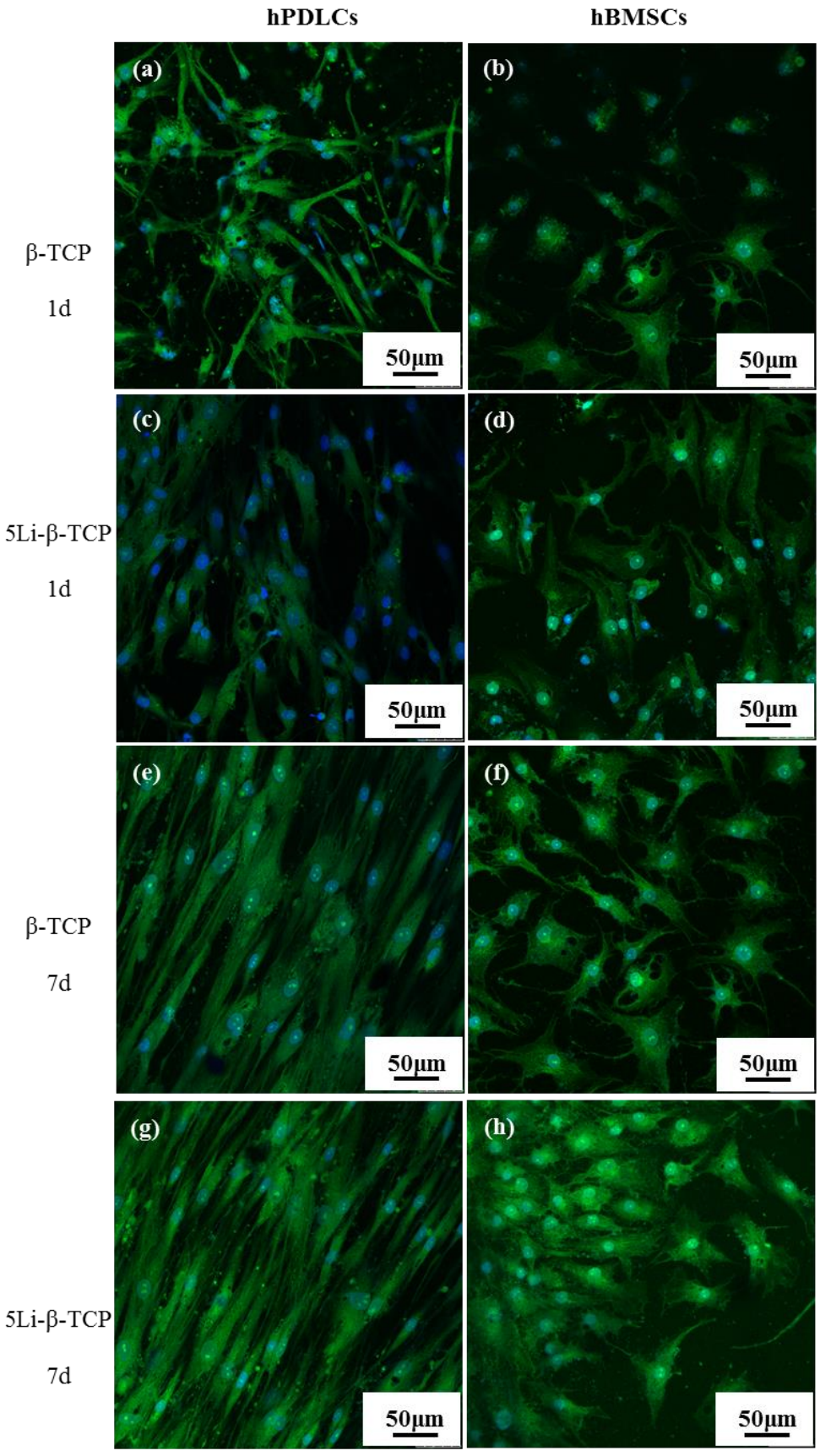

Figure 6-3. The confocal fluorescent images for hPDLCs and hBMSCs on β-TCP and 5Li-β-TCP bioceramics after culture for 7 days.

