THE FUNCTION AND REGULATION OF LIM DOMAIN MINERALIZATION PROTEIN (LMP) IN PERIODONTAL LIGAMENT PROGENITOR CELLS

by

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To My Beloved Family

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TABLE OF CONTENTS

LIST OF TABLES

ABSTRACT

New methodologies to target and deliver osteogenic factors offer significant potential for craniofacial tissue engineering. LIM domain mineralization protein (LMP) appears to be such a candidate for periodontal bone regeneration. The main purpose of this dissertation was to explore the function and regulation of LMP1 in periodontium, specifically in periodontal ligament (PDL) cell, and to evaluate the potential of LMP gene therapy in promoting periodontal bone formation. Using laser capture microdissection, LMP1 was found to be highly expressed in PDL and gingival tissue, and at lower level in mature alveolar bone. During tooth extraction socket healing, LMP1 expression modestly increased over time. However, in the healing of osteotomy defects around implants, LMP1 expression was gradually decreased. In experimental periodontitis model, LMP1 gene expression was upregulated in the inflamed gingival tissue. The physiological function of LMP1 was also investigated by a loss-of-function strategy. Stable knockdown of LMP1 in PDL cell resulted in impaired cell proliferation and subsequent delay in mineralization. Adenoviral gene delivery of LMP1 and LMP3 (a truncated transcription variant without any LIM domain) was performed to assess the potential of LMP gene transduction in enhancing bone formation. AdLMP3 but not AdLMP1 significantly induced matrix mineralization in PDL cell and bone marrow stromal cell *in vitro*. However, AdLMP3 transduced-PDL cells failed to induce ectopic bone formation in

immunocompromised animals. Interestingly, AdLMP1 and AdBMP7 combinatory gene therapy led to increased bone formation above that of AdBMP7 treatment alone. More studies are needed to understand the mechanisms underlying this synergistic effect. The regulatory mechanism of LMP1 gene expression was identified in this thesis as well. LMP1 gene expression is regulated by TGF-β1 in PDL cell and other preosteoblast. TAK1-JNK/p38 kinase cascade was involved in this regulation event. Gene knockdown LMP1 affected the TGF-β1 effect on PDL proliferation. In summary, this dissertation established the gene expression profiles of LMP1 in normal, diseased, and regenerating periodontium, determined the function of LMP1 on PDL cell proliferation and differentiation, investigated the potential of LMP gene therapy in periodontal regeneration, and characterized a regulatory mechanism of LMP1 gene expression.

CHAPTER ONE

INTRODUCTION

1.1 Periodontal disease

Periodontal diseases are characterized by an inflammatory reaction of periodontal tissue in response to bacterial biofilms that accumulated on tooth root surfaces. This leads to destruction of tooth-supporting tissue, including alveolar bone, tooth root cementum, and periodontal ligament [1]. This disease is one of the most common oral inflammatory infectious diseases, of which 31% of the United State population exhibit mild forms, 13% display moderate forms, and 4% have advanced disease symptoms [2]. Periodontal disease is the leading cause of tooth loss in adult and it has also been associated with systemic diseases such as atherosclerosis, heart failure, and diabetes [1].

Lipolysaccharides (LPS), a major component of the outer membrane of Gram-negative bacteria, initiates the cascade of events leading to periodontal tissue destruction [1]. To describe this process briefly, LPS derived from periodontal biofilms on the tooth surface triggers the recruitment of polymorphonuclear leukocytes (PMN) to the site, followed by monocytes and activated macrophages. This inflammatory infiltration from the gingival tissue produces various inflammatory molecules, such as matrix metalloproteinases (MMPs), pro-inflammatory cytokines, and prostaglandins. Proteases

degrade the collagen structure of periodontal tissues and thus lead to further leukocyte infiltration. Interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and receptor activator of nuclear factor-kappa B ligand (RANKL) are elevated in active sites and mediate osteoclastogensis and bone breakdown [1, 3].

Currently, various therapies have been developed to regenerate the lost periodontium, such as guided tissue regeneration (GTR), osseous grafting and application of recombinant growth factors. However, complete and predictable regeneration is still a considerable challenge in periodontology, especially in advanced periodontal diseases, due to the complex microenvironment of the periodontal wound. The following factors contribute to the problems in regeneration: 1) Periodontal wounds are contaminated with tooth-associated biofilms of the anaerobic bacteria; compounding this, the transmucosal hard-soft tissue environment allows entry of pathogens into wounds. 2) Multiple junctional complex and stromal-cellular interactions create difficulty in rebuilding tissue interfaces (e.g., tooth-PDL-bone and epithelium-connective tissue-bone). 3) The effects of occlusal forces deliver intermittent loads in axial and transverse dimensions. 4) Limited blood supply in the cementum [4-6].

1.2 Periodontal tissue engineering

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [7]. Recently, the concept of tissue

engineering has been applied in periodontal regeneration. The factors critical to the outcome of periodontal tissue engineering have been expanded in the last couple of years [8]. To date, it is believed that the following six aspects should, at least, be considered for successful periodontal regeneration: appropriate cells, signals, blood supply, scaffold, mechanical loading, and pathogen control (Figure 1-1). Cells provide the machinery for new tissue growth and differentiation. Growth factors or morphogens modulate the cellular activity and provide stimuli to cells to differentiate and produce matrix toward the developing tissue. A three-dimensional template structure is provided by scaffolds to facilitate the above processes critical for tissue regeneration [8]. New vascular networks promoted by angiogenic signals provide the nutritional base for tissue growth and homeostasis, while appropriate mechanical loading would be essential for the development of highly organized, functional periodontal ligament fibers. Finally, because of the microbial bath in the periodontal sites, strategies in controlling infection are required to optimize periodontal regeneration.

This paper will review current advancements in tissue engineering concerning the areas of periodontal inflammation prevention and treatment. Development of scaffolds designed for periodontal tissue engineering will be discussed and potential cell sources for future clinical periodontal regeneration will be summarized. A review of different gene delivery methods (non-viral and viral) and target genes to promote periodontal soft and hard tissue regeneration will be highlighted, followed by recent attempts in genetic delivery of therapeutic molecules to regulate the pathogen-host response of periodontal disease.

1.3 Cells involved in periodontal regeneration

Cells are the center of new tissue growth and differentiation. In cell-based regenerative medicine, cells are delivered to a donor with the goal of improving the regeneration process. Initial reports in the 1970s by WT Green, a pediatric orthopedic surgeon, demonstrated that implanted spicules and cartilage seeded with chondrocytes into animals could generate new cartilage [9]. Researchers delivered cells to accelerate periodontal regeneration for two purposes: 1) using cells as carriers to deliver growth factors, and 2) providing seed cells which are able to differentiate to multiple cell types in periodontium to promote regeneration. In the first, use of cells as carriers to deliver growth factors can stimulate an endogenous regeneration process. This strategy has been intensively investigated in both soft and hard tissue regeneration in oral and periodontal tissues, and some products in this area are being tested in clinical trials. Although periodontal regeneration can be seen in physiological status or wounding healing, the level and scale of this regeneration are very limited due to several reasons, one of which is lack of stem cells. With the development of stem cell research in the last several years, various stem cell types have been evaluated for their potential in periodontal regeneration [10].

Mesenchymal stem cells (MSCs) are self-renewable and can differentiate into a variety of cell types that form mesenchymal and connective tissues [11, 12]. Bone marrow stromal cells are the most widely investigated MSCs because they are easily accessible. Bone marrow stromal cells were first isolated in the 1960s based on their unique ability to adhere to the plastic substrate of cell culture plates [13]. Since then, this simple protocol has been widely used to isolate MSC from many tissues such as adipose

tissue, muscle, liver, pancreas, and cartilage. MSCs have great promise in regenerative medicine because they are multipotent and capable of forming bone, cartilage, cardiomyocyte, and even hepatic tissue *in vivo*. Mesenchymal stem cells may also be a useful cell source for periodontal regeneration (Table 1).

1.3.1 Non-craniofacial MSCs

Kawaguchi *et al*. showed that bone marrow stromal cells transplantation promoted periodontal regeneration in experimental class III defects in beagle dogs. According to the histomorphometric measurement, bone marrow stromal cells treatment increase new cementum length and bone area up to 20% [14]. Using Green Fluoresent Protein (GFP) label technique, it was further conformed that these cells differentiated to cementum, periodontal ligament, and alveolar bone *in vivo* [15]. In a subsequent small clinical trial, autologous expanded bone marrow stromal cells mixed with Atelocollagen were transplanted into periodontal osseous defects at a periodontal surgery site, and all seven patients were shown to have a good clinical course [16]. Another case report showed that transplantation of bone marrow stromal cells mixed with PRP (Platelet Rich Plasma) resulted in reduced bleeding, reduction of tooth mobility and reduced bone defect depth [17].

Bone marrow stromal cells have also been shown to promote bone healing and dental implant osseointegration. In a series of studies, Yamada et al. used a combination of PRP as an autologous scaffold with *in vitro*-expanded bone marrow stromal cells to increase

osteogenesis in dental implant surgery [18-20]. This "autogenous injectable bone" treatment results in higher marginal bone levels, better bone-implant contact, and increase bone density compared to control. In our on-going clinical trial, bone marrow stromal cells transplantation is being tested to promote bone formation after tooth extraction for implant restoration. Bone marrow stromal cells were harvested from iliac crest and expanded in a special-designed bioreactor. Twelve days later, teeth extraction surgery was performed and the sockets were filled with autologous bone marrow stromal cells. Preliminary data showed that stem cell therapy is safe clinically, and it appears to augment bone regeneration in extraction sockets.

Adipose tissue is another abundant resource for adult MSCs, and their greatest advantage is ease of access. Tobita et al demonstrated that, mixed with PRP, adipose-derived MSCs could promote regeneration in rat periodontal palatal defects [21].

1.3.2. Craniofacial MSCs

Just as periodontal ligament is essential for osteogenesis and cementogenesis in periodontium development, cells derived from periodontal ligament are necessary for regeneration of damaged periodontal tissue [22]. Transplantation of these cells has shown the potential to regenerate periodontal attachment apparatus *in vivo* [23-25]. Akizuki *et al* developed a PDL sheet using temperature-responsive cell culture dish technique and hyaluronic acid carrier [26]. After the transplantation of PDL cell sheet in rat and beagle dog model, significant cementum formation and anchoring PDL fibers were observed, as

well as new alveolar bone formation [26]. Using different labeling techniques, Lekic *et al*. showed that transplanted PDL cells integrate and differentiate into newly formed periodontal tissues [23].

Specific cell types derived from periodontium have also been examined for their potential and roles in periodontal regeneration. Cementoblasts have a marked ability to induce mineralization in an *ex vivo* model [27] and *in vivo* in periodontal wounds [28]. However, when less-differentiated dental follicle cells are delivered in a similar fashion, these cells inhibit periodontal healing [28]. Similarly, progenitor cells isolated from dental follicle fail to form dentin, cememtun or bone *in vivo*, although they express high level of BSP, OCN and ALP [29]. These results suggest the selective behaviors of different cell types in periodontal regeneration. Recently, mesenchymal stem cells were isolated from periodontal ligament. Periodontal ligament stem cells (PDLSCs) express several mesenchymal stem cell markers, such as STRO-1 and CD44, and exhibit osteogenic, adipogenic, and chondrogenic characteristic under defined culture conditions (for reviews, see reference [10, 11]). Implanted into immuno-deficient mice, PDLSCs generated cememtum/PDL-like structures similar to native periodontium apparatus [30-32]. In a porcine model of periodontitis, PDLSCs have been shown to regenerate new bone, cementum, and periodontal ligament in the periodontal defect area, and the height of the new alveolar bone was significantly higher than that in HA/TCP control group [33]. Combining PDLSCs and another stem cell population from the root apical papilla of human teeth (SCAP, stem cells from apical papilla) which contributes to dentin

deposition, Sonoyama et al. generated a bio-root structure encircling with periodontal ligament tissue [34].

1.4. Gene therapy for the periodontal engineering

Gene therapy is defined as the treatment of disease or disorder by transferring genetic materials, which introduce, suppress, or manipulate specific genes that direct an individual's own cells to produce a therapeutic agent [35]. Gene therapies have shown significant promise for the treatment of a wide range of diseases, such as adenosine deaminase deficiency [36], hemophilia B [37], X-linked adrenoleukodystrophy [38], etc. Gene therapy has also emerged as a promising strategy for the modulation of host-response triggered by periodontal microbe and the regeneration of periodontium in periodontal diseases. Compared to traditional treatment (compounds and proteins), gene therapy has the following advantages: 1) Gene therapy provides a greater sustainability than that of single protein/compound application; While the half lives of pharmaceutical compounds or recombinant protein usually range from several hours to several days, viral vector genes can be expressed in vivo from weeks to years [39, 40]. 2) Gene therapy may be able to avoid issues associated with *ex vivo* protein expression and purification, such as palmitoylation and glycosylation [41]. 3) In theory, the temporally regulated delivery of genetic sequences encoding a combinatorial group of regenerative factors, which mimics the biology in natural healing, would be capable of an enhanced periodontal regeneration. 4) Combined with tissue engineering strategies, delivery of different genes

in a spacially regulated manner presents great potential in regenerating the multiple tissue interfaces in periodontium. 5) Gene therapy is more economical to patients.

1.4.1. Gene delivery methods:

The preferred strategy for gene transfer depends on a number of factors: 1) the required duration of protein release (transient versus constitutive expression); 2) target cells (dividing and non-dividing cells, receptor expression); 3) host immune response to vectors; 4) route of gene delivery (*ex vivo* or *in vivo*); and 5) the morphology of the target site. For example, a horizontal one- or two-walled defect may require the use of a supportive carrier, such as a scaffold. Other defect sites may be conducive to the use of an adenoviral vector embedded in a collagen matrix, and a wide variety of viral and nonviral vectors have been developed for gene delivery. Examples of viral vectors are retroviruses, lentiviruses, adenoviruses (Ad) and adeno-associated viruses (AAV), and nonviral vectors include plasmids, DNA polymer complexes, nano/microbubbles and ultrasound, Zinc-finger nuclease and plasmid, etc. (Table 2)

Retroviral vectors are single-strand RNA viruses that are replicated in a host cell through the enzyme reverse transcriptase to produce DNA from its RNA genome, and the resulting reverse-transcribed viral DNA is incorporated into the host cell's DNA strand by an integrase enzyme. When the genetically altered host cell divides later, its descendants contain the viral DNA copy. These vectors have significant advantages for sustained and efficient transgene expression that is ideal for the treatment of life-threatening hereditary

disorders, although most retroviruses can only infect dividing cells. Since the integrase enzyme may insert the DNA copy into an arbitrary position of the target cell DNA, endogenous gene expression maybe disrupted by insertional mutagenesis of a proto-oncogene or tumor suppressor, and carcinogenesis may occur.

Lentiviruses, such as the human immunodeficiency virus (HIV), are a specialized class of the retrovirus family and characterized by a long incubation period [35, 42]. Lentiviral vectors are one of the most efficient methods in gene delivery, being able to transfect both dividing and non-dividing cells [42]. These vectors are integrated into the host cell genome as well. In spite of the evidence that the insertion sites of lentivirus are more restricted than other retroviruses, the carcinogenesis induced by insertational mutation is still a hurdle for clinical application. Additionally, their HIV origin raises many concerns about the possibility that recombination events will lead to replication competent viruses.

Adenoviruses are nonenveloped icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome [35]. In contrast to Lentiviruses, adenoviral vectors are attractive gene delivery vehicles due to a number of features: (1) Ads have high transduction efficiency in both dividing and nondividing cells; (2) Ads do not induce apparent phenotypic changes in transduced cells; and (3) Ads do not integrate into the host genome and remain episomal [43, 44]. These vectors may be advantageous in periodontal tissue engineering because the transient expression of growth factors may

prevent the overgrowth of newly formed tissue. However, in large size craniofacial defects, the short-term gene expression may be insufficient to induce complete tissue regeneration [35]. One major concern regarding Ads gene delivery is the strong host immune response to viral capsid proteins. This has prompted attempts to modify the viral backbone in order to reduce immunogenicity [35]. Recently, several studies suggest that local therapeutic Ads appear to be safe and efficient in diabetic foot ulcer treatment and periodontal regeneration [43-45].

Adeno-associated viurses derive from the parvovirus family and are small viruses with a single-stranded DNA genome [6]. AAV has attracted considerable interest from gene therapy researchers because of several significant advantages: 1) AAV is currently not related to any human disease; 2) AAV presents very low immunogenicity; and 3) AAV infects both dividing and nondividing cells. It has the ability to integrate its genetic material into the host cell genome at a specific site in the human chromosome 19, which makes it more predictable than retrovirus [46]. However, random integration of AAV DNA into the host genome is low but detectable. A recent report raised concerns over the clinical use of AAV vectors when mice developed hepatocellular carcinoma after neonatal injection of an AAV vector, which is associated with the insertion in a 6-kilobase region of chromosome 12 [47]. Types of recombinant AAV have been developed either to remain extrachromosomal or integrate into nonspecific chromosomal sites [6]. One disadvantage of the AAV is that it is small and possesses the capacity to carry target DNA usually less than 5 kb [35].

Besides viral vectors, genetic material can be delivered into host's cell by several nonviral alternatives, including naked plasmid, cationic lipids, polymers, peptides, and physical methods (electroporation and ultrasound) [48-51]. A major disadvantage for nonviral delivery methods is that nonviral gene carriers consistently exhibit significantly reduced transfection efficiency as they are hindered by numerous extra- and intracellular obstacles [51]. However, because of their low immunogenicity, lack of DNA insert size limitation, and potential for large-scale production, nonviral vectors will be given more consideration in the future, especially in the field of siRNA gene therapy [50-53]. In the past decade, a significant amount of research has focused on designing cationic compounds that can form complexes with DNA and can avoid both in vitro and in vivo barriers for gene delivery, and several compounds have been examined in clinical trials, such as Allovectin-7 [51]. It is worth mentioning that, due to the anatomic advantage, some non-invasive physical methods may be useful for delivering DNA to periodontium. Chen *et al*. reported that a gene transfer approach using ultrasound and nano/microbubbles leads to high gene expression in gingival tissue [49].

1.4.2. Scaffold protein LMP1 and osteogenesis

Using insulin receptor (INSR) as a bait in yeast 2-hybrid screen, Wu et al. first cloned a cDNA from HeLa cell encoded ENIGMA, named for its endocytic code recognition properties [54]. The ENIGMA protein is a representative of PDLIM protein family, which has conserved PDZ domain and LIM domains. LIM domains are cysteine-rich double zinc fingers, usually functioning in protein-protein interactions that are critical in

different cellular processes, such as organ development, cytoskeletal organization and oncogenesis [55]. It has been shown that the LIM domains of Enigma interact with several proteins, such as protein kinase C [56], Ret/pct2 oncogene [57, 58], and InSR (Table 3). The PDZ domain of Enigma binds to actin filaments [59, 60]. Although Enigma acts as a scaffold protein in the cytoplasm, its biological function is largely unknown. Enigma is highly conservative in different species. In zebrafish, Enigma knockdown appeared to be embryonic lethal shortly after the end of gastrulation [61]. In few surviving embryos, Enigma knockdown led to reduce tails and deformed somites.

In 1998, Liu et al. cloned LIM mineralization protein-1 (LMP1) as a highly upregulated gene in the early stage of calvarial osteoblast differentiation [62]. Sequencing analysis showed that *LMP1* encodes the same protein as Enigma. The expression of LMP1 was induced by glucocorticoid (GC) and BMP6, both at the mRNA and protein level. The expression pattern of LMP1 during fetal endochondral and membranous bone development was revealed by *in situ* hybridization: at rat embryonic day 14, LMP-1 transcripts were expressed in mesenchymal tissue surrounding the cartilaginous anlage of immature bones and in the future joint spaces; later, as endochondral ossification progressed and the hypertrophic cartilage zone began to be replaced by mineralized bone, LMP-1 expression appeared in the mineralizing portion of the bone. *In vitro* experiments further demonstrated that LMP1 regulated the differentiation of osteoblast precursors. Over-expression of LMP in primary calvarial osteoblasts induced bone specific gene expression, such as osterix, alkaline phosphatase, osteocalcin, further promoting

mineralized nodule formation [63-65]. Blocking the expression of LMP1 by antisense oligonucleotides inhibits the differentiation of calvarial osteoblasts *in vitro* [62].

LMP1 has also been shown to promote bone formation in heterotopic (subcutaneous and intramuscular) and orthotopic (spine fusion) sites (Table 4). Using an adenoviral vector, LMP3, which is an osteogenic transcript variant of LMP1, was injected bilaterally into the exposed triceps surae musculature in mice. Bone formation was seen after 5 weeks in all the mice injected with AdhLMP3, which was confirmed by radiography and Von Kossa staining in tissue sections [64]. The researchers reported that LMP gene transfer to mouse muscle results in rapid and efficient ectopic bone formation, relative to BMP-2 (all the animals injected with AdLMP3 had ectopic bone formation, whereas only half of AdBMP2 group formed bone). LMP1 gene delivery has also been shown to induce new bone formation in a spine fusion model [66]. Bone marrow-derived buffy-coat cells from rabbits were transduced by AdLMP1 adenovirus, and then the cells were implanted during posterolateral arthrodesis. The results showed that LMP1 induces new bone formation and solid spine fusion, and the biomechanical quality of the regenerated bone was further confirmed.

Although LMP1 has been shown to regulate osteogenesis, much is still unknown about the mechanism of LMP1-induced osteogenesis. At least three transcript variants of LMP exist in humans: LMP1, 2, and 3 [63]. LMP1 and LMP3 are osteogenic, whereas the 119-base pair deletion of LMP2 leads to loss of osteogenic capability. It indicated that the 119-base pair area is critical for the osteogenetic characteristic (Figure 1). Recently,

the relative motif of LMP1 in this area has been identified. The motif directly interacts with the *ww* domain of Smurf1, which mediates Smad degradation, and leads to the accumulation of smad1 and smad5 in the cytoplasm which will prolong the BMP signaling effect [67, 68]. Small peptides containing this motif can mimic the ability to block Smurf1 from binding Smads *in vitro*. Overexpression of LMP1 in bone marrow stromal cells increases the amount of cytoplamic phophorylated Smad1. Taken together, LMP1 can increase the cell responsiveness to BMP signals. Other direct mechanisms may also be involved. It was found that BMP-2, BMP-4, BMP-6, BMP-7, and TGF-beta1 expression was elevated in LMP1-overexpressed cells [69, 70]. So it is hypothesized that LMP1 may directly regulate the transcription control of osteogenesis.

Recently, it was found from immunohistological staining that LMP1 expressed in human predentin, odontoblasts, unmineralized reparative dentin, odontoblast-like cells, endothelial cells of blood vessels, and pulp fibroblasts, but not in mineralized mature dentin [71]. The same group also found that the mRNA of LMP1 was upregulated (about 2 fold) in the osteolineage differentiation of human dental pulp cells [72]. However, the biological function of LMP1 in craniofacial and oral tissue development is still largely unknown.

1.5 Statement of Purpose and Dissertation Overview

Periodontal disease is the leading cause of tooth loss in America adults because of severe alveolar (tooth-supporting) bone loss. Successful alveolar bone regeneration approaches for oral reconstruction remain a challenge for oral health care providers.

Periodontal ligament cells have the plasticity to differentiate into multiple distinct cell types including osteoblasts (bone-forming cells). New methodologies to target and deliver osteogenic factors to PDL cells will offer significant potential for alveolar bone regeneration. It has been shown that LMP induces osteolineage differentiation in mesenchymal stem cells. To date, mechanisms underlying LMP-induced osteogenesis remain unknown. My dissertation research continues to explore the biological function of LMP in PDL cells and bone marrow stromal cells, and further define its therapeutic potential for oral bone reconstruction. The research extends beyond a craniofacial bone emphasis to address essential mechanisms in the biology of bone formation.

Four core questions are clarified in my dissertation: 1) What are the gene expression profiles of LMP1 in craniofacial wound healing and periodontal disease? 2) How is the LMP gene regulated in the PDL cell? 3) What is the role of LMP in PDL cell proliferation and differentiation? 4) Will gene transfer of LMP stimulate *ex vivo* bone formation and promote oral implant osseointegration?

Four specific aims are included in this dissertation:

Specific Aim1: To identify the expression profile of LMP1 during craniofacial wound healing and periodontal disease. Hypothesis: The expression of osteogenic molecule LMP1 is regulated during tooth extraction healing and oral implant wound healing, and has an altered expression pattern in periodontal disease. In **Chapter 2**, I used laser capture microdissection (LCM) and real time quantitative RT-PCR to evaluate the

gene expression of LMP1 in healing area. Its expression under disease circumstance was assessed in ligature-induced and *Porphyromonas gingivalis*-lipopolysaccharide (*Pg*-LPS) induced experimental periodontitis. Knowledge about where, when and how LMP1 is regulated in periodontal wound healing and disease helps to shade light on the function of LMP1 in normal and pathological periodontium as well as during periodontal regeneration.

Specific Aim 2: To explore the effect of LMP1 in PDL cell proliferation and differentiation. Hypothesis: LMP is required for PDL cell proliferation and mineralized nodule formation *in vitro.* In **Chapter 3**, a loss-of-function strategy was used to characterize the function of LMP1 in PDL cell. The expression level of LMP in PDL cells was stably knocked down by RNAi. Cell proliferation and DNA synthesis capacities of knockdown cells were measured by crystal violet staining and ^{3}[H]methylthymidine incorporation assay, respectively. Flow cytometry was performed to analyze the cell cycle change after LMP1 knockdown. Mineralization activity was assessed by Alizarin Red staining and Calcium measurement. My results showed that LMP1 gene knockdown impairs cell proliferation and delays osteogenic differentiation in PDL cells.

Specific Aim 3: To investigate the ability of LMP gene delivery to induce mineralization and promote bone formation. Hypothesis: Gene delivery of LMP1 and a truncated transcript variant, LMP3, is able to promote *in vitro* mineralization and stimulate ectopic bone formation. In **Chapter 4**, I first transduced PDL cells with adenoviral LMP1 and LMP3. Our results showed that AdLMP3, but not AdLMP1,

significantly stimulated matrix mineralization *in vitro*, which may be related to increased ALP and BSP expression. Next, I evaluated the potential of AdLMP1 and AdLMP3 to promote bone formation in an e*x vivo* tissue engineering model. LMP3 expressing PDL cells were delivered by polymer or collagen scaffolds and subcutaneously implanted in immunocompromised mice. Unexpectedly, AdLMP3 gene therapy alone or AdLMP3 combined with AdBMP7 was not sufficient to promote ectopic bone formation in our model. Future studies are needed to identify the inhibitory factors which hinder the translation from *in vitro* to *in vivo*.

Specific Aim 4: To determine the regulatory mechanisms of LMP1 gene expression in PDL cells. Because LMP gene expression is up-regulated in the early phase of osteolineage differentiation of PDL cells, <u>the hypothesis is that LMP1 is regulated by early signals in osteogenesis such as TGFβ-1</u>. Studies in **Chapter 5** accomplished the following: 1) Determined the effect of TGFβ-1 on LMP expression *in vitro* and identified dosage and time course of the effect. 2) Identified the signaling pathway mediating this effect. RNA interference (RNAi) and specific pharmaceutical kinase inhibitors were used to knockdown or block key modulators in canonical and non-canonical TGFβ signaling pathways. The effect of TGFβ-1 on LMP1 RNA expression was confirmed by quantitative real time PCR. In addition, I showed that the activation of TAK1-JNK/p38 kinase cascade was necessary for induction of LMP1 gene expression by TGF-β1. TGF-β1 stimulated PDL cell proliferation, however, this effect was compromised when LMP1 was knocked down. I concluded that LMP1 is a downstream

target of TGF-β1, involved in PDL cell proliferation. My findings define a regulatory mechanism of LMP1 in PDL progenitor cells and other MSCs.

Understanding PDL cell biology is of critical importance to the oral health science community. The work described in this dissertation is particularly significant because knowledge gained contributes to establishing cell and molecular mechanisms used by LMP to regulate PDL cell activities, and further direct development of new therapeutics to repair bony defects in the oral and craniofacial complex due to disease, trauma or congenital abnormalities. This knowledge also contributes to better understanding of osteogenic differentiation of mesenchymal stem cells and provide the basis for novel clinical strategy for bone regeneration.

1.6 Figure
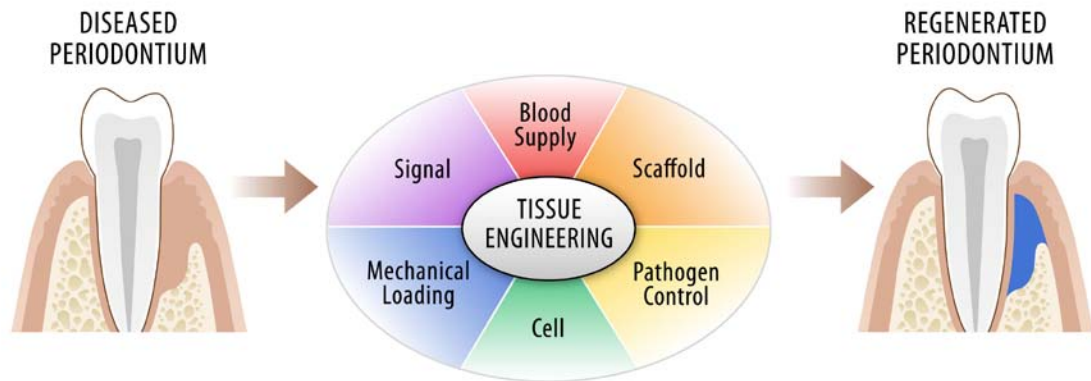
Figure 1-1. Paradigm of periodontal tissue engineering. Six aspects to be considered for successful periodontal regeneration: appropriate cells, signals, blood supply, scaffold, mechanical loading, and pathogen control.

1.7 Tables

Table 1: Selected examples of cell therapies for periodontal regeneration

Cell Type	Auto/Allo graft	Animal model	Surgery model	Reference
Bone marrow stromal cell	Auto	Beagle dog	Class III defects	[14-16]
	Auto	Beagle dog	Periodontal fenestration	[73]
Adipose stromal cells		Wistar rat	Periodontal palatal defects	[21]
Periodontal ligament stromal cells	Auto	Porcine	periodontal defects	[33]
	Allo	Nude mice	Ectopic	[30]
	Auto	Dog	Class II defects	[74]
	Allo	Athymic rat	Periodontal fenestration	[24, 75]
Cementoblasts	Allo	SCID mice	Ectopic	[76]
	Allo	Athymic rat	Periodontal fenestration	[28]
Dental follicle cells	Allo	SCID mice	Ectopic	[28, 76]
	Allo	Athymic rat	Periodontal fenestration	[28]

Table 2. Viral and nonviral gene therapy vectors used in tissue engineering

Vector	Type	Advantages	Disadvantages
Retrovirus	Viral	Nonimmunogenic Constitutive trangene expression	Infects only dividing cells Insertional mutagenesis
Lentivirus	Viral	Infects dividing and non-dividing cells Infect wild range of cell types Low immune response	Insertional mutagenesis Potential pathogenicity Complex large scale preparation
Adenovirus	Viral	Infects dividing and non-dividing cells Does not integrate into target cell genome	Potential immunogenicity Transient expression
Adeno-associated virus	Viral	Infects dividing and nondividing cells Low immunogenicity Nonpathogenic in human	Difficult to produce at high titers Small transgenes
Plasmid	Nonviral	Nonimmunogenic Nonpathogenic	Low transduction efficiency
DNA polymer complexes	Nonviral	Infects dividing and nondividing cells Cell-specific targeting	Low transduction efficiency

Table 3: Scaffold protein LMP1 interacts with several intracellular proteins.

Protein-protein interaction	Binding domain	Function	Reference
InSR (Insulin receptor)	LIM2	NA	[77]
PKC (Protein kinase C)	LIM	NA	[56]
RET (Ret proto-oncogene)	LIM3	Mediate Mitogenic signal	[58]
TPM2 (β-Tropomyosin)	PDZ	NA	[59]
APS (Adaptor protein with PH and SH2 domains)	LIM	Actin cytoskeleton organization (Enigma overexpression in 3T3-L1 adipocytes inhibits insulin-stimulated glucose transportation)	[60]
Smurf1 (SMAD specific E3 ubiquitin protein ligase 1)	Bone motif (Proline rich)	Prevent Smad degradation, osteogenesis	[67]

NA: not available

Table 4. Overview of *in vivo* experiments of LMP inducing bone formation

Therapy type	Plasmid/ Adenovirus	Cell type	Scaffold	Animal species	Bone regeneration model	Resource
Gene therapy	Ad-LMP3	----	----	Mice	Intramuscular injection	[64]
Cell + gene delivery	Rat LMP1 cDNA	Rat bone marrow cells	Devitalized bone matrix	Athymic rats	Subcutaneous (Chest)	[62]
	Rat LMP1 cDNA	Rat bone marrow cells	Devitalized bone matrix	Athymic rats	Posterior arthrodesis of spine	[78]
	Ad-rLMP1	Rat bone marrow derived buffy-coat cells	Collagen-ceramic composite	Rabbit	Posterior arthrodesis of spine	[66]
	Ad-hLMP1	Rabbit buffy-coat cell	Collagen	Athymic rats	Subcutaneous (Chest)	[79]
	Ad-hLMP1	Rabbit buffy-coat cell	Collagen	Athymic rats	Posterior arthrodesis of spine	[79]
	hLMP1-t (truncated form, without LIM domains)	Rat bone marrow cell	Devitalized bone matrix	Athymic rats	Subcutaneous (Chest)	[63]
	AdLMP3	Mouse dermal fibroblast	HA-Collagen	C57BL/6J mice	posterior spine surgery	[80]
	AdLMP3	Rat dermal fibroblast	HA-Collagen	Wistar rats	Rat critical size mandibular defect	[80]

24

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CHAPTER TWO

GENE EXPRESSION PROFILE OF LMP1 IN CRANIOFACIAL WOUND HEALING AND PERIODONTAL DISEASE

2.1 Abstract

LMP1 has been reported to be associated with osteoblast differentiation and bone formation. It remains elusive regarding the expression profiles of LMP1 in physiological and pathological craniofacial tissue. The objective of this study was to explore the gene expression pattern of LMP1 in alveolar bone wound healing processes and an experimental periodontitis model. Laser capture microdissection (LCM) and real time quantitative RT-PCR were performed to investigate the LMP1 expression in normal periodontium, and two alveolar bone healing models: tooth extraction socket healing and oral implant/bone osseointegration. The LMP1 expression was evaluated in inflamed gingival tissue in a rat periodontitis models induced by ligature placement or *Porphyromonas gingivalis*-lipopolysaccharide (*Pg*-LPS) injection. We showed that LMP1 was expressed at higher levels in PDL and gingival tissues compared to mature alveolar bone. After tooth extraction, the LMP1 expression was relatively low in early stage (coagulation stage), and it was slightly increased at day 7, 10 and 14. At an osteotomy defect area around dental titanium implant, LMP1 gene expression was relatively high at day 3, but steadily decreased at late stages. Very interestingly, LMP1

gene expression was up-regulated in the gingival tissue after experimental periodontitis induction. This information will help better understand the function of LMP1 during oral bone healing.

2.2 Introduction

LMP1 (LIM mineralization protein-1) was first cloned as a highly upregulated gene in the early stage of calvarial osteoblast differentiation [1]. Sequencing analysis showed that LMP1 encodes the same protein as ENIGMA, which is a intracellular scaffold protein involved in cell proliferation, differentiation and glucose uptake [2-5]. During embryonic development, LMP1 is expressed in mesenchymal tissue surrounding the cartilaginous anlage of immature bones and in the future joint spaces [1]. Although several studies showed that LMP gene therapy appears to be promising for bone regeneration, very little is known about when, where and how LMP1 gene expression is regulated in physiological and pathological circumstances, specifically, in periodontal disease and wound healing.

Tooth extraction is the most commonly performed surgical procedure in humans. The healing of an extraction socket starts with the formation of coagulum, followed by infiltration of provisional connective tissue which is subsequently replaced by newly formed woven bone, and ultimately by lamellar bone and bone marrow [6]. Tooth extraction socket healing has been intensively studied in the last half century [7-10], however, most of the studies focused on histological aspects of the healing process. Very few studies attempted to understand the dynamic gene expression profiles in the wound healing site after tooth removal.

Oral implants have become a standard treatment modality for tooth loss caused by periodontal disease, trauma, or some other reasons. The long-term success of implant

anchorage in alveolar bone is believed to be dependent on "osseointergration", which histologically is defined as 'direct bone-to-implant contact' without soft/connective tissue intervention [11, 12]. The processes of osseointegration involves a series of events including early protein and cell apposition, necrosis and resorption of traumatized bone around the titanium body, subsequent de novo bone formation and maturation to achieve the primary and secondary dental implant stability [11, 12].

The purpose of this study was to investigate the gene expression profile of LMP1 on craniofacial wound healing (tooth extraction socket healing and oral implant/bone osseointegration) and an experimental periodontitis model. In addition, the expression profile of a group of genes associated with bone healing, such as growth factors, transcription factors and chemokines, was also evaluated. Knowledge from this study will help us to better understand the function of LMP1 and shed light on the design of future LMP1-based gene therapies.

2.3 Results

Stronger LMP1 expression in periodontal ligament and gingiva compared to mature alveolar bone

At first we tested the gene expression profile of LMP1 in normal periodontium. Because suitable LMP1 antibodies are not available for immunohistological staining, we used laser capture microdissection (LCM) to collect PDL, gingival and alveolar bone tissues from decalcified rat maxillae sections. In order to validate the technique, genes with distinct expression profiles in these three tissues were tested (Figures 2-3).