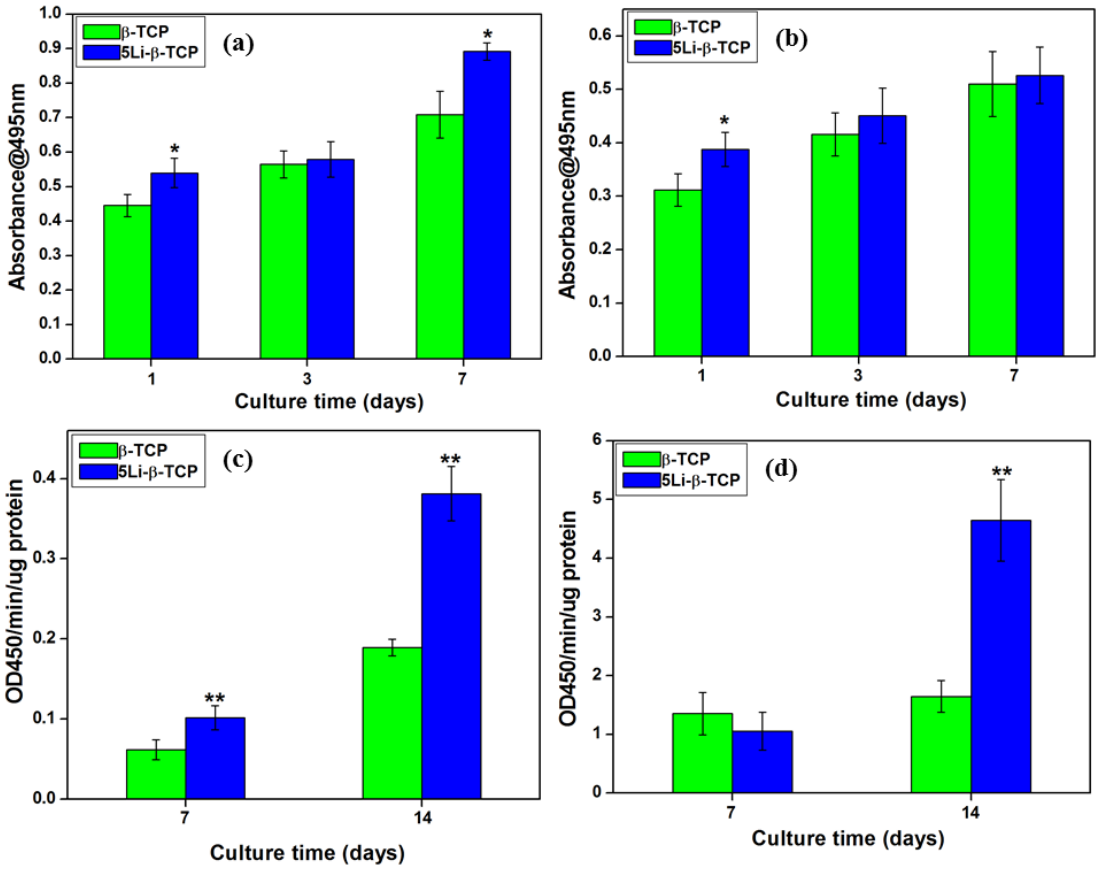

Figure 6-4. The cell proliferation and relative ALP activity for hPDLCs (a, c) and hBMSCs (b, d) on β-TCP and 5Li-β-TCP bioceramics. *: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group with respect to cell proliferation rate. **: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group regarding relative ALP activity.

The relative ALP activity of hPDLCs on the 5Li-β-TCP ceramics showed a significant increase compared to β-TCP group at both 7 and 14 days ($p<0.05$, Fig. 6-4). 5Li-β-TCP ceramics enhanced the relative activity of ALP in hBMSCs compared to other two groups at day 14.

6.3.3 Cementogenic/osteogenic gene expression of hPDLCs and hBMSCs on 5Li-β-TCP bioceramic disks

At day 7, RT-qPCR analysis showed that the 5Li-β-TCP ceramic disks significantly stimulated bone-related genes expression of *ALP*, *OCN* and cementum-specific genes of *CEMP1* and *CAP* compared to those of other groups for both hPDLCs and hBMSCs. 5Li-β-TCP ceramics could significantly enhance the osteogenic/

cementogenic gene expression for both hPDLCs and hBMSCs compared to β-TCP ceramics (Fig. 6-5).

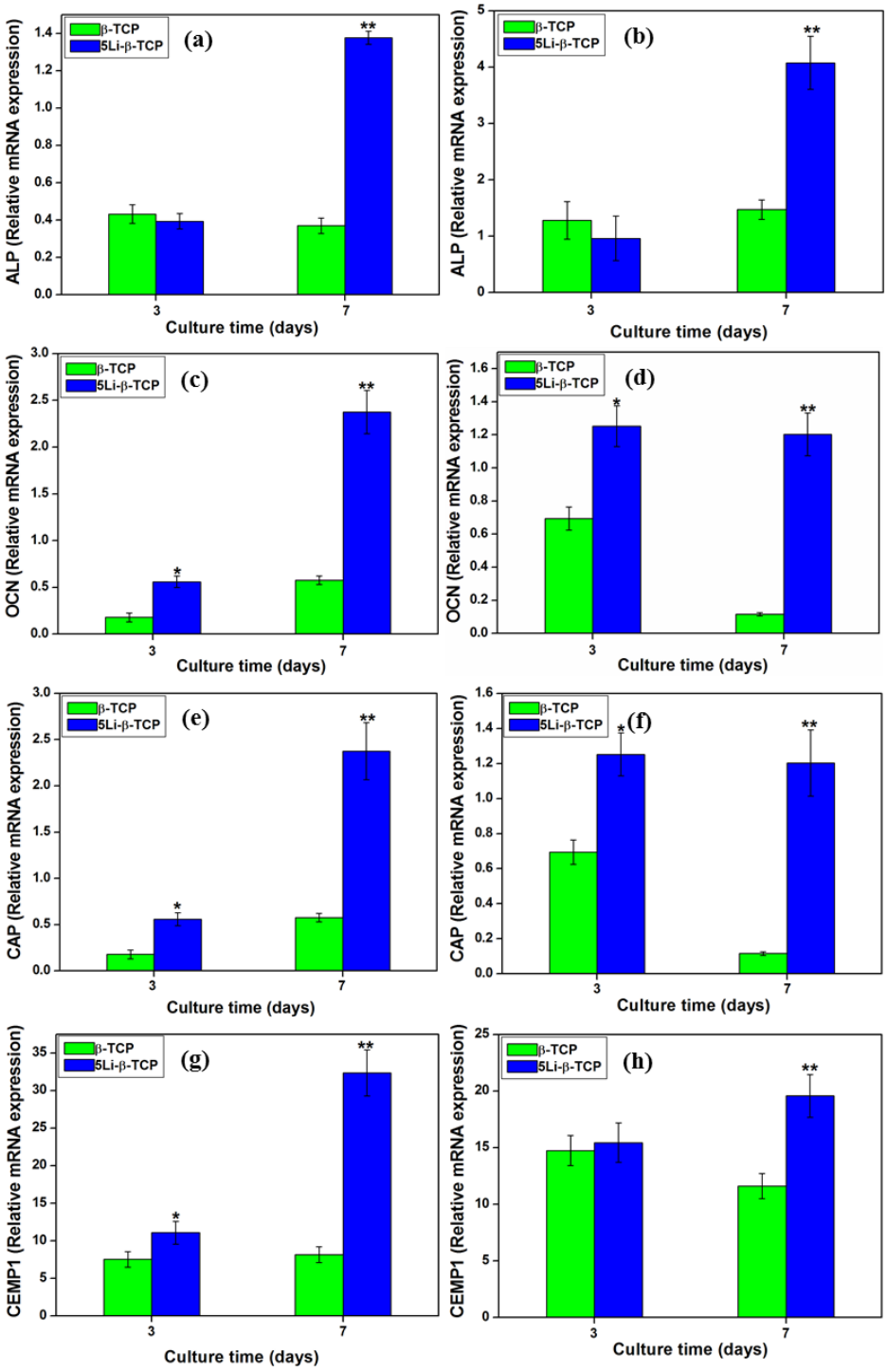

Figure 6-5. The bone-related gene expression of ALP, OCN and cementum-specific markers of CAP and CEMP1for hPDLCs (a, c, e, g) and hBMSCs (b, d, f, h) on β-TCP and 5Li-β-TCP bioceramics. *: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group at day 3. **: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group at day 7.

6.3.4 Wnt-related signalling gene expression for hPDLCs and hBMSCs on 5Li-β-TCP bioceramic disks

The analysis of the expression of canonical Wnt-related genes such as *WNT3A*, *AXIN2* and *CTNNB* was performed by RT-PCR after culturing both hPDLCs and hBMSCs on two ceramics disks and blank group for 3 and 7 days. The results showed that the expression of *WNT3A* and *AXIN2* of both hPDLCs and hBMSCs on 5Li-β-TCP bioceramics was significantly higher than β-TCP ceramics group at day 7 (Fig. 6-6).