Keratin14, an epithelial marker, was highly expressed in gingiva but present only at low levels in bone and PDL; Osteocalcin (OCN), a primary bone marker, was highly expressed in alveolar bone; Periostin, a marker for ligature and PDL, was highly expressed in the PDL samples. These results suggested that RNA retrieved from laser capture microdissection was still suitable for gene expression analysis. LMP1 gene expression in the above three tissues was also investigated. We showed that LMP1 is expressed at higher levels in PDL and gingival tissues compared to mature alveolar bone (Figures 2-3).

LMP1 expression during tooth extraction socket healing

Next, we looked for possible regulation of LMP1 during wound healing following tooth extraction. The rat first maxillary molars were extracted, and after 3, 7, 10 and 14 days, animals were sacrificed. Histologically, the extraction sockets followed a well-defined healing sequence (Figures 2-4A). At day 3, large clots were seen in the sockets, surrounded by scattered neutrophils and a large amount of mesenchymal cells (severed PDL). At day 7, the coagulum area became relative small, and more fibroblasts appeared in sockets, and newly formed bone which is less stained could be easily seen. At day 10, clots were replaced by fibroblasts and new bone. At day 14, the sockets were completely filled by new bone and bone marrow.

Tissues in the extraction sockets were dissected by laser capture at the above time points. We found that LMP1 expression was relatively low in early stages (coagulation stage), and it was slightly increased at days 7, 10 and 14 (Figure 2-5). We also analyzed

the expression profile of genes associated with wound healing. Four categories of genes were examined: growth factors, extracellular matrix proteins, chemokines, and transcription factors. In the 19 genes we tested, three expression patterns were evident (Figure 2s-5): 1) genes that are highly expressed at early time points and are down-regulated at later stages. Chemokines IL-1β, CXCL2 and CXCL5 belong to this category; Wnt5a and Wnt4 seemed to decrease as well during healing; 2) Genes that are slowly increased during the healing process: Growth factors (BMP4, BMP7, Wnt10b and VEGF), transcription factors (Runx2), and extracellular matrix proteins related to mineralized tissue (OPN and OCN) are in this group; Very interestingly, CXCL12 (SDF-1) gradually increases during extraction socket healing. TGF-β1 increases at a mid stage of healing (day 10) and then decreases. Similarly, Periostin, a target gene of TGF-β1, had the same expression pattern; 3) Genes that are constitutively expressed. Tendon specific transcriptional factor Scx appeared to be in this group.

LMP1 gene expression gradually decreased during healing after osteotomy around implants

We also examined the LMP1 gene expression during the bone defect healing around titanium implants. Histologically, the healing after osteotomy is similar to extraction socket healing except for the following differences: 1) the healing seems delayed; 2) more infiltration by inflammatory cells is seen in early stages (Figure 2-4B). The same 19 genes were also analyzed (Figure 2-6). Interestingly, most genes follow the same pattern as of extraction socket healing. However, LMP1 gene expression was relatively high at day 3, but steadily decreased at late stages.

LMP1 gene expression was up-regulated in the experimental periodontitis

In a ligature-induced periodontitis model, LMP1 expression in gingival tissue was upregulated 3 days after ligature placement, peaked at day 7, and dropped down to baseline at 14 days (Figure 2-7A). Similarly, in *Pg*-LPS-induced-periodontitis models, LMP1 gene expression in gingival tissue increased significantly 24 hours after LPS injection, and the effect lasted until 48 hours (Figure 2-7B). It was suggested that LMP1 may be related to the immune response induced by pathogens.

2.4 Discussion

LCM is a method to procure pure subpopulations of tissue cells of interest under direct microscopic visualization [13, 14]. Combined with other sophisticated molecular biology technologies such as DNA sequencing, cDNA array, DNA microarray, real time PCR and two-dimensional polyacrylamide gel electrophoresis, etc, this technology is a powerful tool to analyze in vivo cell function and gene expression [15, 16]. LCM has been extensively used in soft tissue samples, however it is still a challenge for mineralized hard tissue due to the need for decalcification. Although it has been reported that successful RNA extraction and RT-PCR could be performed from fresh frozen undecalcified tissue [17], this method is not very practical for bone and teeth tissue. Some studies had reported that RNA can be retrieved from decalcified samples [15]. Here, we were able to use LCM to dissect decalcified maxillae samples and successfully analyze gene expression from different area of interest. LCM provides a unique opportunity to clearly dissect the tooth extraction healing areas and implant osseointegration sites. In the

future, if we perform fluorescent bone labeling techniques after surgical procedures, it will provide a clearer border to guide the laser capture microdissection.

In this study, we found that, in normal periodontium, the expression level of LMP1 is higher in PDL and gingival tissue than in mature alveolar bone, which is somehow surprising to us. However, this result is consistent with our *in vitro* finding that LMP1 is expressed at lower levels in the final stage of pre-osteoblast differentiation, specifically in PDL cells (see Chapter 3). Taken into account the fact that LMP-1 was expressed in higher amounts and showed a higher degree of variation in bone samples from young patients than old individuals [18], it is suggested that LMP1 may not be critical in mature bone.

So far there is very limited information regarding gene expression profiles during the tooth extraction socket healing, and no information was reported about the regulation of LMP1 during bone wound healing. Taking advantage of LCM, we found that LMP1 appear to be slightly increased during tooth extraction healing although there was no statistical significance due to the large variability in the small sample. At the same time, valuable information about the gene expression pattern of key players in alveolar bone healing were provided in this study. We found that chemokines are highly expressed at the early stage of healing which is consistent with histological finding. Growth factors and angiogenesis factors showed significant increases after the early inflammation stage and kept in a relative high level until the wound healed. Very interestingly, we found that Wnt10b and CXCL12/SDF-1 were gradually increased during bone healing. More studies

are needed in the future to characterize the function and possible application of these morphogen and stem cell chemoattractants in alveolar bone healing.

We found that the gene expression pattern of LMP1 in the osteotomy defect area around titanium implants was different compared to the tooth extraction socket. We still don't understand this phenomenon. One possible explanation is that more immune cells infiltrate into the implant defect area which might lead to increased LMP1 expression in this area because LMP1 is highly expressed in leukocytes.

We found that LMP1 is upregulated in inflamed gingival tissue. This suggests that LMP1 may play a role in host immune modulation. It has been shown by Liu et al. that LMP1 is highly expressed in portions of the immune system, such as lymph nodes, thymus, tonsils, spleen and leukocytes [19] . Recently it was reported that LMP-1 has an anti-inflammatory effect which is due to the inhibition of NO production by suppression of NF-kappaB activation [20]. Another clue suggesting that LMP1 is associated with immune cells relies on the fact that LMP1 is regulated by TGF-β1 [21], which is a potent immune suppressor and plays an important role in immune cell differentiation. Taken together, it will be very interesting to determine the role of LMP1 host response to pathogens, especially in periodontal disease.

In summary, for the first time, this study provided the gene expression profiles of LMP1 in normal periodontium. We also report its expression pattern in bone wound

healing and disease situations. This information will help better understand the function of LMP1 during oral and craniofacial bone repair.

2.5 Materials and methods

Experimental design

A total of 36 male Sprague–Dawley rats were used in this study and the general timeline is shown in Fig 2-1. Briefly, the first molar teeth (M1) at one side of maxillae in all rats were extracted. After 1 month, osteotomy was performed on the healing sites and implants were placed. At the same time, first molar teeth (M1) at the other side of the maxillae were extracted. 3, 7, 10, 14 day following the surgeries, rats were euthanized and the maxillae were removed. In each time point, samples from 6 animals were used for laser capture microdissection and RNA extraction followed by qRT-PCR. Histological evaluation was performed on the other 3 animals (H&E staining for tooth extraction sites and back-scatter SEM for bone-implant osseointergration). All procedures were approved by the University of Michigan Committee of Use and Care of Animals. Animals were anesthetized under general anesthesia with ketamine (50 mg/kg) and xylazine (10 mg/kg).

Tooth extraction, defect creation, implant placement

The entire surgical procedure is shown in Figure 2-2. Briefly, the maxillary first molar teeth (M1) were extracted using an atraumatic technique. The extraction sockets and soft tissues were allowed to heal for approximately 30 days. After healing, an osteotomy was created using a custom drill-bit as previously described [11]. The drill-bit

was designed with a 0.95-mm diameter, 1mm long-apical portion and a 2.2-mm diameter, 1 mm long at the coronal aspect. The apical part of the drill created an osteotomy for initial fixation and the coronal part of the drill created a circumferential osseous defect before dental implant installation. Custom-fabricated, sterile, commercially pure, solid-cylinder titanium implants with SLActive® surface (chemically modified surface by extensive hydroxylation/hydration with an average 4.1-4.7 μm roughness) designed (Institut Straumann AG, Waldenburg, Switzerland) to the appropriate dimensions for placement into the rat maxillae (2 mm in length and 1 mm in diameter). The implants were press fit into position and evaluated for primary stability. The surgical field was closed by means of tissue glue (PeriAcryl, n-Butyl Cyanoacrylate, GluStitch Inc., Delta, B.C., Canada). The animals were observed post-operatively on a heating pad until fully alert to ascertain their response to surgery. To maintain energy and prevent infection, animals were given a 10% dextrose solution containing 268 g/L ampicillin for one week post-surgery.

Laser capture microdissection (LCM)

The animals were sacrificed by CO_2 euthanasia at the designated time points following surgery. Block biopsies were harvested, and immediately fixed with 10% phosphate-buffered neutral formalin for 24 hours. Biopsies were decalcified for 14 days in 10% EDTA solution. After implants were gently removed, biopsies were embedded in paraffin, cut sagittally along the axis of the tooth into 7 µm sections by microtome. Laser capture microdissection (LCM) was performed to dissect out the areas of interest (Fig 2). Six different tissues from each animal were collected: osteotomy defect area (A),

osseointegration site (B), tooth extraction healing site (C), gingival tissue (D), periodontal ligament (E), native alveolar bone in non-surgical area (F).

Animal model of experimental periodontal disease

Two models were used to induce periodontal disease and bone loss in Sprague-Dawley rats. 1) Ligature model. The use of ligatures elicits the rapid loss of approximately one-half of the bone support over a period of 3 to 6 weeks, which is related to the inflammatory process induced by increased microbial biofilm formation around the cervix of the teeth and an acute physical irritation factor as a consequence of the subgingival placement of the ligature. 3/0 cotton ligatures were placed bilaterally into the gingival sulci of the mandibular first molar teeth. The ligatures were evaluated twice weekly, gently displaced apically into the gingival sulci to ensure a subgingival position, and replaced when necessary. 2) *Porphyromonas gingivalis*-lipopolysaccharide (*Pg*-LPS)-induced periodontal disease model. Experimental periodontal disease induction was performed by administering 10 μl of *Pg*-LPS (1.0 mg ml^{-1}) into four palatal gingival tissue sites (total of 40 μl per animal) at the base of the interproximal gingival papillae between maxillary molars bilaterally [22]. The injections were performed three times weekly using custom-designed 0.375 in $\times$ 33 ga, 30° bevel needles attached to a 50 μl Hamilton syringe (Hamilton Company, Reno, NV, USA). Gingival tissue biopsies were harvested from a standardized region of the palatal region of the maxillary molar teeth, comprising an ~5 $\times$ 2 mm rectangular area from the medial of the first molar to the distal of the third molar extending from the gingival margin to the palatine suture.

Quantitative RT-PCR

Total RNA samples were extracted with RNAeasy kit (Qiagen, Maryland) according to the manufacture's instruction. RNA was subjected to reverse transcription in a 50μl RT reaction using TaqMan Reverse transcription reagents (Applied Biosystems, Foster city, CA). cDNA was generated using random hexamer primers and oligo-T primers with 2:1 ratio). After that, a preamplification kit was used to boost the low cDNA amount from LCM dissection (Applied Biosystems). For quantitative real-time PCR, the generated cDNA was analyzed, in triplicate, with the Master Mix (Applied Biosystems) in the ABI7500 Sequence Detection System. The results were normalized with 18s transcript. The primers and probes were ordered from Applied Biosystem.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. All data are presented as the mean ± SD. The significance of the differences was determined by using the two-tailed Student's t-test and one-way ANOVA. P-values less than 0.05 were considered statistically significant.

2.6 Figures
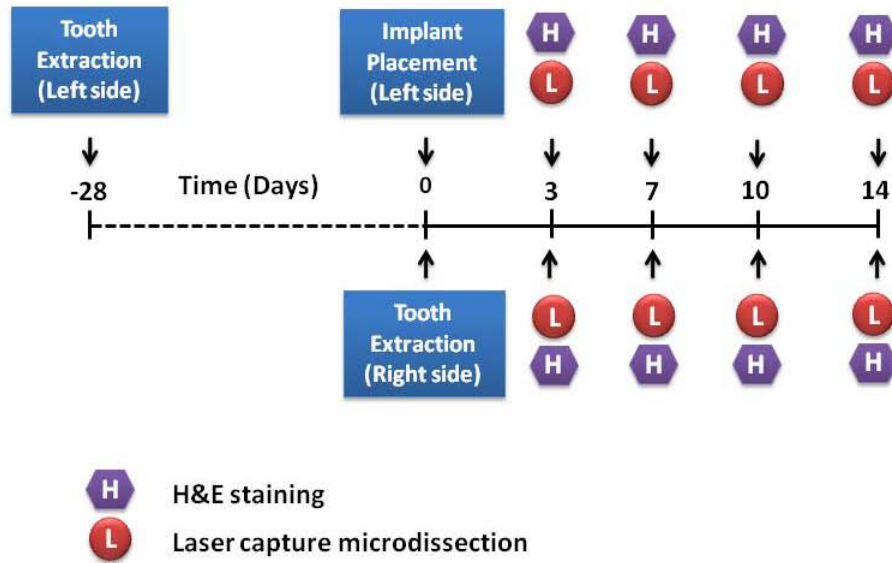
Figure 2-1 Experimental design for investigation of gene expression profiles in craniofacial wound healing. The left maxillary first molar (M1) were extracted using an atraumatic technique. The extraction sockets and soft tissues were allowed to heal for 28 days. After healing, an osteotomy was created using a custom step-drill and the right maxillary molars were extracted at the same time. The animals were euthanized at day 3, 7, 10, 14 days, and the maxillae were fixed. For the implant placement site, backscattered SEM measurements, H&E staining, and laser capture microdissection were performed. For the tooth extraction sites, only H. & E. staining, and laser capture microdissection were done.
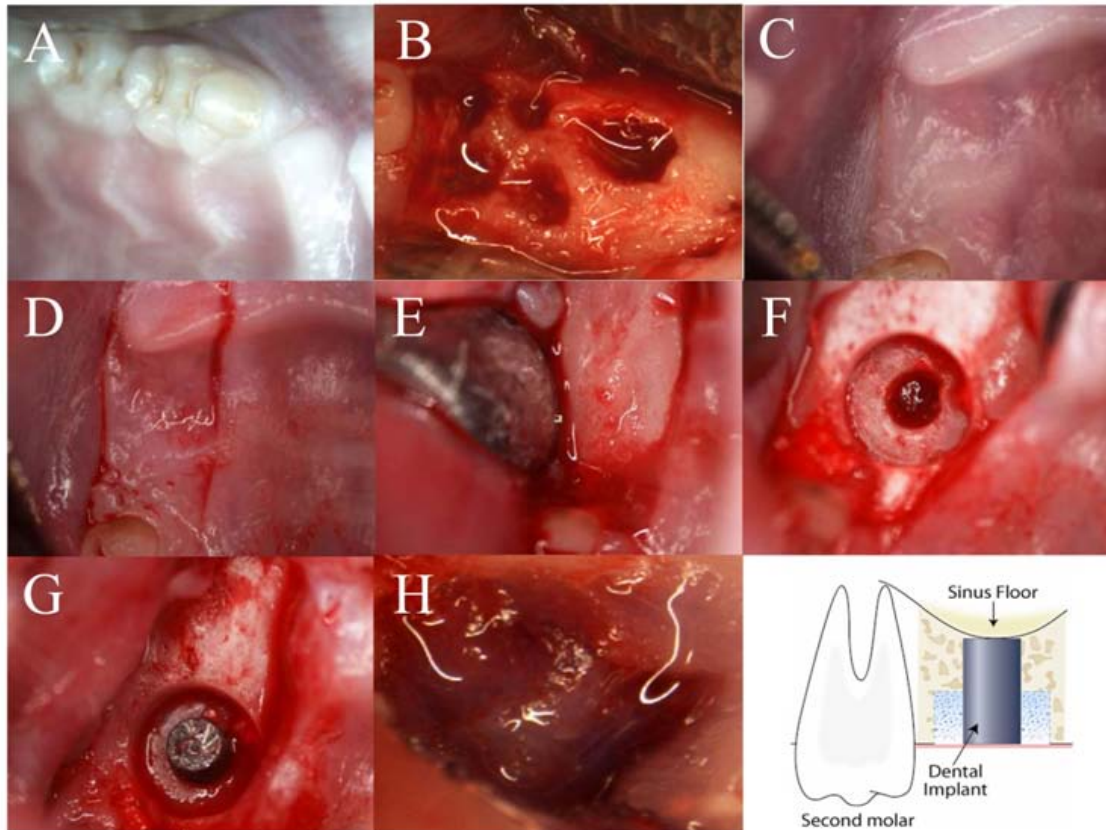
Figure 2-2 Surgical procedures of tooth extraction and titanium implant placement.
(A) Healthy maxillary first molar (M1) before extraction. (B) M1 was extracted, and five root sockets were clearly seen. (C) After 1 month, the mucosa and alveolar bone heal. (D) A full thickness flap was created from in the direction of palatal to buccal. (E) Alveolar bone was exposed. (F) An osteotomy was created using a custom step-drill. The drill-bit was designed with a 0.95-mm diameter, 1 mm long-apical portion and a 2.2-mm diameter, 1 mm long at the coronal aspect. (G) Implant was placed. (H) The flap was place back and glued. The scheme shows the position and size of the osteotomy. The apical part of the drill created an osteotomy for initial fixation and the coronal part of the drill created a circumferential osseous defect before dental implant installation.
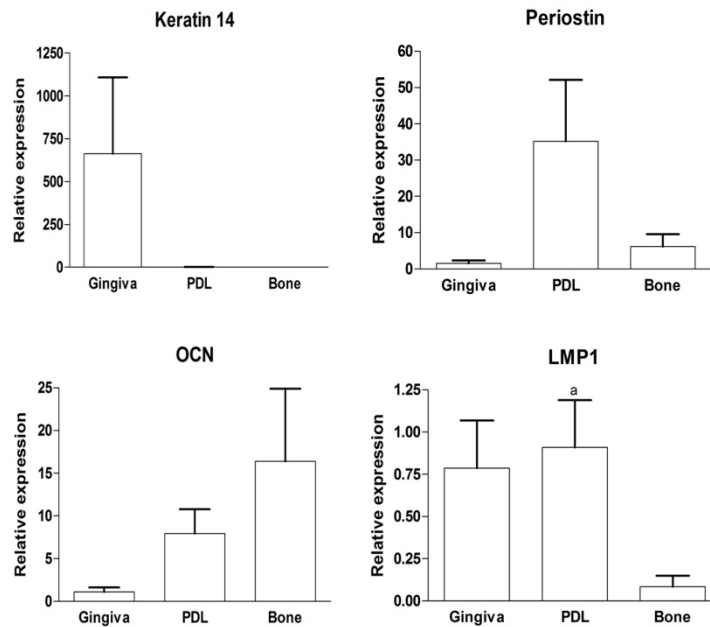
Figure 2-3. Gene expression pattern of LMP1 in healthy periodontium. LCM was utilized to collect gingiva, periodontal ligament, alveolar bone tissues from decalcified maxillae sections. RNA extraction and RT-PCR were performed to detect genes markers in different tissue. Keratin 14: an epithelial marker. OCN: osteocalcin, a primary bone marker. Periostin, a marker for ligature and PDL. Stronger LMP1 expression in periodontal ligament and gingiva compared to mature alveolar bone. a: $p<0.05$ compared to Bone. n=4.
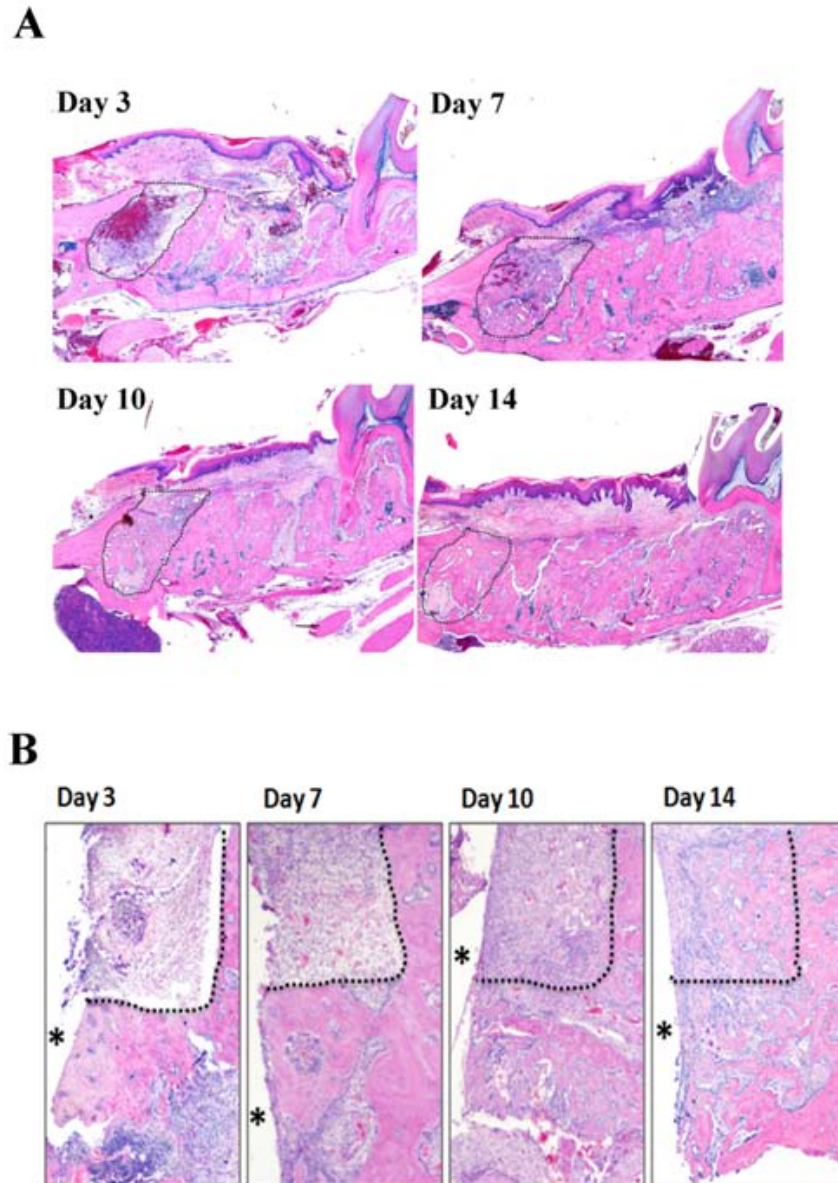
Figure 2-4 Histology view of the healing after tooth extraction (A) and osteotomy and implant placement (B). (A) H. & E. staining images of extraction socket 3, 7, 10, 14 days after surgery. (B) H. & E. staining images of osteotomy defect around implant. Implants were pushed out already. *: original implant sites. The dotted lines show the area which were dissected by LCM.

Group 1: Growth Factors
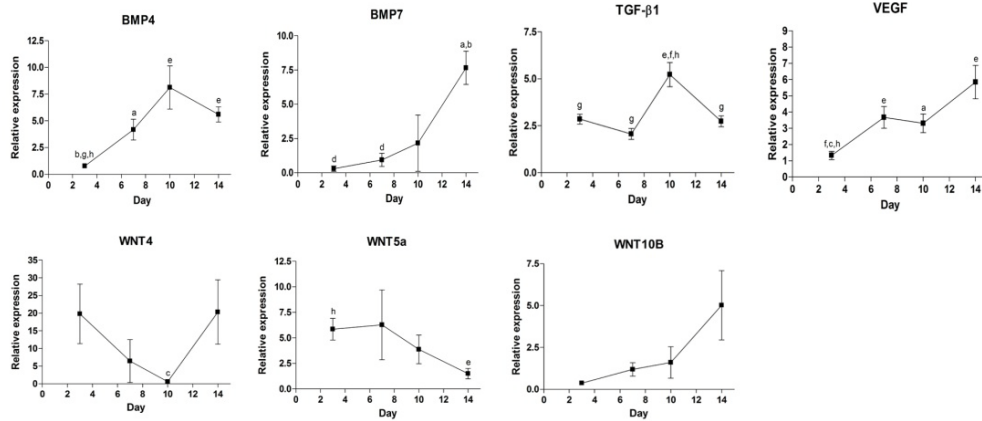
Group 2: Extracellular Matrix Proteins
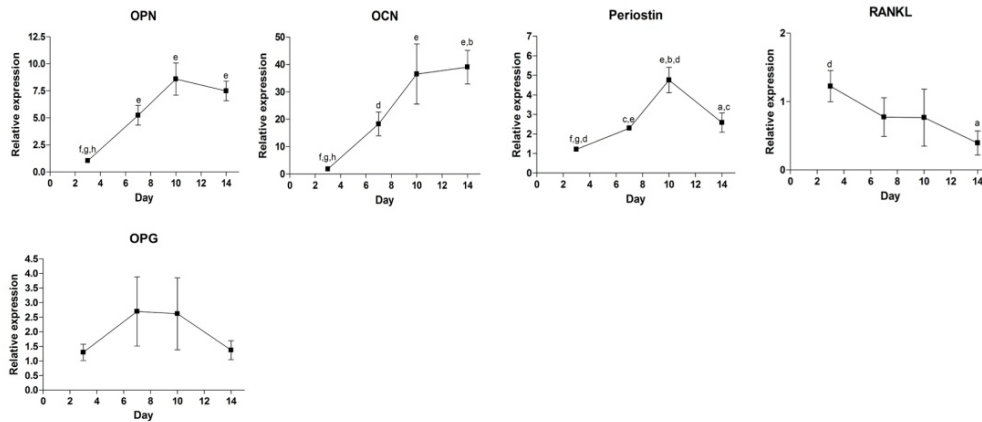
Group 3: Chemokines

Group 4: Transcriptional Factors and Scaffold Protein
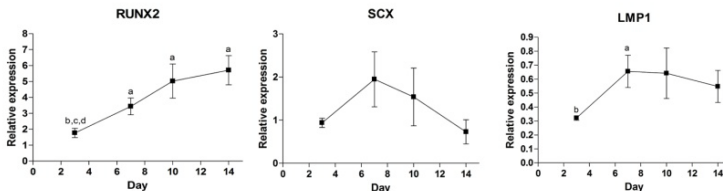
Figure 2-5 Gene expression profiles during tooth extraction socket healing. LCM was performed to dissect the extraction socket 3, 7, 10 and 14 days after tooth removal. qRT-PCR was used to analyze genes related to wound healing. Group 1: growth factors. Group 2: extracellular matrix proteins. Group 3: chemokines. Group 4: transcription factors and other intracellular scaffold protein. LMP1 expression was relatively low in

early stage (coagulation stage), and it was slightly increased at day 7, 10 and 14. a: $p<0.05$ compared to day 3; b: $p<0.05$ compared to day 7; c: $p<0.05$ compared to day 10; d: $p<0.05$ compared to day 14; e: $p<0.01$ compared to day 3; f: $p<0.01$ compared to day 7; g: $p<0.01$ compared to day 10; h: $p<0.01$ compared to day 14. n=4-6/time point.

Group 1: Growth Factors
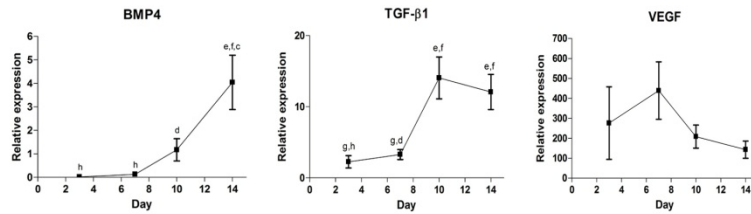
Group 2: Extracellular Matrix Proteins
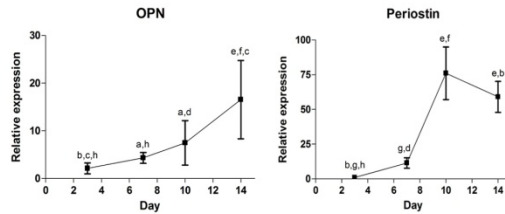
Group 3: Chemokines
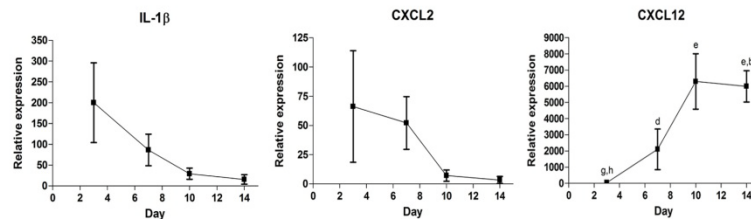
Group 4: Transcription Factors and Scaffold Protein
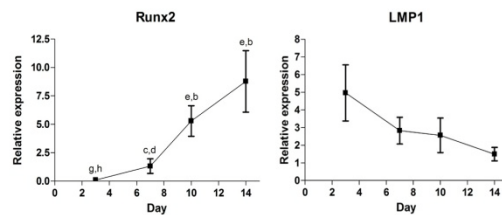
Figure 2-6 Gene expression profiles during healing in the osteotomy area around titanium implants. LCM was performed to dissect the defect area around implants. RT-PCR was used to analyze genes related to wound healing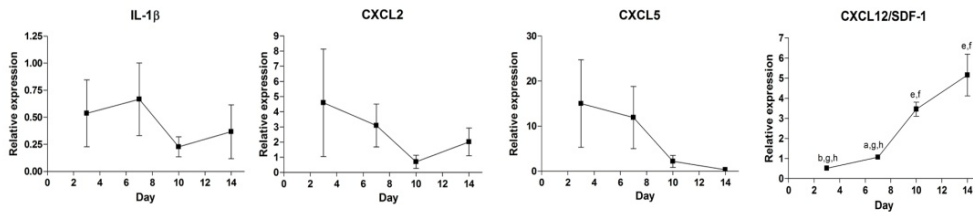. Group 1: growth factors. Group 2: extracellular matrix proteins. Group 3: chemokines. Group 4: transcription factors and other intracellular scaffold protein. *LMP1* gene expression gradually decreased during healing after osteotomy around implants. a: $p<0.05$ compared to day 3; b: $p<0.05$ compared to day 7; c: $p<0.05$ compared to day 10; d: $p<0.05$ compared to day 14; e: $p<0.01$ compared to day 3; f: $p<0.01$ compared to day 7; g: $p<0.01$ compared to day 10; h: $p<0.01$ compared to day 14. n=4-6/time point.
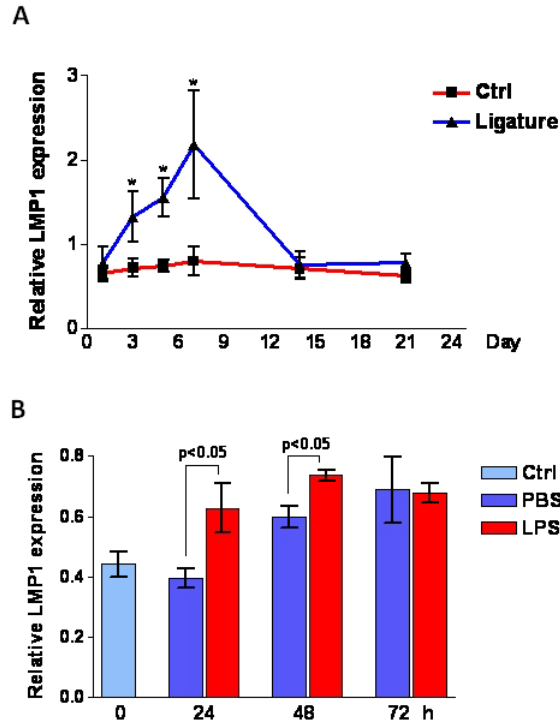
Figure 2-7 *LMP1* gene expression is up-regulated in experimental periodontitis. Two models were used to induce periodontal disease and bone loss in Sprague-Dawley rats. (A) Cotton ligatures were placed bilaterally into the gingival sulci of the mandibular first molar teeth. The use of ligatures elicits the rapid bone loss due to the inflammatory process induced by increased microbial biofilm formation around the cervix of the teeth and an acute physical irritation factor as a consequence of the subgingival placement of the ligature. (B) *Porphyromonas gingivalis*-lipopolysaccharide (*Pg*-LPS was injected into four palatal gingival tissue sites at the base of the interproximal gingival papillae between maxillary molars. Gingival tissue biopsies were harvested from a standardized region of the palatal region of the maxillary molar teeth. RT-PCR was performed to evaluated the LMP1 gene expression in these tissues.

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CHAPTER THREE

LMP1 REGULATES PERIODONTAL LIGAMENT PROGENITOR CELL PROLIFERATION AND DIFFERENTIATION

3.1 Abstract

LMP1 is an intracellular scaffold protein that contains a PDZ domain and three LIM domains. LMP1 has multiple functions including regulating mesenchymal stem cell (MSC) osteogenesis. Gene delivery of *LMP1* induces bone formation *in vivo* in heterotopic and orthotopic sites. However, little is known about the physiological function of *LMP1* in MSCs. Periodontal ligament (PDL) cells are a unique progenitor cell population that can differentiate into multiple cell types, including osteoblasts, adipocytes or chondrocytes. This study sought to determine the physiological function of *LMP1* in PDL cells. We show that *LMP1* is upregulated in early stage of PDL cell osteogenic differentiation. Stable gene knockdown of *LMP1* by shRNA inhibits DNA synthesis and corresponding cell proliferation in PDL cells, and further leads to decreased mineralization *in vitro*. Overexpression of LMP1 increases cell proliferation, and PDZ and ww-interacting domains are not enough to mediate this effect. We conclude that *LMP1 is* involved in PDL cell proliferation. Our findings advance the understanding of the physiological function of *LMP1* in PDL progenitor cells and other MSCs.

3.2 Introduction

The intracellular protein LMP1 (LIM domain mineralization protein) belongs to the PDLIM protein family, which consists of a PDZ domain in the N-terminus and three LIM domains at the C terminus [1, 2]. Increasing evidence suggests that *LMP1* regulates the osteogenesis program in MSCs. For example, overexpression of *LMP1* in bone marrow stromal stem cells, calvarial osteoblasts, and dermal fibroblasts initiates osteolineage differentiation *in vitro* [2-5]. Gene delivery of *LMP* induces efficient bone formation *in vivo* in heterotopic (subcutaneous and intramuscular) and orthotopic (spine fusion and bone fracture healing) sites [5-8]. Although the potential application of LMP1 in bone regenerative medicine, the physiological roles of *LMP1* in MSCs remain to be established. So far, *LMP1* knockout mice still haven't been developed, and *LMP1* knockdown in zebrafish is embryonically lethal [9].

Periodontal ligament (PDL) cells are a unique mesenchymal stem cell population that can differentiate into multiple cell types, such as osteoblasts, adipocytes, and neurons [10, 11]. The PDL cell is a promising cell source for periodontal hard and soft tissue regeneration [12, 13]. This study sought to determine the physiological function of *LMP1* in PDL cells proliferation and differentiation. We stably knocked down *LMP1* by shRNA. Gene knockdown of *LMP1* inhibits cell proliferation and DNA synthesis in PDL cells, and further impairs osteogenic differentiation. Overexpression of LMP1 in PDL cells stimulates proliferation, which is not dependent on its PDZ and ww-interacting domains. Our findings may help in the better understanding of the role of LMP1 in PDL cells proliferation and differentiation.

3.3 Results

LMP1 is upregulated in the early stage of osteogenic differentiation of PDL cells.

PDL cells are a mixed cell population from the tooth-supporting apparatus. It is well established that these cells can differentiate to multiple cell types [10], and we also confirmed that PDL cells from different patients can differentiate to osteoblasts and adipocytes. We next analyzed the gene expression of *LMP1* during osteogenic differentiation. As shown in Figure 3-1A, *LMP1* expression is upregulated at 3 d and decreases at later time points. The same pattern was seen at protein level as well (Figure 3-1B). This result reveals that LMP1 is involved in the early stage of osteogenic differentiation of PDL cells.

shRNA-mediated silencing of LMP1 impairs PDL cell proliferation.