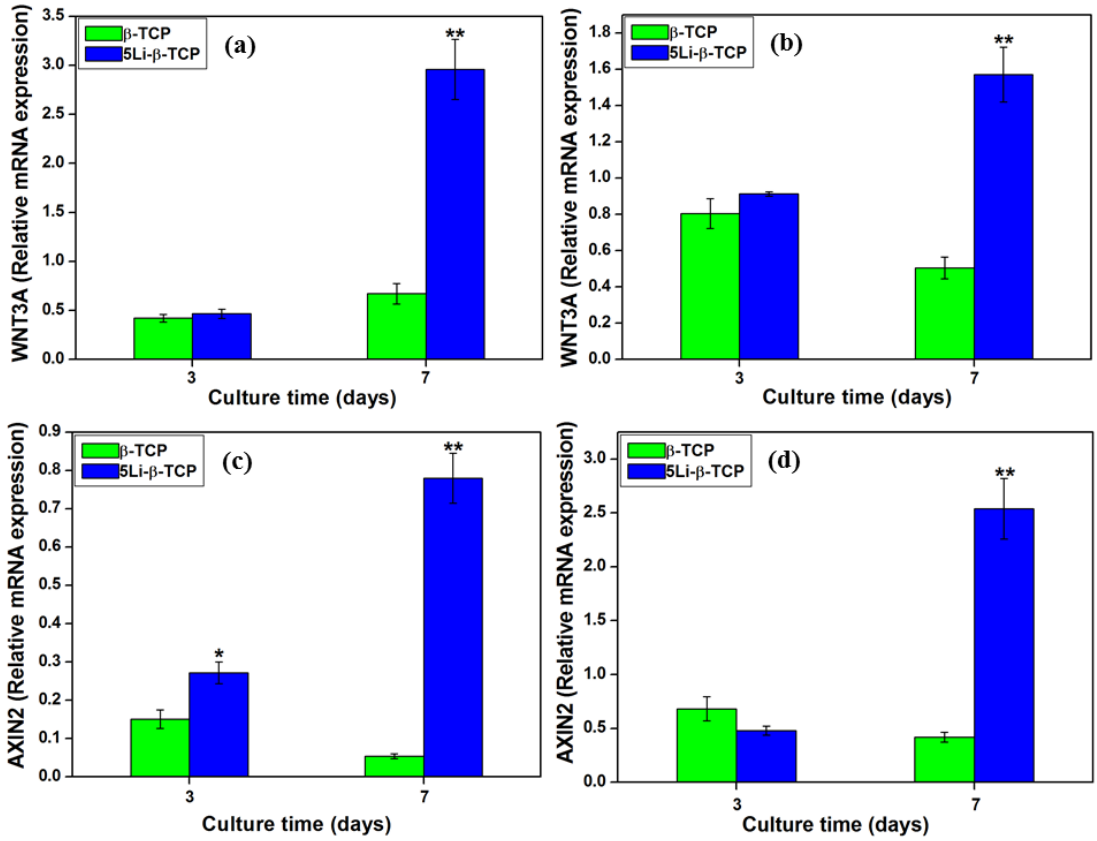

Figure 6-6. The canonical Wnt-related gene expression of WNT3A and AXIN2 for hPDLCs (a, c) and hBMSCs (b, d) on β-TCP and 5Li-β-TCP bioceramics. *: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group at day 3. **: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group at day 7..

6.3.5 Ionic concentration analysis for the cell culture medium after culture 5Li-β-TCP bioceramic disks

It was noted that there is no Li ion release in DMEM and β-TCP medium. However, there were obvious Li ions in the medium released from 5Li-β-TCP bioceramic disks

culture for hPDLCs (Table 6-2) and hBMSCs (Table 6-3) at day 3, 7 and 14. Interestingly, the released Li ions from hBMSCs cultured on 5Li-β-TCP disks were more than that from hPDLCs cultured on 5Li-β-TCP disks. There was no significant difference for P ions between 5Li-β-TCP and β-TCP groups cultured with two cells; however, the concentrations of Ca ion for 5Li-β-TCP was a slightly lower than those for β-TCP groups cultured with two cells (Table 6-2 and 6-3).

Table 6-2 Ions release of 5Li-TCP ceramic disks in DMEM cultured with hPDLCs

Culture time	Materials	Li (mg/L)	P(mg/L)	Ca(mg/L)
3d	DMEM	0	323.9±5.148	78.03±0.505
	β-TCP	0	304.1±1.374	65.37±0.243
	5Li-β-TCP	0.34±0.013	301.2±1.172	62.58±0.183
7d	DMEM	0	295.4±0.751	75.38±0.259
	β-TCP	0	280.4±2.051	65.12±0.41
	5Li-β-TCP	0.44±0.019	272±1.223	63.14±0.295
14d	DMEM	0	221.4±2.479	76.99±0.551
	β-TCP	0	203.2±1.305	67.87±0.805
	5Li-β-TCP	0.34±0.026	205.5±1.375	59.02±0.125

Table 6-3. Ions release of 5Li-TCP ceramic disks in DMEM cultured with hBMSCs

Culture time	Materials	Li(mg/L)	P(mg/L)	Ca(mg/L)
3d	DMEM	0	317±3.2	74.94±0.832
	β-TCP	0	285.6±1.986	62.55±0.036
	5Li-β-TCP	1.23±0.014	281.7±5.773	54.89±0.249
7d	DMEM	0	290.2±1.589	67.26±0.815
	β-TCP	0	269.5±0.702	62.59±0.206
	5Li-β-TCP	0.89±0.047	269.3±0.611	57.8±0.449
14d	DMEM	0	206.1±0.989	59.85±0.651
	β-TCP	0	203.4±1.617	65.63±0.305
	5Li-β-TCP	0.79±0.046	205.4±1.127	59.38±1.463

6.3.6 The effects of ionic extracts from 5Li-β-TCP powders on bone/cementum-related protein expression of hPDLCs and hBMSCs

The ionic concentrations of Li, Ca and P in the extracts from β-TCP and 5Li-β-TCP powders were shown in Table 5. At the concentration of 200 mg/mL, it was obvious that the Li concentrations reached 7.42 mg/L; however, there were no Li ions in β-TCP extracts (Table 6-4). The concentrations of Ca and P ions in β-TCP and 5Li-β-TCP groups were comparable.