In order to better understand the function of *LMP1* in PDL cells, we used RNAi technology to knock down *LMP1* gene expression *in vitro*. Two double-stranded shRNAs targeting *LMP1* and a scrambled shRNA were designed and cloned into a retroviral system. After retrovirus infection and puromycin selection, resistant clones were pooled. The LMP1 expression was verified at mRNA (Figure 3-2A) and protein (Figure 3-2B) levels. *LMP1* knockdown of PDL cells demonstrated lower proliferation rates when compared to controls. When we seeded the same number of cells in 12-well-plates and induced them towards osteolineage differentiation, *LMP1* knockdown cells demonstrated a slower proliferation rate compared to control (Figure 3-2C). At day 10, cells were fixed and stained with crystal violet staining and less staining was found in knockdown cells (Figure 3-2D, E). Consistent with this obervation, *LMP1* knockdown in PDL cells

inhibited DNA synthesis which was shown by ^{3}H methyl thymidine incorporation assay (Figure 3-2F). Since this effect can also be explained by the increase of apoptotic cells while LMP1 was knocked down, we tested the expression of an apoptosis marker Caspase-3. Caspase-3 is a critical executioner of both intrinsic and extrinsic apoptosis, as it is responsible for the proteolytic cleavage of many key factors involved in apoptosis [14]. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments [14]. There is no significant increase of cleaved caspase-3, which indicates that the LMP1 knockdown effect may be related to impaired proliferation (data not shown). Actually, by RT-qPCR, we confirmed that LMP1 knockdown resulted in less cell expression of Cyclin D1 and Cyclin B1 compared to control (Figure 3-2G). FACS analysis further showed that greater degree of LMP1 knockdown of cells were blocked at G1 phase compared to scrambled control cells (Table 1). Taken together, knockdown expression of LMP1 in PDL cells impairs cell proliferation.

LMP1 silencing delays osteogenic differentiation.

We further determined whether gene knockdown of *LMP1* affects osteogenic differentiation in PDL cells. Control shRNA showed similar levels of ALP staining and mineralization capability when compared to non-treatment controls (data not shown). However, less ALP positive cells were seen when *LMP1* was stably knocked down by shRNA, and the ALP activity was lower in knockdown cells (Figure 3-3A). Consistent with this, less mineralized nodules were observed in *LMP1* knockdown PDL cells at late stage of PDL osteogenic differentiation, which was shown by Alizarin Red staining and

extracellular measurement (Figure 3-3B). We further used RT-qPCR to examine several gene markers involved in PDL differentiation. We found that LMP1 knockdown of PDL cells demonstrated delayed expression of *Runx2* and *Osterix* (Figure 3-3C). *Collagen1A1 (Col1A1)* and *Bone sialoprotein (BSP)* tended to decrease in LMP1 knockdown cells as well. These results suggest that *LMP1* knockdown retards the early osteogenic differentiation of PDL cells *in vitro*.

PDZ and ww-interacting domains are not sufficient to stimulate cell proliferation

Our results suggest that LMP1 is required for PDL cell proliferation. To examine whether LMP1 transgene can enhance PDL cell proliferation, we stably overexpressed *LMP1* in PDL cells using a retroviral system. We also established stable PDL cell lines expressing a truncated form of LMP1 only containing the first 144 amino acid residues, which consists of PDZ domain and ww-interacting motifs, but not any LIM domain (Figure 3-4A). The antibody we used can recognize the N-terminus of LMP1, which made it possible to detect both forms in Western blot (Figure 3-4B). The full length LMP1 is about 50 kD, and the truncated form in about 16kD. As shown in Fig 4C, expression of the full length LMP1 significantly promoted PDL cell proliferation, however, the truncated form had limited effect. We didn't see significant cell death during cell culture. Consistent with this, there were no significant increase in the cleaved fragments of caspase-3, which are the active forms, in control and LMP-t PDL cells (data not shown). By ^{3}H methyl thymidine incorporation assay we further showed that DNA synthesis is upregulated in LMP1 overexpression PDL cells (Figure 3-4D). Therefore, our result suggests that PDZ and ww-interacting domains are not enough to stimulate the

mitotic effect. This is also supported by Durick *et al* reporting that LMP1 mediates the mitogenic signaling in mouse fibroblasts [15].

3.4 Discussion

Although it has been reported that *LMP1* plays a role in osteoblast differentiation [4, 16, 17], its physiological function remains unclear. Because LMP1 is highly expressed in periodontal ligament tissue and it is up-regulated at early stages of osteogenic differentiation in PDL cells, we further explore the possible function of LMP1 in PDL cells. By stable expression of two shRNAs in PDL cells, we observed that the proliferation and DNA synthesis capability decreased in *LMP1* knockdown PDL cells compared to non-target shRNA control. LMP1 knockdown appears to lead to longer G1 phase in PDL cells. On the other hand, using a "gain-of-function" strategy, we showed that LMP1 overexpression significantly promotes PDL cell proliferation. Consistent with this finding, Yoon *et al* showed that *LMP1* transfection induced mild but significant increased in DNA synthesis in intervertebral disc annulus cells [18]. These results suggest that LMP1 is necessary and sufficient for PDL cell proliferation.

It is not clear how LMP1 participates in cell proliferation. By the truncated mutation experiment, we found that PDZ and ww-interacting domains are not enough to induce the mitogenic effect of LMP1. This finding is consistent with the hypothesis that LMP1 exerts its function as a scaffold protein that mediates mitogenic signaling activated by growth factors. Durick *et al* showed that LMP1 mediates the mitogenic signaling by Ret/ptc2 in mouse 10T1/2 fibroblasts. LMP1 binds to Ret/ptc2 via its second LIM

domain and functions as an adaptor protein, with the PDZ domain of LMP1 anchoring the LMP1-Ret/ptc2 complex to the cell periphery [1, 15]. On the other side, overexpression of a truncated form of LMP1 without LIM domains inhibited the mitogenic effect of Ret/ptc2 [15]. In future study, it will be important to identify the binding partners of LMP1 in order to characterize its mechanism in PDL cell proliferation.

Osteogenesis is a complicated process that involves cell proliferation, differentiation and subsequent nodule formation and mineralization. Our data showed that LMP1 gene knockdown impairs PDL proliferation, and consequently the mineralization was delayed. This is consistent with the observation from Boden *et al* [2]. On the contrary, when stably overexpressed in PDL cells, LMP1 did not seem to promote mineralization nodule formation *in vitro* and bone formation *in vivo* (data not shown). Actually, constitutive expression of LMP1 tends to inhibit mineralization in PDL. More study should be done to further demonstrate why this happens. One possible explanation is that LMP1-overexpressing PDL cells tend to maintain in a proliferating stage, thus less cells will exit the cycle into differentiation.

Besides this role in osteogenesis, LMP1 might be involved in the adipocyte differentiation as well. It has been shown that LMP1 mRNA expression increases in adipose tissue of diabetic obese patients. LMP can bind to insulin receptor, and it also interacts with adaptor protein through PH and SH2 domains (APS) to control insulin-induced actin cytoskeleton remodeling and glucose transporter 4 translocation in

3T3-L1 adipocytes. In the future it will be interesting to explore the function of LMP1 in adiopogensis.

We conclude that *LMP1 is* required for PDL cell proliferation and osteolineage differentiation. With the limits of the lack of an *in vivo* LMP1 knockout model, our findings suggest a possible physiological function of *LMP1* in PDL progenitor cells.

3.5 Materials and methods

Cell culture

The isolation of human periodontal ligament (PDL) cells for these studies was approved by the University of Michigan Health Sciences Institutional Review Board. PDL cells were obtained from extracted third molar or premolar teeth of healthy patients and cultured in 100 mm tissue culture dishes in a DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin [10]. PDL cells from 5 patients (Age range 20-50 years) were pooled together and were used at passages 2 to 6.

Short Hairpin RNAs (shRNA) and Retroviral Infection and Constructs

Retrovirus-based shRNA knockdown system (pSIREN-RetroQ vector, from Clontech (Mountain view, CA) was utilized to stably knock down endogenous *LMP1* expression. Target sequences were selected with software available on the Dharmacon web sites. Oligonucleotides synthesized by Invitrogen (Carlsbad, CA) were annealed and subcloned into retroviral vectors at EcoRI and BamHI sites. The two target sequences to *LMP1* identified were: si1: 5'-gtttgagtttgctgtgaagtt-3' and si2: 5'-gcaagagccgagataaagcca-3'.

Non-target scramble shRNA sequence is: 5'-aaaaccgacggctatctct-3'. shRNA expression vectors were delivered into PDL cells using retroviral transduction according to the manufacturers directions. Briefly, PDL cells were transfected by retrovirus twice over 36 hours, with a 12 hour interval between infections. Next, puromycin (1μg/ml) was added for 3 days. Resistant clones were pooled together for subsequent experiments. At least 6 independent transfections had been performed and the efficiency and specificity of suppression by shRNAs were evaluated with analyses of protein and/or RNA levels as indicated.

LMP1 gene overexpression in PDL cells

Full length LMP1 gene was cloned from MG63 cells by RT-PCR, then was inserted into retrovirus vector pQC-XIN (Clotech, Mountain view, CA). A truncated form without any LIM domain was generated by PCR. After that, retrovirus production and transfection was performed following the similar protocol, and PDL cells were selected by G418 for 10 days.

[methyl-^{3}H]thymidine incorporation assay

PDL cells with stable shRNA expression were seeded in 12-well-plates with 1×10^4 cells per well. The next day, medium was changed to serum-free DMEM. After 24 h, 2×10^5 cpm (count per minute) [methyl-^{3}H]thymidine were added to each well. At day 5, the medium was removed and each well was washed twice with cold PBS. The DNA in each well was precipitated with 5% cold trichloroacetic acid at for 2 h 4 °C, solubilized with 1% SDS solution for 2 h at 55 °C, followed by measurement of [methyl-^{3}H]thymidine

radioactivity in the solution via a scintillation counter (Wallac 1410, Perkin-Elmer, Waltham, MA).

Flow cytometry

3×10^4 PDL cells cultured on 10cm dishes were washed with phosphate-buffered saline, trypsinized, and fixed in cold 70% ethanol for 0.5 h. Ethanol was removed by centrifugation, and the pellets were resuspended in 1 ml of phosphate-buffered saline (PBS) containing 50 µg/ml propidium iodide and RNAse A (10 ug/ml) and incubated for 30 min at 37 °C before FACS analysis.

Cell Lysates and Immunoblotting

Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). SDS-PAGE gels were run and transferred to PVDF membranes (Bio-Rad, Richmond, CA). After blotting, the membranes were incubated overnight with primary antibodies and appropriate secondary antibodies (anti mouse IgG or anti rabbit IgG, Amersham, Buckinghamshire, UK) for 1 h. The membranes were washed and visualized by an ECL chemiluminescence detection kit (Amersham). Monoclonal antibody for LMP1 (1:1000) was from Abcam and monoclonal antibody for alpha-tubulin (1:1000) was from Sigma-Aldrich.

RNAi

For PDL cells or hBMSCs RNAi experiments, cells were seeded in 6-well-plates at 2×10^5 cells per well, and transfected with 100 nM siRNA for 72 h in serum-free and

antibiotic-free DMEM. Next, media were changed and cells were stimulated with or without TGF-β1. siGENOME SMARTpool siRNA targeting *Smad2, Smad4 and TAK1*, and scramble control siRNA were purchased from Dharmacon.

Quantitative RT-PCR

Total RNA samples were extracted with RNAeasy Mini kit (Qiagen, Maryland) according to the manufacture's instruction. 1 ug RNA was subjected to reverse transcription in a 50μl RT reaction using TaqMan Reverse transcription reagents (Applied Biosystems, Foster city, CA). cDNA was generated using random hexamer primers and oligo-T primers with 2:1 ratio). For quantitative real-time PCR, the generated cDNA was analyzed, in triplicate, with the Master Mix (Applied Biosystems) in the ABI7500 Sequence Detection System. The results were normalized with 18s transcript. The primers and probes were ordered from Applied Biosystem. The probe sequences were: 18S: Hs99999901_s1, TCCATTGGAGGGCAAGTCTGGTGCC; *LMP1*: Hs01103928_g1, CAAACCGCAGAAGGCCTCCGCCCCC.

Determination of cell number by crystal violet staining

$3x10^3/cm^2$ PDL cells were seeded in 12-well plates in triplicate with osteogenic induction media. Media were changed every 3 days. Two weeks later, the cells were fixed with ice-cold methanol for 10 minutes. After PBS washing, 0.5% crystal violet solution was added for 10 minutes. Crystal violet was removed and the plates were washed carefully with water 5 times. Photographs were taken using a Nikon digital camera. For crystal violet quantification, Sorenson's buffer (0.1 M sodium citrate, 50% ethanol, 50%

H$_2$O) was used to extract the dye and further measured using a spectrometer (Beckman Coulter, Mason, MI) at A540. The optical density readout is positive correlated to cell numbers.

In vitro mineralization assay

PDL cells with stably expressed shRNAs were seeded in 6-well plates in triplicate at the density of $3 \times 10^3/cm^2$. In order to induce PDL cells to mineralize, 50 ug/ml ascorbic acid, 5 mM beta-glycerol phosphate, and 10^{-8}M dexamethasone were added to the medium for 2-3 weeks. Alkaline phosphatase (ALP) staining was performed as previous described [14]. Matrix mineralization was evaluated by Alizarin Red staining and Von Kossa staining.

Statistical analysis

All data are presented as the mean ± SD. The significance of the differences was determined by using the two-tailed Student's t-test and one-way ANOVA. In each figure, representative results from 2-3 repeated independent experiments were shown.

3.6 Figures

A

B
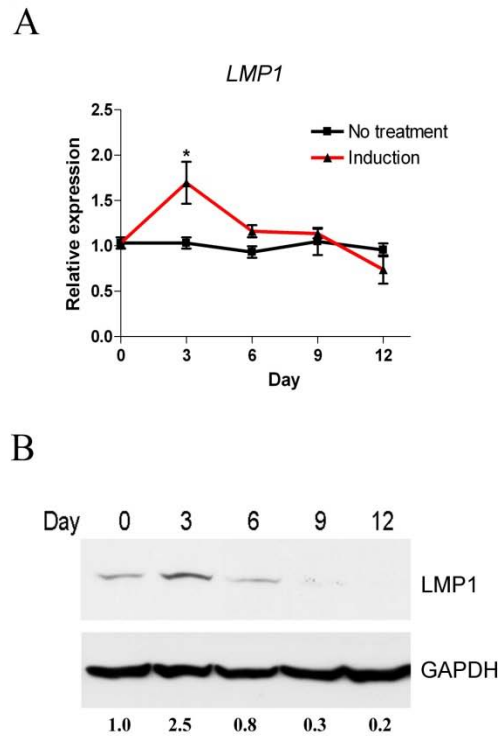
Figure 3-1 LMP1 is upregulated at early stage of osteogenesis in PDL cells. Primary PDL cells were induced for osteogenic differentiation. (A) RT-qPCR was used to evaluate *LMP1* gene expression. A representative result of three independent experiments is shown. **, $p<0.01$; *, $p<0.05$ *vs.* non-induced control. (B) The LMP1 protein expression is shown by Western Blot. Relative expression ratios after normalization to GAPDH are shown at the bottom.
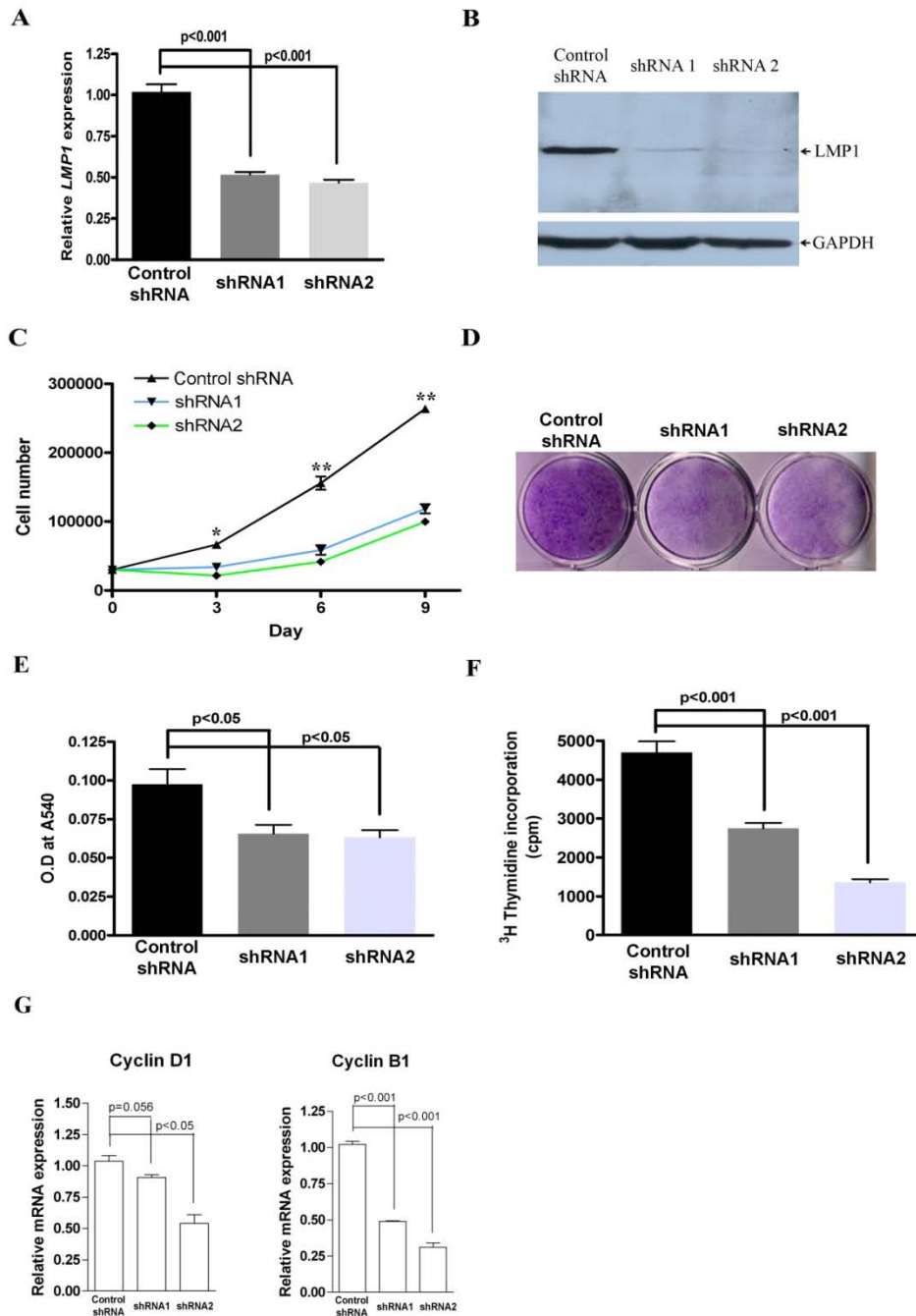
Figure 3-2 LMP1 is required for PDL cell proliferation. Two double-stranded shRNAs targeting *LMP1* and a scramble shRNA were designed and cloned into a retroviral system. After retrovirus infection and puromycin selection, all the survival cells were pooled. *LMP1* expression was evaluated by RT-qPCR (A) and Western Blot (B). (C) *LMP1* stably knocked down and control PDL cells were seeded in 6-well-plates at low density (3 x 10^3/cm^2). Osteogenic media were added to the cells, and media were changed every 3-4 days. At day 3, 6, and 9, cells were harvested and counted by hemocytometry, n=6 per