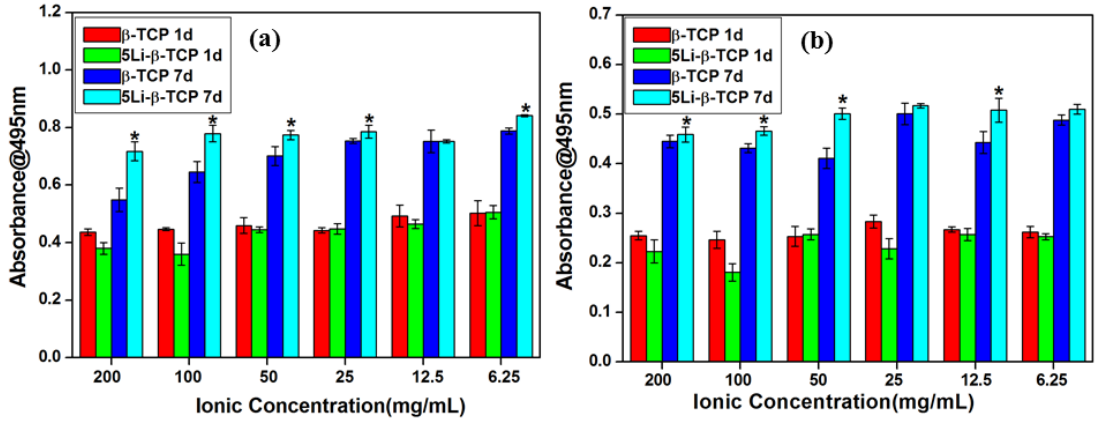

Figure 6-7. The proliferation for hPDLCs (a) and hBMSCs (b) after culture different concentrations of β-TCP and 5Li-β-TCP powder extracts for 7 and 14 days. *: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group at day 7.

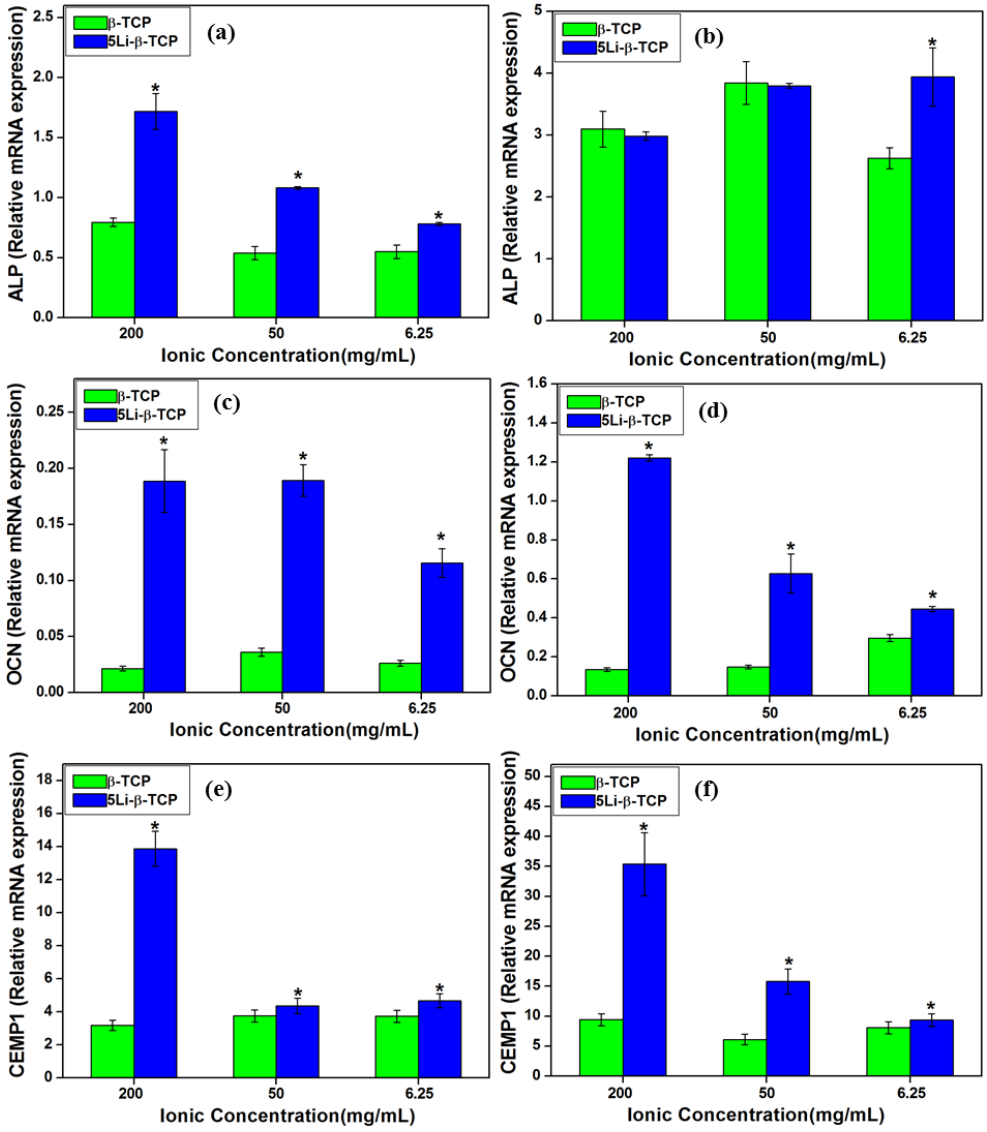

Figure 6-8. The relative bone-related gene expression of ALP, OCN and cementum-specific markers of CEMP1 for hPDLCs (a, c, e) and hBMSCs (b, d, f) after culturing with different

concentrations of β-TCP and 5Li-β-TCP extracts for 7 days. *: significant difference ($p<0.05$) for 5Li-β-TCP group compared to β-TCP group.

Table 6-4. The ionic concentrations of the extracts from β-TCP and 5Li-β-TCP powders

Extract concentrations (mg/mL)	Materials	Li(mg/L)	P(mg/L)	Ca(mg/L)
200mg/mL	β-TCP	0	205.1±3.156	55.53±1.056
	5Li-β-TCP	7.42±0.046	202.2±1.011	54.45±1.752
50mg/mL	β-TCP	0	291.3±3.125	71.3±2.024
	5Li-β-TCP	1.86±0.023	290.5±4.235	71.0±1.652
6.25mg/mL	β-TCP	0	316.3±5.321	75.9±1.365
	5Li-β-TCP	0.23±0.009	316.4±4.216	75.8±0.956

As determined by MTT assay, the cell proliferation rate of both hBMSCs and hPDLCs was significantly higher cultured in presence of 5Li-β-TCP extracts with a wide range of concentrations (200, 100, 50 and 6.25mg/mL) than that cultured with β-TCP group at day 7 (Fig. 6-7a, b).

Results of bone/cementum-related gene expression analysis of ALP, OCN and CEMP1 showed that the 5Li-β-TCP extracts at all the range concentrations enhanced hPDLCs osteogenic and cementogenic differentiation compared to β-TCP extracts group at day 7 (Fig. 6-8a, c, e). At day 7, the expression of ALP for hBMSCs was reduced when cultured with 5Li-β-TCP at the concentration of 200 and 50mg/mL compared to those grown in β-TCP extracts (Fig. 6-8b). The expression of OCN and CEMP1 for both hPDLCs and hBMSCs was increased by 5Li-β-TCP extracts at all the range concentrations compared with that in β-TCP extracts group (Fig. 6-8c-f).

6.4 DISCUSSION

The present study conducted a systematic investigation of the cell attachment, proliferation, bone/cementum and canonical Wnt related gene expression for both hPDLCs and hBMSCs cultured on 5Li-β-TCP ceramic disks or with 5Li-β-TCP powder extracts. To our best knowledge, this was the first study in preparation of Li-containing β-TCP bioceramics. Li ions have been successfully incorporated into β-TCP by a conventional chemical precipitation method. The results showed that the incorporation of 5% Li into β-TCP bioceramics did not change the crystal phase structures. ICP analysis for both Li-β-TCP ceramics disks and powder extracts further confirmed that Li ions had been incorporated into β-TCP.

It is well known that hBMSCs can be potentially used for regenerating and repairing bone defects that normally cannot undergo spontaneous healing [251-252]. hPDLCs, another population of mesenchymal cells, have shown a promising potential for periodontal regeneration as they are easily isolated from the patients' tooth extractions [253]. Many studies suggested that conventional β-TCP bioceramics have been proven to be osteoconductive for bone/periodontal regeneration application [143, 254-258]; Li ions could enhance the *in vitro* cell proliferation and osteogenic differentiation by activating their canonical Wnt signalling pathway. However, it remains unclear whether Li-β-TCP bioactive ceramics can be used for potential bone and cementum regeneration. In this study, the cells cultured on β-TCP bioceramic disks were used for controls. Our results showed that both hPDLCs and hBMSCs are well attached on the surface of 5Li-β-TCP and β-TCP bioceramics through SEM and CLSM analysis.

ALP is known as an early marker for osteoblastic differentiation; OCN is another important gene related to the bone mineralization [259-260]. CEMP1 and CAP are the cementum-specific markers which have been identified to have limited and specific expression in cementum or cementum-like tissues [217-218, 261]. Our results showed that 5Li-β-TCP had enhanced ostegenic and cementogenic differentiation for both cell lines over time, suggesting it is reasonable that gene activity is not always matched with enzyme activity. These data indicate that 5Li-β-TCP ceramic disks possess excellent *in vitro* osteogenic and cementogenic stimulation for both hPDLCs and hBMSCs. However, in our study, hPDLCs showed a slightly enhanced proliferation rate than that of hBMSCs which may be due to the age difference from the donor donated periodontal ligament and bone marrow stromal cells (i.e. 18-25 vs. 65 years).

Strontium (Sr) is an element proposed to induce bone and previous studies have indicated that Sr might have a positive effect on bone formation by inhibiting bone resorption and through enhancing endosteal and trabecular bone formation [262-265]. Silicon/strontium (Si/Sr)-containing β-TCP ceramics have been proved to stimulate bone cell proliferation and osteogenic differentiation by releasing Si/Sr ions products [234, 266]. There are increasing studies about the stimulatory effect of Sr^{2+} ions on the osteogenic differentiation of osteoblast-like cell and regeneration of lost bone [267]. It was reported that Si-containing β-TCP bioceramics had enhanced

osteogenic differentiation through increasing relative ALP activity and bone-related gene expression (Col1a, BSP and OCN *et al.*) for hBMSCs by 0.5-1 fold change compared to β-TCP only [268-269]. Li is another element and it is reported that Li may enhance fracture healing in mice, while clinically it reduces the incidence of fractures with increasing clinical accumulated dose use of Li. In this study, Li^+ ions released from 5Li-β-TCP biomaterials had an increased osteogenic and cementogenic differentiation for both hPDLCs and hBMSCs in a general trend. Notably, Li^+ ions could stimulate canonical Wnt signalling pathway, suggesting 5Li-β-TCP biomaterial could be potentially applied for periodontal regeneration due to its excellent osteogenic/cementogenic differentiation via activation of canonical Wnt signalling pathway.

It is known that the chemical composition and surface property of bioceramics are two important factors to influence cell response. Our results showed that the incorporation of Li into β-TCP ceramic disks did not influence the surface morphology [241, 270-273]. Previous study showed the crystal size of biocramics also significantly influenced the cell attachment and proliferation [274-275]. Therefore, it is reasonable to speculate that the decreased crystal size of Li-containing β-TCP ceramics may be the other important factor to influence the cell differentiation. In this study, 5Li-β-TCP ceramics have smaller particles size and grains which may influence the cell differentiation. While the crystal sizes is still in the range for the cells ingrowth and differentiation. To further investigate whether the composition of 5Li-β-TCP may play the key role to stimulate osteogenic/cementogenic differentiation of hPDLCs and hBMSCs, the ionic extracts of 5Li-β-TCP powders were prepared, which mainly contains Li^+, Ca^{2+} and PO_4^{3-} ionic products dissolved from 5Li-β-TCP (Table 6-5). Similar results were obtained, ionic products from 5Li-β-TCP also significantly promoted bone/cementum-related gene expression of both hPDLCs and hBMSCs. Therefore, it is reasonable to speculate that the Li-containing ionic products play an important role to stimulate osteogenic/cementogenic differentiation for both hPDLCs and hBMSCs. It is known that effect of LiCl has a dose-dependence manner on cell proliferation and differentiation. Recent findings showed that osteogenic differentiation of primary osteoblasts was enhanced by LiCl at the concentration of 1 mM [276]; however, higher concentrations of 10 mM showed inhibited effect for osteoblastic