group. (D) At day 10, cells were fixed and stained with crystal violet. (E) Subsequently, the crystal violet staining was washed and quantified. The optical density readout which correlates to cell numbers are shown. (F) *LMP1* stably knocked down and control PDL cells were seeded in 12-well-plate at 3 x 10^3/cm^2 and cultured in osteogenic induction media. ^{3}H methyl thymidine was added after overnight attachment. At 5 d, the DNA was harvested and the ^{3}H methyl thymidine incorporation was measured by scintillation counter. (n=4 per group). (G) PDL cells were cultured in 6-well-plates in serum free medium. 10% FBS was added, and RT-qPCR was used to examine the expression of Cyclin D1 and Cyclin B1 (n=3 per group).
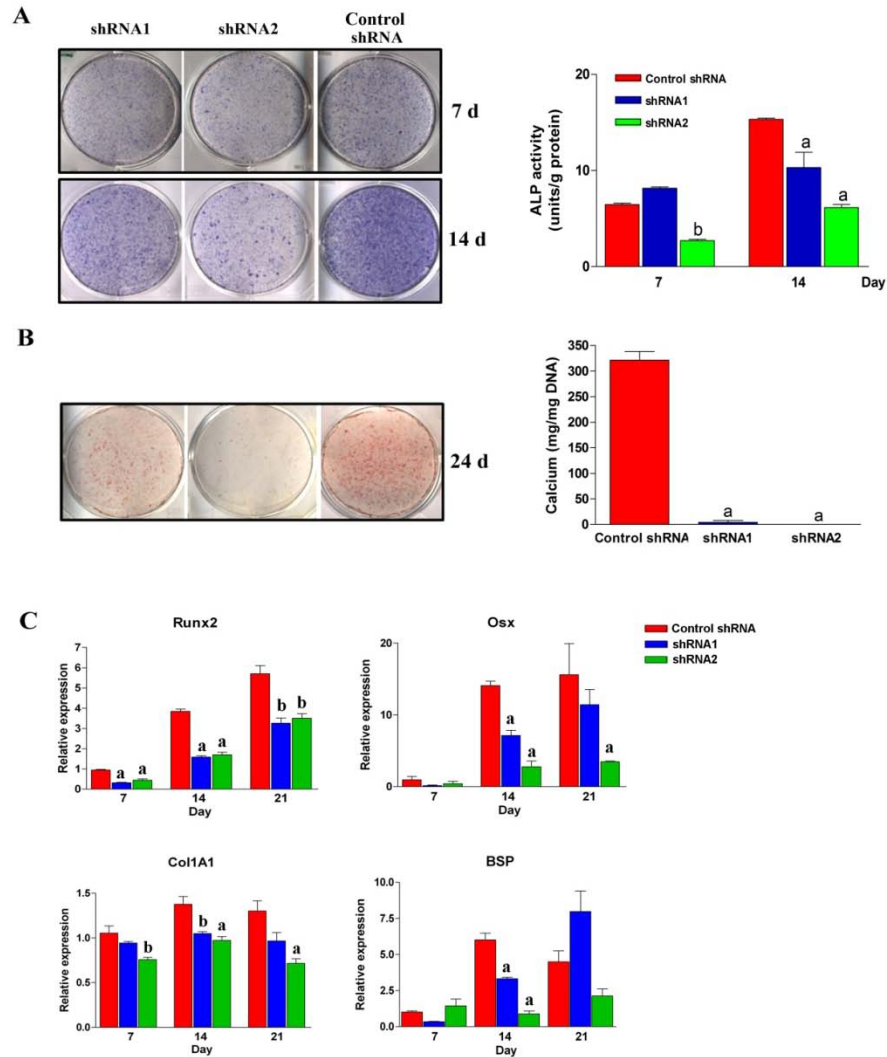
Figure 3-3 LMP1 silencing decreases osteogenic differentiation in PDL cells. *LMP1* stably knocked down and control PDL cells were seeded in 12-well-plates at low density $(3 \times 10^3/cm^2)$. Osteogenic medium was added to the cells, and media were changed every 3-4 days. (A) At indicated time points, ALP activity was measured by ALP staining (left panel), and quantified assay (right panel). (B) Mineralization was assessed by Alizarin Red staining (left), and extracellular calcium concentration was quantified (right). (C) RT-qPCR was performed at d 7, 14, and 21 to evaluate the gene expression of several gene markers. a: $p<0.01$ compared to scramble shRNA in the same time point; b: $p<0.05$ compared to scramble shRNA in the same time point. n=3 per group.
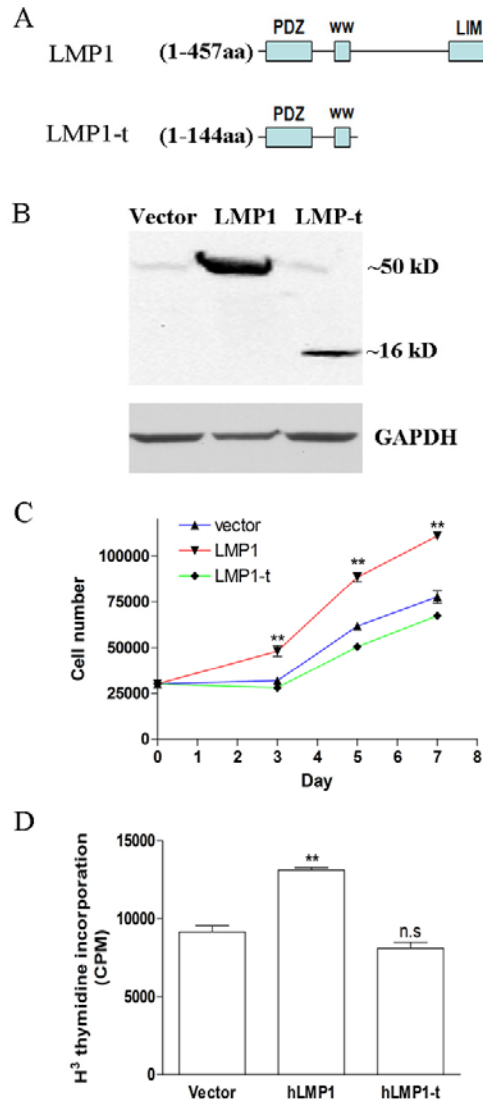
Figure 3-4 PDZ and ww-interacting domains are not sufficient to induce PDL cell proliferation. Full length LMP1 cDNA and a truncated form without any LIM domain were constructed into retroviral expression vector. PDL cells were transfected by retrovirus and selected by G418 for 10 days. Survived cells were pooled for the following experiments. Stable cell lines overexpressing LMP1 and LMP1-t were established in PDL cells from two different individuals. Representative data from 1 patient were shown here. (A) Truncated LMP1 only contains the first 144 aa including PDZ and ww interacting domains. (B) Endogenous and exogenous LMP1 proteins were detected by western blot. This antibody can detect the truncated form LMP1-t as well. (C) PDL cells were seeded in 6-well-plates at low density (3 x 10^3/cm^2). Cells were harvested by trypsin and counted using hemocytometry, n=6 per group. (D) PDL cells were seeded in 6-well-plate at 3 x 10^3/cm^2 and ^{3}H methyl thymidine was added. At 5 d, the DNA was harvested and the ^{3}H methyl thymidine incorporation was measured by scintillation counter. (n=4 per group).

3.7 Table

Table 3-1: FACS analysis for cell cycle of PDL cells following *LMP1* gene knockdown

	G1	S	G2/M
Control shRNA	$36.17 \pm 1.41^{a,b}$	$45.28 \pm 1.37^{a,b}$	$18.54 \pm 0.75^{a,b}$
shRNA 1	58.77 ± 1.16	30.07 ± 1.96	11.15 ± 0.89
shRNA 2	44.24 ± 3.51	28.55 ± 1.80	27.02 ± 2.24

PDL cells were cultured in 10 cm petri dishes in serum free medium overnight. 10% FBS was added for 24h. Cells were fixed and stained by PI, analyzing by FACS. n=4 per group. a: $p<0.01$, compared to -shRNA 1; b: $p<0.01$, compared to -shRNA 2

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CHAPTER FOUR

THE POTENTIAL OF LMP GENE THERAPY IN PROMOTING
MINERALIZATION *IN VITRO* AND *IN VIVO*

4.1 Abstract

Gene transfer of key regulators of osteogenesis for mesenchymal stem cells (MSCs) represents a promising strategy to regenerate bone. The intracellular protein LMP1 (LIM domain mineralization protein) and a transcription variant LMP3 which is lack of any LIM domain are reported to induce osteogenesis both *in vitro* and *in vivo*. However, little is known about the effect of LMP gene therapy on periodontal ligament (PDL) cell osteogenic differentiation. This study sought to explore whether gene delivery of LMP1 and LMP3 promotes PDL cell mineralization and bone formation. We found that stably overexpressed LMP1 in PDL cell by retroviral vector delayed mineralization. AdLMP3 treatment, but not AdLMP1, induced significant matrix mineralization in PDL cells and hBMSCs. This effect was related to upregulated ALP and BSP gene expression. However, PDL cell transduced with AdLMP3 alone failed to induce ectopic bone formation *in vivo*. AdLMP1 and AdBMP7 combinatory gene therapy led to increased bone formation than AdBMP7 treatment alone. Future study will be needed to characterize inhibitory factors preventing the osteogenic effect of LMP3 in PDL cell under *in vivo* situations.

4.2 Introduction

Gene transfer of key regulators of osteogenesis for mesenchymal stem cells (MSCs) represents a promising strategy to regenerate bone. The intracellular protein LMP1 (LIM domain mineralization protein) belongs to the PDLIM protein family [1, 2]. At least three transcript variants of LMP exist in humans: LMP1, 2, and 3 [3]. LMP1 is the longest, encoding a 457 AA protein. LMP-2 has a 119-base deletion between +325 and +444 and a 17-base insertion at +444. The resulting derived protein contains 423 AA with the LIM domains intact. Human LMP-3 has the same 17 nucleotide insertion at +444, resulting in a shift in the reading frame that causes an early stop codon which encodes a 153 AA protein without LIM domains [3]. It has been reported that *LMP1* and *LMP3,* but not *LMP2* promote the osteogenesis program in MSCs. Overexpression of *LMP1/3* in bone marrow stromal stem cells, calvarial osteoblasts, and dermal fibroblasts initiates osteolineage differentiation *in vitro* [2-5].

LMP gene delivery has been shown to promote bone formation in heterotopic (subcutaneous and intramuscular) and orthotopic (spineal fusion) sites. Intramuscular injection of AdLMP3 induced ectopic bone formation, which was shown by radiography and Von Kossa staining in tissue sections [5]. AdLMP3 transduced dermal fibroblasts formed ectopic bone in HA-collagen gel carriers. The same group also used AdLMP3 to repair critical size bone defects in rat mandibular ramus [6]. *LMP1* gene delivery has been shown to induce new bone formation in a spine fusion model [7]. Bone marrow-derived buffy-coat cells from rabbits were transduced by AdLMP1 adenovirus, and then the cells were implanted during posterolateral arthrodesis. The results showed that LMP1 induces new bone formation and solid spine fusion [7].

74

So far two theories have been proposed regarding the mechanism underlying the osteogenic effect of LMP1/3. First, LMP1/3 increases cell responsiveness to BMP signaling by preventing Smad degradation. Motifs directly interacting with the *ww* domain of SMURF1 were identified in LMP1 and LMP3. SMURF1 is a negative regulator in BMP signaling through mediating SMAD proteins degradation. The interaction between LMP1/3 and SMURF1 leads to SMAD1/5/8 accumulation in the cytoplasm, therefore the signaling effect induced by BMPs will be prolonged [8, 9]. Small peptides containing this motif can mimic the ability to block Smurf1 from binding to Smads *in vitro*. Second, LMP1/3 may be directly involved in the transcriptional regulation of osteogenesis. It was reported that BMP-2, BMP-4, BMP-6, BMP-7, and TGF-β1 expression was elevated in LMP1-overexpressing cells [10, 11].

Based on the potential of LMP in bone regeneration medicine, in this study, we hypothesized that LMP gene therapy will stimulate the osteogenic differentiation of periodontal ligament (PDL) cells, and promote in vivo bone formation. Using human PDL cells and bone marrow stromal cells (BMSC), we will compare the effect of LMP1 and LMP3 in these two different mesenchymal progenitors.

4.3 Results

LMP1 overexpression in PDL cell fails to induce mineralization *in vitro*

We first tested whether *LMP1* gene delivery promotes PDL cell osteogenic differentiation *in vitro.* We stably overexpressed *LMP1* in human PDL cells by retroviral vector, and the exogenous LMP1 expression was confirmed by Western blot analysis

(Figure 4-1A). After that, PDL cells were transfected into osteogenic medium. Compared with vector control, less ALP staining positive cells were seen in *LMP1*-overexpressed PDL cells, and less mineralization was shown at the late stage (Figure 4-1B). We further tested whether LMP1 has direct effects on the late stage of mineralization by seeding PDL cells with very high cell density. High cell density resulted in rapid *in vitro* mineralization in control cells, however, little mineralization was seen in stable *LMP1*-overexpressing PDL cells (Figure 4-1C). By using adenoviral vector, LMP1 was expressed in PDL cells as well. Similarly, we found that AdLMP1 had limited effect on promoting PDL cell mineralization compared to AdLacZ (Figure 4-1D).

LMP3 stimulates strong mineralization in PDL in vitro

The effect of LMP3 on *in vitro* PDL cell osteogenic differentiation was also examined. Cells were transduced with a LMP3-expressing adenovirus (AdLMP3). As examined by Western blot analysis, AdLMP3 induced reasonable amount of protein expression at MOI 200 (Figure 4-2A). Increased MOI resulted in stronger ectopic protein expression, however, the toxic effect was stronger as well (data not shown). So MOI 200 was the highest dose we used in our experiments. AdLMP3 gene transduction in PDL cells induced dramatic ALP expression at 1 week (Figure 4-2B). Compared with AdGFP control, the matrix mineralization also significantly increased after AdLMP3 treatment, which was shown by Von Kossa staining (Figure 4-2C). We also test the expression of some genes related to osteogenesis. Interestingly, we found that AdLMP3 treatment induce significant increase in ALP and BSP gene expression. However, Runx2, OCN, and Col1A1 were inhibited by both AdGPF and AdLMP3 adenovirus. Although 200

MOI is almost the minimal dosage for us to detect reasonable exogenous LMP3 protein in Western blot, considering the possible viral toxicity, we repeated the mineralization assay by using low MOI such as 50 and 100. At 100 MOI, AdLMP3 induced ALP activity and mineralization, but very limited effect was seen at 50 MOI (data not shown).

LMP3, but not LMP1 stimulated matrix mineralization in BMSC in vitro

We also examined the effect of LMP1 and LMP3 gene delivery in the osteogenic differentiation of human BMSC. A specific population mesenchymal stem cell with strong osteogenic and angiogenic capabilities was tranduced by AdLMP1, AdLMP3 and control adenovirus. As shown in Fig 3A, MOI 200 induced reasonable expression of LMP1 and LMP3. Like the results with PDL cells, we found that expression of LMP3 significantly upregulated matrix mineralization in vitro, compared with cells transduced with AdGFP and AdLacZ (Figure 4-3B, C).

AdLMP1 and AdLMP3 gene delivery alone are not able to induce ectopic bone formation ex vivo

Because of the promising *in vitro* result regarding the effect of LMP3 in PDL cell and BMSC osteogenic differentiation, we further examined whether AdLMP3 can induce bone formation. PDL cells were transduced with AdLMP3, AdLMP1, and negative control adenovirus AdGFP. We also chose AdBMP7 and AdRunx2 as positive controls to mimic two possible mechanisms which LMP3 may use to stimulate bone formation. After adenovirus transfection, PDL cells were subcutaneously transplanted into immunocompromised mice. 4 weeks later, AdBMP7 and AdRunx2 treatment resulted in

solid ossicles, which were confirmed by X-ray (Figure 4-4A) and micro-CT (data not shown). Newly formed bone with bone marrow were clearly shown in von Kossa staining in samples that received AdBMP7 and AdRunx2 treatment (Figure 4-5B). This result suggested that PDL cells transduced with AdBMP7 and AdRunx2 were able to induce new bone formation in our model. However, both AdLMP3 and AdLMP1 transduced PDL cells showed no sign of mineralization *in vivo.* Similar results were observed in low dose or high dose gene transduction, from MOI 50-200 (data not shown) . Similar results were also obtained at shorter (2 week) and longer time points (8 week) (data not shown). In order to rule out the possible effect related to different scaffolds, we tested both PLGA and type I collagen scaffolds. PDL cells and BMSCs transduced by AdLMP1 or AdLMP3 alone failed to promote ectopic bone formation in both scaffolds (Figure 4-4A, Figure 4-5A, C). Since the differentiation stage of the cell due to the carrier may be a critical issue, two different strategies were used in order to promote more mature PDL cells before transplantation: 1) PDL cells were transfected with AdLMP3 and then treated with osteogenic media for 7 days before transplation; 2) PDL cells were treated with osteogenic media for 7 days and then transfected with AdLMP3 before transplantation.. However, AdLMP3 gene delivery using either strategy failed to induce bone formation (data not shown). In summary, AdLMP3 alone does not appear to be able to induce ectopic bone formation by PDL cells *in vivo*.

AdLMP1 and AdLM3 transduced BMSCs and ectopic bone formation

We also examined whether LMP gene delivery to BMSCs can stimulate ectopic bone formation. BMSCs were transfected with adenovirus expressing LMP1 or LMP3, then

cells were transplanted into immunocompromised mice by PLGA and collagen carriers. We found that AdLMP1 or AdLMP3 alone was not able to induce bone formation. However, BMSCs transfected by AdBMP7 stimulates significant bone formation.

AdLMP1/3 and AdBMP7 combinatorial gene therapy showed limited effect to promote in vivo bone formation.

Since LMP1/3 may work cooperatively to amplify BMP signaling, we determined if it is possible that BMP expression is prerequisite for LMP gene therapy. Therefore, we co-tranduced PDL cells with AdBMP7 and AdLMP3, or AdBMP7 and AdLMP1. Using type I collagen scaffolds, BMSCs were subcutaneously implanted into immunocompromised animals. AdBMP7 induced minimal ectopic bone formation at low dose (MOI 50), whereas significant new bone formation were seen in high dose (MOI 200) (data not shown). Combined with AdLMP1, low dose AdBMP7 induced more new bone formation than AdBMP7 alone, which was shown radiographically. Using 3-D μCT, we further confirmed the synergistic effect of LMP1 and BMP7. Cotransfection of AdLMP3 and AdBMP7 showed similar trends. We also tested whether cotransfection of the combinatory gene therapy in PDL cells can promote bone formation. Similar synergistic effects were observed between BMP7 and LMP1 although the data was not statistically significant due to our small sample number. (Figure 4-5).