differentiation [277]. In this study, Li^+ ions concentration was only 0.2 mM released from 5Li-β-TCP bioceramic disks; however, it showed higher Li^+ ions release with the concentration of 1.1mM when 5Li-β-TCP extracts were at the concentration of 200 mg/L. Therefore, the concentration range for Li ions is around 0.2-1.1mM, which is comparable to that reported by Galli *et al.* We further analysed the ionic composition of cell culture medium for β-TCP and 5Li-β-TCP ceramic disks. Interestingly, it is also found that the concentrations of Li^+ ions in the culture medium containing 5Li-β-TCP ceramic disks with hPDLCs are obviously lower than those with hBMSCs as shown in Table 2 and 3. The possible reason is that the different cell types may lead to different surface dissolution in the microenvironment of cell and material surfaces. Furthermore, two types of cells may also possess different up-taking ability for Li^+ ions. The pH value of cell culture medium with hBMSCs was slightly lower than that of hPDLCs. The lower pH value may be the main reason to improve the Li^+ ions release from materials. However, further study is needed to confirm the difference between hPDLCs and hBMSCs on these material surfaces.

The effect of 5Li-β-TCP bioceramics on the Wnt signalling pathway for both hPDLCs and hBMSCs was further investigated to explore possible underlying mechanisms for 5Li-β-TCP bioceramics to stimulate osteogenic/cementogenic differentiation of hPDLCs and hBMSCs. The Wnt signalling pathway plays a major role in various processes during development including cell proliferation and differentiation [278]. WNT3a is a secreted glycoprotein that interacts with cell membrane-associated proteins and plays an important role in intracellular canonical Wnt signal transduction through the phosphorylation and accumulation β-catenin, which is another critical member of Wnt/β-catenin signalling pathway [243, 279]. Axin2, also known as axin-like protein (Axil) or axis inhibition protein 2 (Axin2), is recognised as the most accurate reporter gene in the canonical Wnt pathway since it is a direct target gene of Wnt ligand binding and activation of the Wnt signalling pathway [83]. Up to date, there are few reports involving the interaction of the canonical Wnt signalling and hPDLCs osteogenic differentiation. Our results showed that 5Li-β-TCP bioceramics significantly enhanced the Wnt canonical-related genes expression (WNT3A and AXIN2) of both hPDLCs and hBMSCs compared to β-TCP bioceramics, suggesting that Li^+ ions from 5Li-β-TCP ceramics may be involved in

the activation of canonical Wnt signalling pathway for enhancing the osteogenic/cementogenic differentiation.

It is known that Li^+ ionic concentrations within a certain range concentrations (1mM-10mM) can induce cell proliferation and osteogenic differentiation. In this study, the Li^+ ions concentrations of 5Li-β-TCP extracts were ranging from 0.03 mM to 1.1 mM; while there was only approximately 0.05 mM and 0.1 mM with culturing hPDLCs and hBMSCs on 5Li-β-TCP bioceramics, which is over the optimal range concentrations. This may lead to contradictory results for relative ALP activity and proliferation for 5Li-β-TCP extracts and 5Li-β-TCP bioceramics. However, our results showed a general enhanced trend on 5Li-β-TCP bioceramics for gene expression of key osteogenic and cementogenic differentiation markers compared to that of β-TCP bioceramics, suggesting that 5Li-β-TCP bioceramics might be a promising biomaterial for periodontal tissue engineering.

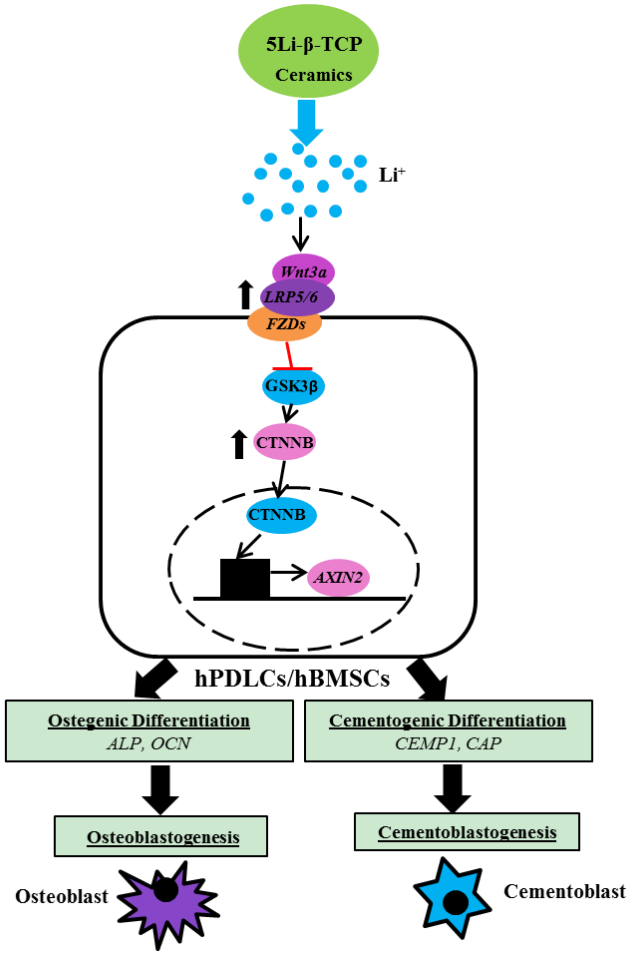

Figure 6-9. Schematic illustration for the effect of 5Li-β-TCP bioactive ceramics on osteogenic and cementogenic differentiation of both hPDLCs and hBMSCs.

Therefore, it is speculated that 5Li-β-TCP bioceramics possess strong osteogenic/cementogenic stimulation property for promoting the bone/cementum-related gene expression. The scheme illustration for the possible mechanism was shown in Figure 6-9, in which canonical Wnt signalling pathways in both hPDLCs and hBMSCs have been distinctively regulated by 5Li-β-TCP bioceramics. However, this concept needs to be further investigated by other Wnt pathway regulators and a future *in vivo* evaluation of bone/cementum forming capacity is also needed to demonstrate that the 5Li-β-TCP bioceramics scaffolds can initiate bone/cementum regeneration.

6.5. CONCLUSION

In summary, Li-containing β-TCP bioceramics were successfully prepared. 5Li-β-TCP bioceramics supported cell attachment and proliferation, and significantly enhanced bone/cementum-related gene expression maybe via the regulation of canonical Wnt signalling pathways in both hPDLCs and hBMSCs. Li-β-TCP bioceramics show potential application in bone/cementum tissue regeneration due to their distinctively osteogenic and cementogenic stimulation property.