4.4 Discussion

Although it was reported that LMP1 can stimulate the ostegenic differentiation of rat primary calvarial osteoblasts and buffy coat cells [3, 7, 10], to our surprise, AdLMP1 was

not able to induce mineralization *in vitro* and *in vivo* in our hands, neither in PDL cells nor hBMSC. Similar findings were also seen in a retroviral gene delivery system, in which full length human LMP1 were stably expressed in PDL cells. Constitutive expression of LMP1 failed to induce the osteogenic differentiation of PDL cells as well. One possible explanation is that the LMP1 effect is very cell type specific. Another possibility is that LMP1 only works in a small dosage range and in a transient expression pattern. High dose, sustained LMP1 expression is inhibitory to osteogenic differentiation. This is supported by the fact that LMP1 only increases at the early stage of PDL cell differentiation, and rapid drops down to and even lower than basal level. In our gene expression pattern study, the expression level of LMP1 in mature mineralized tissue is far lower than PDL and gingival tissues. A more controllable "switchable" gene delivery system may be helpful to elucidate these possibilities.

In our study, we found that AdLMP3 is a positive regulator of *in vitro* matrix mineralization, both in human PDL cells and a specific population of human BMSC. Using retroviral a gene delivery strategy, we also showed that a truncated LMP1 without any LIM domains, which was highly similar to LMP3, demonstrated the capability to promote *in vitro* mineralization in PDL cells from curtain patients. This result was consistent with the observation by Pola *et al.* that AdLMP3 gene therapy induced osteogenic differentiation in dermal fibroblasts, embryonic fibroblasts (NIH3T3), and human BMSCs [5, 6]. We also found that AdLMP3 gene delivery significantly induced ALP and BSP gene expression in PDL cell. However, some other genes related to osteogenesis, such as Runx2 and OCN, didn't show significantly increase because of the

possible toxic effect of adenovirus. In order to further characterize whether the increased ALP expression was specifically related to LMP3, we measured ALP activity after adenovirus treatments at different dosages. Control viruses inhibited ALP activity at high dosages. However, AdLMP3 induced ALP activity with a positive correlated manner (data not shown).

In spite of its potent effect on mineralization *in vitro*, AdLMP3 failed to induce ectopic bone formation *in vivo* in our model. This result was inconsistent with the literature [5, 6]. Several possible explanations to our data set are listed here: 1) difference in cell delivery carrier. Other published work used Hydroxyapatite (HA)-collagen to deliver cells. However, we used either PLGA or collagen because these materials are transparent to x-rays. It is possible that osteoinductive HA is critical for the AdLMP3 effect *in vivo*. 2) We used the subcutaneous implantation model because we considered this to be a logical model to evaluate ectopic bone formation [12-16]. However, the subcutaneous condition is very different from actual bone formation circumstance because of lack of related growth factors and extracellular signals. Although potent osteogenic factors such as AdBMP7 and AdRunx2 were able to induce new subcutaneously bone formation, less effective molecules may need more stringent conditions. Gene delivery in an *in situ* bone defect model may help clarify this issue. 3) Immunocompromised mice were used in our experiments because of the xeno-immune reaction between human cells and hosts. However this will compromise the AdLMP3 effect on ectopic bone formation. In the literature, AdLMP3 was reported to successfully induced bone formation in autologous transplantations in immunocompetent animals. 4)

It is possible that the effect of AdLMP3 is cell type sensitive. PDL cell may not respond to AdLMP3 treatment in vivo.

AdBMP7 and AdLMP1 combinatory gene therapy resulted in more bone formation than AdBMP7 treatment alone. Thus far, it is not clear how LMP1 and BMP7 work together to promote bone formation. One possible mechanism is that LMP1 binds to Smurf1 and subsequently prevents Smad degradation. Thus, LMP1 prolongs the BMP signaling. If this is the case, it will be very important to identify the binding motifs which will be a potential pharmaceutical target for bone regeneration. Another possible mechanism is that LMP1 stimulates osteogenesis under high BMP7 levels. It would be important to identify the fate of the transplanted cells with LMP1 and BMP7 co-transfection.

In summary, AdLMP3 gene delivery, but not AdLMP1, induced significant matrix mineralization in PDL cells and hBMSCs. This effect is related to upregulated ALP and BSP gene expression. However, PDL cell transduced with AdLMP3 alone couldn't induce ectopic bone formation in vivo. We also found that AdLMP1 gene transfection promotes bone formation synergistically with AdBMP7. Future study will be needed to characterize the mechanisms underlying this synergistic effect.

4.5 Materials and methods

Cell culture

The isolation of human periodontal ligament (PDL) cells and human bone marrow stromal cells (BMSC) was approved by the University of Michigan Health Sciences Institutional Review Board. PDL cells were obtained from extracted third molar or premolar teeth of healthy patients and cultured in 100 mm tissue culture dishes in a DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin [17]. PDL cells from 5 patients (Age range 20-50 years) were pooled together and were used at passages 2 to 6. Human BMSC cells were extracted from iliac crest and expanded in a bioreactor. MG63 osteosarcoma cells were maintained in DMEM with 10% FBS.

Gene transduction

Full length cDNA of rat LMP1 was subcloned from osteosarcoma cell line ROS and inserted into an adenoviral vector. cGMP (current the Good Manufacturing Practice Regulations promulgated by the US Food and Drug Administration) grade adenoviral vectors for LMP1 was generated by University of Michigan Vector Core Laboratory (Ann Arbor, MI, USA). AdLMP3 and AdGFP were provided by Dr. Paul Robbins from the University of Pittsburgh. AdLMP1 was Ad-lacZ and AdBMP7 were purchased from the University of Michigan Vector Core Laboratory. AdRunx2 was kindly provided by Dr. Renny Franceshi. For *in vitro* transduction of cells, adenovirus was added to cells in serum-free medium. The next day, medium was removed and osteogenic medium was added with 10% FBS, 50 µg/ml ascorbic acid and 5 mM β-glycerol phosphate.

LMP1 gene overexpression in PDL cells

Full length LMP1 gene was cloned from MG63 cells by RT-PCR, then was inserted into retrovirus vector pQC-XIN (Clotech, Mountain view, CA). After that, retrovirus production and transfection was performed following the similar protocol described in Chapter 3, and PDL cells were selected by G418 for 10 days.

Quantitative RT-PCR

Total RNA samples were extracted with RNAeasy Mini kit (Qiagen, Maryland) according to the manufacture's instruction. 1 ug RNA was subjected to reverse transcription in a 50μl RT reaction using TaqMan Reverse transcription reagents (Applied Biosystems, Foster city, CA). cDNA was generated using random hexamer primers and oligo-T primers with 2:1 ratio). For quantitative real-time PCR, the generated cDNA was analyzed, in triplicate, with the Master Mix (Applied Biosystems) in the ABI7500 Sequence Detection System. The results were normalized with 18s transcript. The primers and probes were ordered from Applied Biosystem.

In vitro mineralization assay

PDL cells with stably expressed shRNAs were seeded in 6-well plates in triplicate at the density of $3x10^3/cm^2$. In order to induce PDL cells to mineralize, 50 ug/ml ascorbic acid, 5 mM beta-glycerol phosphate, and $10^{-8}M$ dexamethasone were added to the medium for 2-3 weeks. Matrix mineralization was evaluated by alizarin red staining and Alizarin Red staining.

Cell Lysates and Immunoblotting

Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). SDS-PAGE gels were run and transferred to PVDF membranes (Bio-Rad, Richmond, CA). After blotting, the membranes were incubated overnight with primary antibodies and appropriate secondary antibodies (anti mouse IgG or anti rabbit IgG, Amersham, Buckinghamshire, UK) for 1 h. The membranes were washed and visualized by an ECL chemiluminescence detection kit (Amersham). Monoclonal antibody for LMP1 (1:1000) was from Abcam and monoclonal antibody for alpha-tubulin (1:1000) was from Sigma-Aldrich.

PLGA scaffold fabrication

We processed poly (dl-lactic-co-glycolic acid: 85:15) (PLGA) 3D scaffolds into porous foams by an established solvent-casting, particulate-leaching technique as previously described [18]. The resultant PLGA blocks were scaffolds containing 95% porosity and pore sizes in the range of 250–425 µm. These composites were cut into 5x5x2 mm blocks, sterilized with UV light, and stored until use.

Cell seeding in PLGA scaffold

Sterile PLGA blocks were incubated overnight in DMEM supplemented with 10% FBS, 100 units/mL penicillin–streptomycin, and 2 mM glutamine at 37°C. Before use, the medium in PLGA blocks was removed by autoclaved Whatman filter paper. Cells transduced with adenovirus were removed by trypsin, centrifuged at 1000 rpm for 5 minutes, and resuspended in medium after the supernatant is aspirated. Subsequently, one million cells in 15 µL medium were dropped into PLGA scaffold blocks, cultured in

DMEM for 24 h in plastic dishes at 37°C. Then, PLGA blocks were implanted subcutaneously into the dorsa of immunodeficient (SCID).

Cell seeding in type I collagen gel.

BMSCs or PDL cells were trypsinized and harvested. Type I collagen (BD Biosciences) was neutralized and mixed with 1 million cells. 125 µl cell-gel mixture was added into 96-well plate and placed in incubator for 1 h (37^0C, 5% CO_2).

Implantation of scaffolds in immunocompromised mice

All procedures were approved by the University of Michigan Committee of Use and Care of Animals. General anesthesia was administrated to NIH-III nude mice (5-8 week in age) by Isoflurane (Mallinckrodt Veterinary, Mundelein, IL, USA) for all surgical procedures. Two midsagittal incisions were made on the dorsa, and two subcutaneous pockets were created with forceps at both sides of each incision. Thus, each mouse allowed 4 blocks. The PLGA blocks or collagen gels containing different treated cells were inserted into the pockets and the incisions were closed with surgical staples. Implants were harvested 2, 4, or 8 weeks after surgeries.

Radiography and histology

Radiographic analysis was performed utilizing a microradiography apparatus (Faxitron X-Ray Corp., IL, USA). For histology analysis, implants were removed from mice and fixed in 4% formaldehyde overnight. Samples were decalcified by 10% EDTA, subsequently were embedded in paraffin and sectioned at 5 µm. Hematoxylin and eosin

staining was used to determine tissue morphology. For von Kossa staining, undecalcified

samples were sectioned and stained with 1% silver nitrate.

4.6 Figures
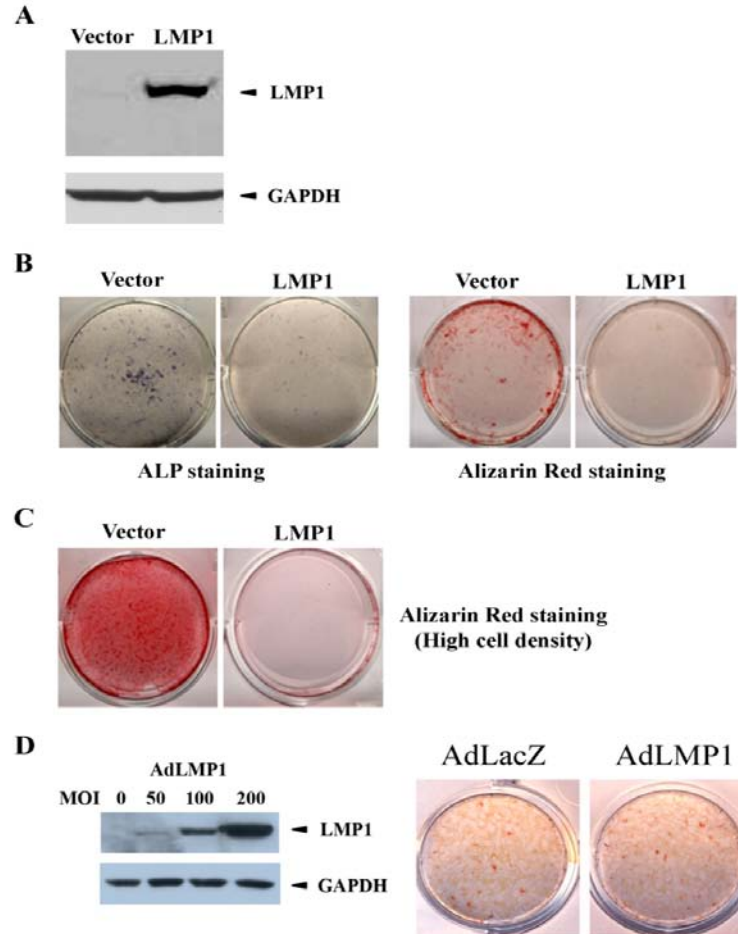
Figure 4-1 LMP1 gene transduction alone inhibits *in vitro* mineralization in PDL cells. Full length LMP1 cDNA were constructed into retroviral expression vector. PDL cells were transfected by retrovirus and selected by G418 for 10 days. Survived cells were pooled for the following experiments. (A) Exogenous LMP1 proteins were detected by western blot. (B) PDL cells were seeded at low density (3×10^3 cells/cm^2). Cells were induced to osteogenic differentiation. ALP staining was performed at Day 10, and Alizarin Red stainings were shown at Day 21. (C) PDL cells were seeded in culture plates at high density (5×10^4 cells/cm^2). Cells were induced to osteogenic differentiation. Alizarin Red stainings were shown at Day 14. (D) PDL cells were tranduced with AdLMP1 at different MOI, and the exogenous LMP1 expression was shown by Western blot analysis. Mineralization was shown at Day 14 by Alizarin Red staining (MOI=200).
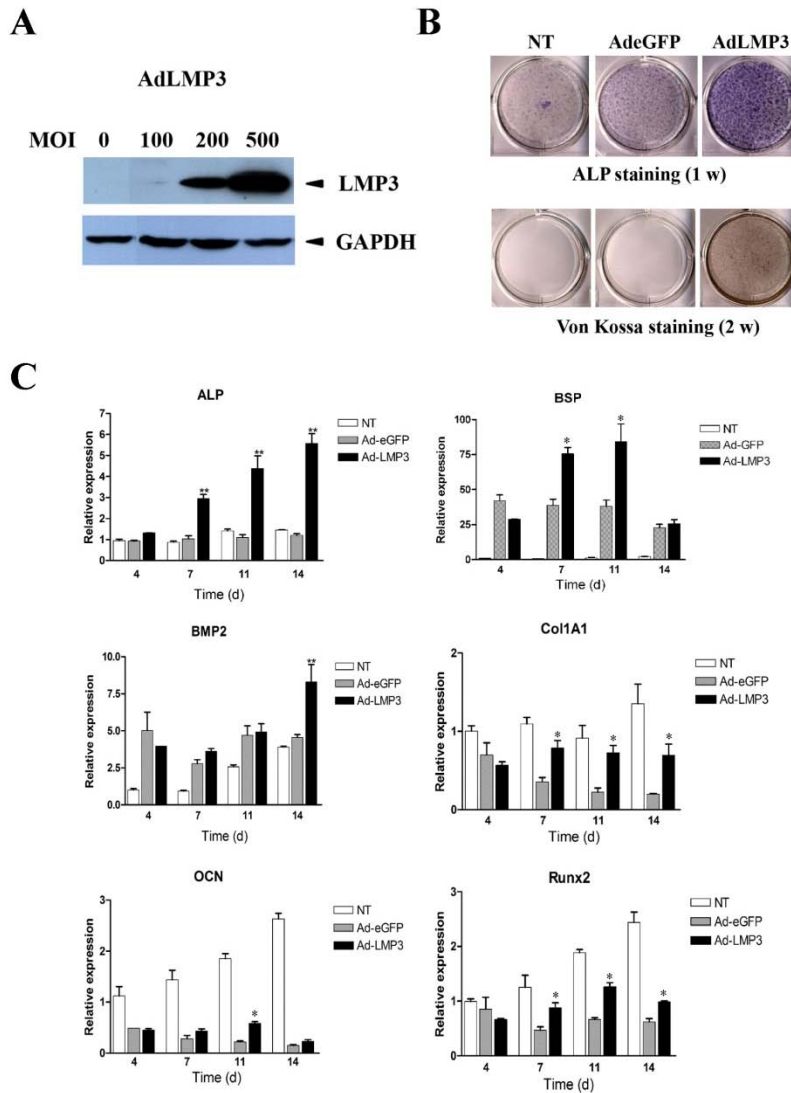
Figure 4-2 LMP3 stimulates strong mineralization in PDL cells *in vitro*. (A) PDL cells were transduced with ALMP3 at different MOI. Western blot was used to test the LMP3 expression. (B) After AdLMP3 and AdGFP transduction, PDL cells were induced to osteogenic differentiation. At 1w, cells were fixed and ALP staining was performed. At 2w, von Kossa staining was used to assess the matrix moralization. (C) Real time PCR was used to test some gene markers related to osteogenesis. (*: p<0.05 compared to AdGFP; **: p<0.01 compared to AdGFP)
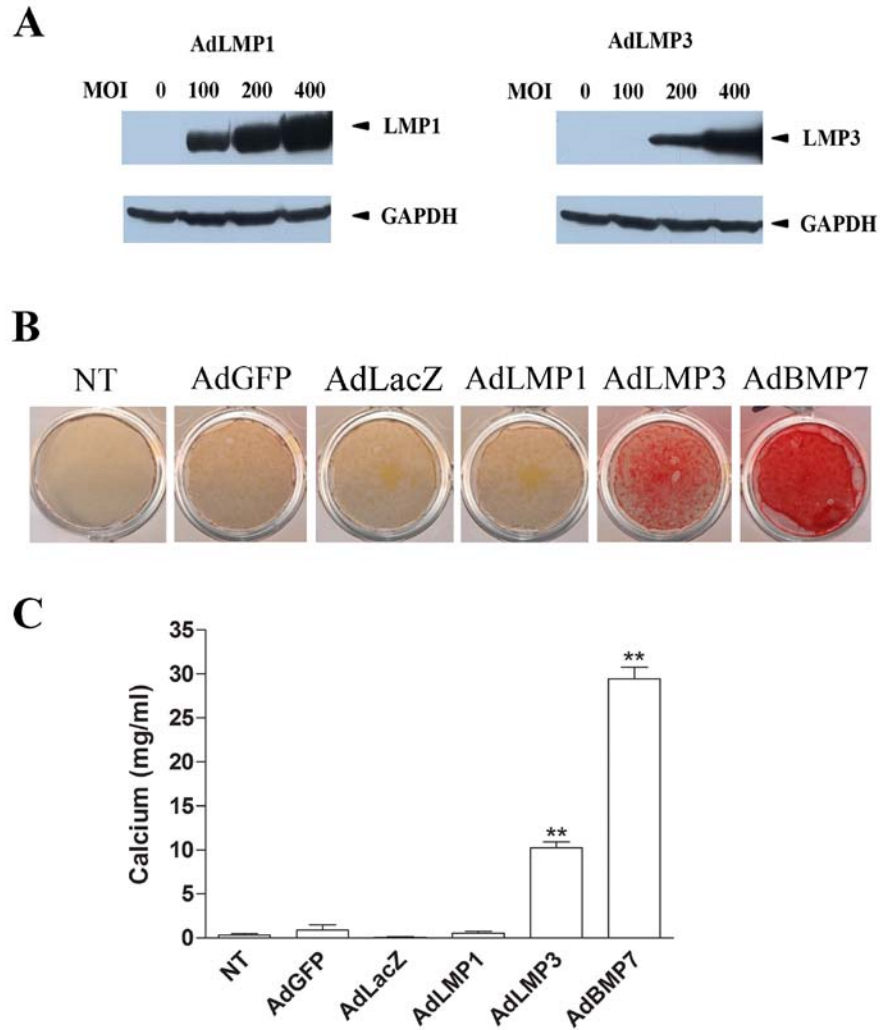
Figure 4-3 LMP3, but not LMP1 stimulated matrix mineralization in BMSC *in vitro.* (A) hBMSC cells were transduced with AdLMP1 and ALMP3 at different MOI. Westerblot was used to test the LMP1 and LMP3 expression. LMP1 is about 50 kDa, and LMP3 is about 16 kDa. (B) After AdLMP3 and AdGFP transduction, hBMSC cells were induced to osteogenic differentiation. At 2w, Alizarin Red staining was used to assess the matrix mineralization. (C) Extracellular calcium concentration was measured 2 weeks after adenovirus transduction. NT: no treatment. (**: p<0.01 compared to NT and AdGFP, Adâ-Gal, and AdLMP1)
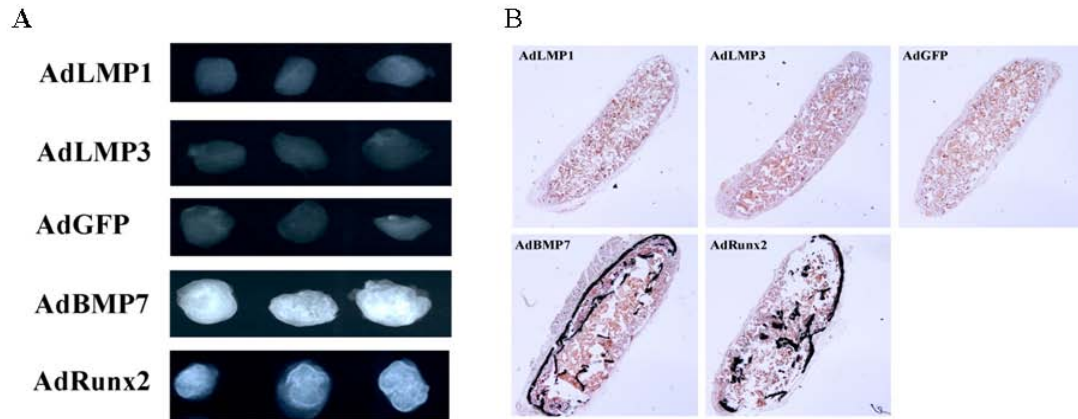
Figure 4-4 Gene delivery of AdLMP3 alone failed to stimulate ectopic bone formation *in vivo*. (A) PDL cells were transduced with the indicated adenovirus (MOI 100). 24 h after transduction, 1×10^6 cells were suspended into PLGA polymer scaffolds and implanted subcutaneously into immunodeficient mice as described under Materials and Methods. Implants were harvested and analyzed after 4 weeks. Typical microradiographic images from multiple repeating experiments are shown. (B) Von kossa stainings were performed. Mineralized areas in Von kossa-stained sections are black. Typical images from multiple repeated experiments are shown. Original magnification, 40X.
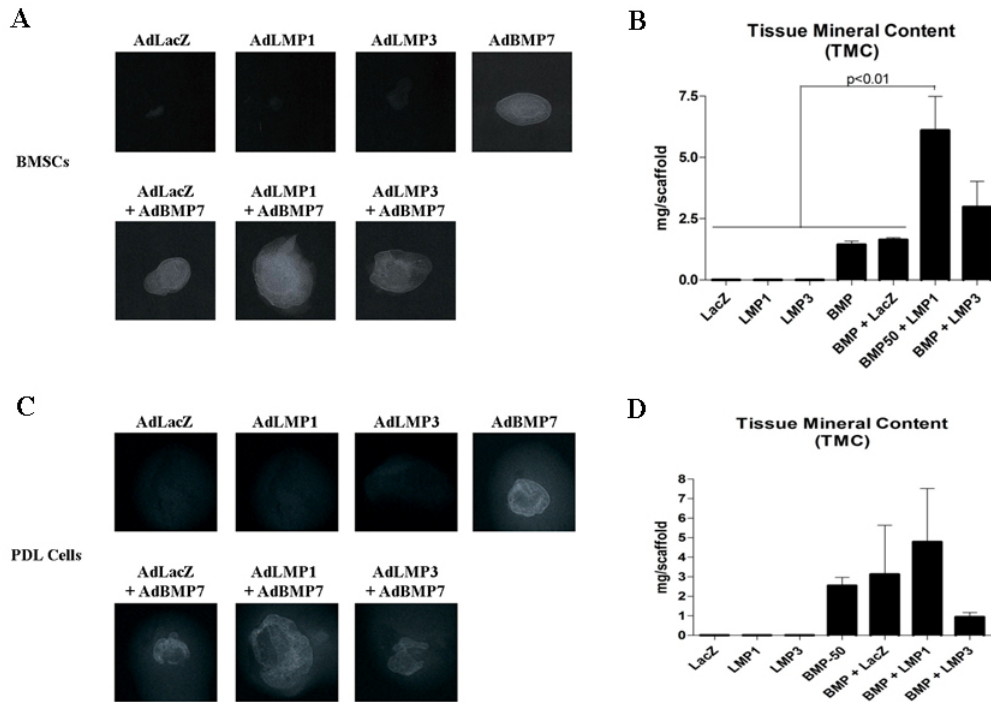
Figure 4-5 AdLMP1 gene transduction promote bone formation synergistically with AdBMP7. BMSCs (A-B) and PDL cells (C-D) were co-tranduced by AdBMP7 (MOI 50) and AdLMP1 (MOI 200), or AdBMP7 (MOI 50) and AdLMP3 (MOI 200). 24 h after transduction, $1x10^6$ cells were suspended into type I collagen scaffolds and implanted subcutaneously into immunodeficient mice. Implants were harvested and analyzed after 4 weeks. (A) Typical microradiographic images from BMSCs experiments are shown. (B) Samples were scanned by µ-CT and quantitative analysis was performed to measure tissue mineral content in each samples. (n=4). (C) Typical microradiographic images from PDL cells are shown. (B) Samples were scanned by µ-CT and quantitative analysis was performed to measure tissue mineral content in each samples. (n=3).