6.6 ACKNOWLEDGEMENTS

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Chapter 7: Conclusion and Discussion

7.1 INTRODUCTION

Periodontal regeneration is a complex physiological process requiring spatial and temporal coherence for the coordinated formation of alveolar bone, cementum, and functional periodontal ligament [280]. Periodontal tissue regeneration entails the induction of cementogenesis along the exposed and denuded root surfaces with newly-generated Sharpey's fibres inserted [281-282]. However, despite considerable research efforts in this area, regeneration of the periodontium remains a subject of intense interest to dentists and dental scientists, with the molecular and cellular bases of PDL formation, repair, and regeneration still poorly understood [283]. Therefore, it is of great importance to better understand the molecular mechanism underlying in this condition in order to develop therapies capable of regenerating the periodontium. In order to achieve these periodontal regeneration outcomes, the specific biological factors responsible for periodontal tissue regeneration, must be identified and targeted. The initial rationale for this project was to investigate the role of particular cell signalling pathway during periodontal regeneration, especially regarding cementum regeneration, and then to test their ability to aid periodontal regeneration tissue engineering.

Among cellular signallings, the canonical Wnt signalling pathway has been proven to play essential roles in cell maintenance, proliferation and differentiation during the entire tooth development including embryonic odontogenesis and tooth root formation. There is a direct relationship between tissue induction and morphogenesis in embryonic development and postnatal tissue regeneration; fracture repair may be considered to recapitulate events that occur in the normal course of embryonic development. In this study, it was also investigated whether the canonical Wnt signalling pathway might be responsible for cementum regeneration *in vivo* and cementogenic differentiation *in vitro*, and whether the canonical Wnt signalling can work in combination with biomaterials for cementum/ periodontal regeneration tissue engineering.

At the time that this project was conceived, several reports had been made efforts to

understand the interaction between the Wnt signalling pathway and cementum regeneration *in vivo*; although several findings demonstrated that apparent periodontal regeneration is present at a macroscopic observation scale. This project sought to investigate the activation of canonical Wnt signalling, both in a rat periodontal defect animal model *in vivo* and human periodontal ligament cells culture *in vitro*, with respect to the cementum regeneration and the molecular mechanism underlying this process at a cellular level. The ultimate goal was to apply the canonical Wnt signalling pathway in cementum regeneration tissue engineering combined with biomaterials as a potential treatment carrier and strategy to regenerate periodontal tissues.

7.2 THESIS DESIGN

Firstly, in Chapter 4 of this thesis, activation of the Wnt signalling pathway was highlighted with respect to cementum repair using a rat periodontal defect model. The objective of this study was to evaluate the role of canonical Wnt signalling during primary periodontal healing progress after periodontal defect surgery. Successful rat periodontal defect was established until the root surface exposed as seen by macroscopic and micro-CT observation. The canonical Wnt signalling pathway was locally activated in the periodontal defect area by local injection of the canonical Wnt activators (LiCl and Scl-Ab) or LV-Ctnnb. The findings from this study indicated that local activation of Wnt signalling can induce significant new cementum formation and well-organized periodontal ligament fibres, suggesting a vital role for canonical Wnt signalling during cementum regeneration. Subsequently, another interesting finding from Chapter 4 was that activation of Wnt signalling by LiCl and LV-Ctnnb could promote hPDLCs differentiation towards cementogenic with enhanced expression of cementum-related markers, which hints at a possibility that canonical Wnt signalling can stimulate the PDL fibres to form cementum *in vivo* and *in vitro*. However, this concept still requires further confirmation by different aspects of experiments that will be discussed in details in the following section of "limitation and future direction".

Due to Scl-Ab are growth factors with short half-life and being costly, whereas LiCl is inexpensive and easier to be controlled. Thus, LiCl was performed as main character in next two chapters as LiCl showed similar promising results with induced

cementum regeneration with Sharpey's fibres compared to that from Scl-Ab injection. The general purpose of Chapter 5 was to design possible suitable biomaterials for periodontal/cementum regeneration tissue engineering. Li^+ ions were incorporated into the mesoporous bioactive glass (MBG) scaffolds to activate canonical Wnt signalling in the scaffolds. It showed that Li-MBG scaffolds possess a favorable composition, microstructure and mesopore properties for cell attachment, proliferation, and cementogenic differentiation of human periodontal ligament-derived cells (hPDLCs). The findings in this part indicate that incorporation of Li^+ ions into bioactive scaffolds is a viable means of enhancing canonical Wnt signalling pathway to stimulate cementogenic differentiation of hPDLCs, suggesting that Li-containing MBG scaffolds might have potential for the application of periodontal tissue engineering.

In Chapter 6, the aim of this study was to investigate the interactions of human periodontal ligament cells (hPDLCs) and human bone marrow stromal cells (hBMSCs) with Li-β-TCP bioceramic bulks and their ionic extracts, and further explore the osteogenic and cementogenic stimulation of Li-β-TCP bioceramics and possible molecular mechanisms. To our best knowledge, this study was the first study in preparation of Li-containing β-TCP bioceramics and investigated the effect of Li-β-TCP bioceramics on cementogenic/osteogenic differentiation for both hPDLCs and hBMSCs. The results indicate that the stimulatory effect of 5Li-β-TCP bioceramics on the osteogenic/cementogenic differentiation of hPDLCs and hBMSCs is related to the regulation of canonical Wnt signalling pathway. This study provides some insight into the notion that hBMSC might be another alterative cell source for periodontal tissue engineering. The results suggest that 5Li-β-TCP bioceramics possess excellent osteogenic/cementogenic stimulation property for both hPDLCs and hBMSCs and can be a promising bioactive material for bone/ periodontal tissue regeneration.

Taken together, it is the first time in this report that local injection of LiCl, Scl-Ab and LV-Ctnnb improve periodontal regenerative therapy with new cementum formation *in vivo* through activation of canonical Wnt signalling. The observations from this work suggest that canonical Wnt signalling plays a vital role during cementum regeneration *in vivo* and i*n vitro*. Importantly, it demonstrated that activation of canonical Wnt signalling can induce significant cellular cementum

formation in a rat periodontal defect model, which highlights the novelty of potential periodontal tissue engineering applications. More interestingly, these results revealed that Li-MBG and Li-β-TCP biomaterials can induce the cementogenic differentiation of hPDLCs *in vitro,* via activation of canonical Wnt signalling pathway, suggesting they may be promising bioactive materials for cementum regeneration. Based on the results in the present study, strategies directly targeting Wnt signalling pathway may provide new approaches for periodontal regenerative tissue engineering.

7.3 LIMITATIONS OF PRESENT STUDY

The limitation of the particular research approach in this study has been addressed in each part of this work. One overarching limitation, however, related to the ability to form more new cementum *in vivo* as the original cementum in rat is only a thin layer along the tooth root. The cementum-specific markers are not defined which make it challenging to confirm the newly-formed mineralized tissues along the tooth root surface possess physiological functions of cementum. I did perform a combination of different experimental approaches in order to validate the findings to observe the formation of new cellular cementum; however, other new approaches are still needed to further confirm the newly-formed cementum can mimic the function of physiological cementum. Furthermore, it demonstrated Li-MBG and Li-β-TCP biomaterials can be promising periodontal regeneration *in vitro*; it is still unclear whether they can be applied *in vivo* to induce new cementum formation via activation of canonical Wnt signalling pathway. In addition, it is still unknown if canonical Wnt signalling pathway had cross talk with other signalling pathways during periodontal regeneration.

7.4 FUTURE DERECTIONS

In this thesis, it is noted that activation of canonical Wnt signalling can induce new cementum regeneration *in vivo*, with the aid of a local injection with the concern concentration of LiCl, anti-sclerotin antibody and LV-Ctnnb at the periodontal defect area. However, it is unknown whether other concentrations of these two reagents still have the same effect for cementum regeneration and whether they would be toxic for the tissues around the defect area. Therefore, further studies are required to identify such issues, probably by local injection of different concentrations of LiCl and anti-

sclerotin antibodies using the same rat periodontal defect model.

In addition, additional studies should be designed to understand whether there is a cross talk between canonical Wnt signalling and other soluble molecules during the cementum regeneration progress. It is of great interest to identify whether there is any potential link between cementum regeneration and other signalling networks including the Bmp signalling pathway, Fgf signalling pathway and Hh signalling pathways. Furthermore, it is of great importance to test, whether the newly-formed cementum has the same mechanical properties as physiological cementum. Subsequently, the chemical components analysis of newly-formed cementum needs to be analysed to determine whether the newly-formed cementum can function well uner normal physiological conditions *in vivo*.

As mentioned previously, Li-MBG and Li-β-TCP biomaterials can significantly enhance the cementogenic differentiation of hPDLCs *in vitro* via activation of canonical Wnt signalling pathway. However, it is still unclear whether these materials can induce new cementum formation *in vivo*. An animal study would also have to be designed to investigate the potential efficacy of these biomaterials in relation to cementum regeneration *in vivo* conditions.

7.5 CONCLUDING REMARKS

The study presented in this thesis highlights the important role of canonical Wnt signalling pathway during cementum regeneration *in vivo* and *in vitro*; and that Li-MBG and Li-β-TCP biomaterials may be promising bioactive materials for cementum/periodontal tissue engineering via activation of canonical Wnt signalling. A systematic targeting of this molecular event by canonical Wnt signalling pathway may provide a new scientific rationale for the potential development of novel therapeutic strategies for periodontal diseases treatment.

Appendix

University Animal Research Ethics Committee

ANIMAL ETHICS APPROVAL CERTIFICATE

QDPI Scientific Use Registration Number 0052

Date of Issue: 12/11/12 (supersedes all previously issued certificates)

Dear Ms Pingping Han

UAECs must advise investigators and teachers of their decisions in writing as promptly as possible and activities must not commence until written approval has been obtained from the UAEC. This Approval Certificate serves as your written notice.

Within this Approval Certificate are:

* Activity Details
* Animal Details
* Conditions of Approval (Specific and Standard)

The following standard conditions are particularly important:

(a) prompt notification to the UAEC of any adverse or unexpected effects that impact on animal wellbeing;

(b) submission to the UAEC for consideration, any changes to the project as detailed in the proposal application.

Further information regarding your ongoing obligations regarding animal based research can be found via the Research Ethics website (http://www.research.qut.edu.au/ethics/) or by contacting the Research Ethics Coordinator on 07 3138 2091 or ethicscontact@qut.edu.au.

If any details within this Approval Certificate are incorrect please advise Research Ethics within 10 days of receipt of this certificate.

Activity Details

Approval Number:	1100000141
Activity Title:	Investigation of the cell signaling pathways during cementum repair and regeneration in tooth root wound healing rat modelings
Activity Summary:	Tooth is a common research model used in the study of the molecular mechanisms of organ development. The four classical signaling pathways including Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factor (FGFs), Hedgehog proteins (Hhs) and Wnt signaling pathways are known as an imperative guiding signal system in the embryonic course of pattern formation and differentiation responsible for organ formation. Research in the area of periodontal regeneration has been revealed similar cellular interactions and cell signaling activation, which resembles with the tooth development. In the process of periodontal tissue regeneration, one of the most critical and difficult component is cementum regeneration. In this proposed research project, we are aiming to investigate the role of several cell signaling pathways in cementum regeneration.
Date Approved:	02-Jun-2011 **Approved Until:** 02-Jun-2014 (subject to annual reports to the UAEC)
Location(s):	MERF, HMRC

Investigator Details

Chief Investigator: Ms Pingping Han

Other Staff/Students:

Investigator Name	Type	Role
A/Prof Yin Xiao	Internal	Ethics - Alternative Investigator
Miss Xufang Zhang	Student	Associate Investigator
Mr Zetao Chen	Student	Associate Investigator
Dr Zhibin Du	Internal	Associate Investigator

Researcher	Prof Yin Xiao [00463975]
Approval Number	0800000490
Ethics Status	Approved / In Progress
Ethics Category	Human [Human Negligible-Low Risk]
Ethics Title	The potential application of human dental tissue derived mesenchymal stem cells in tissue regeneration
Ethics Summary	Summary
Applied Date	21/07/2008
Approved Date	22/09/2008
Approved Until	31/12/2013
Standard Conditions of Approval	See www.research.qut.edu.au/ethics/humans/stdconditions.jsp
Special Conditions of Approval	Special Conditions Recruitment is via the clinic receptionist. Information sheets can be made available to clients who will peruse information / ask questions before entering dentist room. The receptionist can notify the dentist which clients have confirmed consent, or who seek further information.
QUT Lead Investigator	Prof Yin Xiao
Ethics Investigators	Investigator List

Investigator No.	Investigator Code	Name	Role	Organisation	Country
1	00463975	Yin Xiao	Chief Investigator		

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