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CHAPTER FIVE

THE REGULATORY MECHANISM OF LMP1: A DOWNSTREAM GENE OF TGF-β1

5.1 Abstract

LMP1, also called LIM domain mineralization protein or Enigma, has been reported to improve the osteo-lineage differentiation in mesenchymal cells. Little is known regarding the regulation of LMP1 in human osteoblasts. This study sought to determine gene regulatory mechanisms of *LMP1* at the molecular level. We show that *LMP1* is a downstream target gene of TGF-β1 that is an early signal critical in preosteoblast proliferation and differentiation. TGF-β1 stimulates *LMP1* expression in human PDL cells and other pre-osteoblasts, both at the mRNA level and protein level. However, neither BMP2 nor BMP6 could stimulate the *LMP1* mRNA expression. We further demonstrated that *LMP1* induction by TGF-β1 was specifically mediated by TGFβR because it was inhibited by the TGFβ type I receptor kinase inhibitor SB-431542. When *SMAD4* and *SMAD2* were knocked down by siRNA, there was no effect on *LMP1* expression levels when induced by TGFβ-1. We further identified that the activation of TAK1-JNK/p38 kinase cascade is involved in the *LMP1* gene regulation by TGF-β1. TGF-β1 stimulates PDL cell proliferation, however, this effect is compromised when *LMP1* is knocked down. We concluded that *LMP1 is* a downstream gene of TGF-β1,

involved in PDL cell proliferation. Our findings define a regulatory mechanism of *LMP1* in PDL progenitor cells and other MSCs.

5.2 Introduction

LMP1 is a member of PDLIM protein family, which has conserved PDZ domain in the N-terminus and three LIM domains at the C terminus [1, 2]. LIM domains are cysteine-rich double zinc fingers, usually functioning in protein-protein interactions that are critical in different cellular processes, such as organ development, cytoskeletal organization and oncogenesis [3]. The PDZ domain of LMP1 binds to actin filaments [4, 5]. Increasing evidence suggests that LMP1 functions as a scaffold protein, interacting with several proteins, such as protein kinase C [6], Ret/pct2 oncogene [1, 7], and InSR [1, 7]. Recently, it has been shown that LMP1 is involved in the proliferation and differentiation of mesenchymal stem cells [2, 8-10]. It is reported that the expression of LMP1 was induced by glucocorticoid (GC) and BMP6, both of the mRNA and protein level [2]. However, the regulatory mechanism of LMP1 gene expression in mesenchymal stem cell is still largely unknown.

TGF-β1 is one of the most abundantly deposited growth factors sequestered in bone matrix [11]. It has multiple functions in osteogenesis, regulating osteoblast precursor proliferation, differentiation and migration [11-14]. It is strongly expressed in proliferating osteoblasts during intramembranous ossification, and is strongly expressed in proliferating chondrocytes during chondrogenesis and endochondral ossification [15]. TGF-β1 knock-out mice display a 30% decrease in tibial length and a reduction in bone mineral content [16]. Recombinant TGF-β1 administration increases bone formation and promotes fracture healing *in vivo[11]*. TGF-β1 exerts cellular functions and affects gene expression through binding to two transmembrane serine/tyrosine kinase receptors (type I

and type II). When the type I receptor is activated, Smad dependent and Smad independent signaling pathways are utilized to mediate the extracellular stimulus to the nucleus. In Smad dependent signaling, Smad2 and Smad3 are phosphorylated by type I receptors, forming a trimeric complex with Smad4, subsequently translocating into the nucleus to activate target gene transcription [17, 18]. Besides the Smad dependent pathway, other signaling pathways are used by TGF-β1 including the Erk, JNK and p38 MAPK kinase pathways. [17].

This study sought to determine gene regulatory mechanisms of *LMP1* in PDL cells and other mesenchymal progenitor cells such as bone marrow stromal cell. We also demonstrate that *LMP1* is a downstream target gene of TGF-β1. Canonical Smad signaling pathway is not involved in the *LMP1* gene regulation by TGF-β1. However, TGF-β1 regulates LMP1 through the activation of TAK1-JNK/p38 kinase cascade. Furthermore, LMP1 knockdown inhibits the proliferation effect mediated by TGF-β1. Our findings may help, for the first time, define a regulatory mechanism of *LMP1* at the molecular level.

5.3 Results

LMP1 gene and protein expression is regulated by TGF-β1.

It is not clear how *LMP1* gene expression is regulated in osteoblast progenitors. Based on its role in the early stage of PDL progenitor cell osteogenesis, we hypothesized that *LMP1* may be regulated by early signals critical to proliferation and differentiation, such as TGF-β1, BMP2, BMP6, and PDGF-BB. To test our hypothesis, PDL cells were

98

treated with different growth factors and we first confirmed that all the growth factors can activate downstream signaling molecules, for instance, smad1/5/8 and smad2 (Figure 5-1A). Subsequently, we found that TGF-β1 but not BMP-2/6/ stimulated *LMP1* gene expression (Figure 5-1B). PDGF-BB also stimulated *LMP1* expression, however, the effect is very limited compared to TGF-β1 (data not shown). Of the doses that we tested, 2 ng/ml TGF-β1 induced consistent high level *LMP1* gene expression (Figure 5-1C). We next examined the temporal profile of *LMP1* in response to TGF-β1 in PDL cells. *LMP1* expression was induced by 6 h following TGF-β1 treatment, peaking at 24 h, and slowly returned to basal levels by 72 h (Figure 5-1D). Using Western blotting, we further found that LMP1 protein was increased at 24 post-treatment in PDL cells (Figure 5-1E). These results indicate that LMP1 is regulated by TGF-β1 in PDL cells. We further confirmed that TGF-β1-induced *LMP1* expression occurs in other osteoblast progenitor cells as well, such as hBMSCs and MG63 cells, however, BMP2 and BMP6 had very little effect on regulating LMP1 gene expression (Figure 5-2A, 2C). Again, the TGF-β1 effect appears at around 6 hours after treatment, and peak at 24 hour (Figure 5-2B, 2D).

TGF-β1 induction of LMP1 is specifically mediated by TGF-βI receptors.

TGFβ signaling is initiated by ligand binding leading to the formation of receptor complexes, which comprises type II and type I serine/threonine kinase receptors. The type II receptor phosphorylates and activates the type I receptor which further phosphorylates various Smad molecules. Seven known type I receptors, also called activin receptor-like kinase (ALKs), have been divided into two categories: ALK-4, -5, and -7, corresponding to the TGFβ/Activin/Nodal branch through phosphorylating

Smad-2 and -3, while ALK-1, -2, -3, and -6 corresponds to the BMP/GDF branch and mediate Smad-1, -5, and -8 phosphorylation. SB-431542 is a selective inhibitor to ALK-4, 5, and 7, without affecting ALK-1, -2, -3, and -6 and corresponding to BMP signaling. SB-431245 suppressed TGF-β1 induced *LMP1* expression in PDL (Figure 5-3A). These results suggest that TGF-β1 induces *LMP1* expression through TGF-βRI activation. In order to rule out the non-specific effect of SB-431245, we used siRNA to knockdown ALK5 expression in PDL cells. ALK5 knockdown partially blocked *LMP1* expression stimulated by TGF-β1 (Fig. 3B). The partial inhibition may be explained by incomplete knockdown of ALK5 and the involvement of other receptor species such as ALK4 and ALK7. It has been reported that SaOS2 cells possess type I but no type II TGFβ receptors on the cell surface [19]. This led to the very limited effects by TGF-β1 on cell proliferation and proteoglycan synthesis of SaOS2 cells. We found that *LMP1* is not induced by TGF-β1 in SaOS2 cells which further supports that the TGF-β1 effect on *LMP1* expression is specifically through TGF-βI receptors (Figure 5-3C).

TAK1-JNK/p38 cascade is involved in TGF-β1 induction of LMP1.

To further identify the signaling pathway that TGF-β1 uses to stimulate *LMP1* gene expression, we first knocked down the canonical Smad4 and Smad2 signaling by siRNA (Figure 5-4A, 4C). To our surprise, both Smad4 and Smad2 knockdown did not affect the up-regulation of *LMP1* after TGF-β1 stimulation in PDL cells (Figure 5-4B, 4D). It is known that activated TGFβ receptors also trigger a Smad independent signaling pathways such as the mitogen-activated protein kinase (MAPK) signaling cascade [17, 20-22]. ERK, JNK and p38 are three members of MAPK signaling. U0126 is a MAP kinase

inhibitor selectively blocking the ERK1/2 phosphorylation but not JNK and p38. However, U0126 pretreatment failed to block TGF-β1-induced *LMP1* gene expression (Figure 5-5A). Consistent with this result, another Ras-Erk signaling inhibitor PD98059 had very limited effect on TGF-β1-induced *LMP1* gene expression (data not shown). However, when we pre-treated PDL cells with SB203580 (p38 kinase inhibitor) and SP600125 (JNK kinase inhibitor), the *LMP1* gene expression stimulated by TGF-β1 was significantly blocked (Figure 5-5B). To further confirm the roles of JNK and p38 in LMP1 gene expression, we used siRNA to knock down their upstream regulator TAK1 (TGF-β-activated kinase 1). When TGF-βRI is activated, TAK1 phosphorylates JNK and p38, but not Erk1/2 [23]. After siRNA transfection, the expression of *TAK1* was successfully knocked down ~90%. The gene knockdown of TAK1 inhibits the *LMP1* gene expression ~50% (Figure 5-5C). Taken together, non-canonical pathways, particularly TAK1-JNK/p38 cascade, play an important role in TGF-β1-induced *LMP1* upregulation. The phosphorylation of JNK and p38 kinase regulates downstream target genes indirectly through activating AP-1 or ATF2 transcription and translation. Using bioinformatics analysis, it was shown that there is an AP-1 binding site in the *LMP1* promoter, which suggested that TGF-β1 induction of LMP1 requires *de novo* protein synthesis [24]. To test this, we utilized Cycloheximide (CHX) to inhibit protein synthesis. Pre-treatment with CHX 1 h prior to addition of TGF-β1 effectively blocked *LMP1* mRNA induction in PDL cells (Figure 5-5D). This is consistent with the observation that *LMP1* is not an early response gene of TGF-β1, and *LMP1* mRNA begins to increase several hours after TGF-β1 treatment. Taken together, the activation of TAK1-JNK/p38 kinase cascade is used by TGF-β1 to regulate *LMP1* gene expression.

LMP1 knockdown attenuates the TGF-β1 effect on PDL cell proliferation.

The effect of TGF-β1 on PDL cell proliferation appears to depend on TGF-β1 dose and cellular context. In the PDL cells we used, TGF-β1 stimulus significantly induced cell proliferation (Figure 5-6A). Next, we evaluated whether LMP1 is involved in the TGF-β1 effect on PDL cells proliferation. LMP1 knockdown of expression by shRNA in PDL cell resulted in a blockage of TGF-β1 dependent proliferation (Figure 5-6B). This further suggests that LMP1 may be involved in the proliferation effect of TGF-β1 in the early stage of PDL differentiation.

5.4 Discussion

To date, the regulatory mechanism of *LMP1* remains "enigmatic". Because of the upregulated expression of LMP1 in early stages of MSCs osteogenesis and the significant effect of LMP1 in PDL progenitor cell proliferation, we hypothesized that *LMP1* is regulated by some of the mitogenic growth factors that provides early signals for osteogenesis. It is widely accepted that TGF-β1 stimulates MSCs proliferation during endochondral ossification and the early phase of bone fracture healing [11]. In this study, we identified that *LMP1* is a downstream gene of TGF-β1 in human MSCs including PDL cells, bone marrow MSCs and the preosteoblast cell line, MG63. It is worth mentioning that TGF-β1 induces ~10 fold increase in LMP1 expression in MG63, and it would be interesting to further investigate its biological mechanisms. Although Boden *et al.* reported that *LMP1* is regulated by BMP6 in rat calvarial osteoblasts [2], none of the BMPs that we tested can stimulate *LMP1* expression in PDL cells and hBMSCs (data not shown). Though *LMP1* may respond to different TGFβ superfamily members in different

species, our data strongly suggested that TGF-β1, but not BMPs, is the main regulator of *LMP1* gene expression in human preosteoblastic cells. Besides TGF-β1, PDGF-BB is a mitogenic growth factor involved in periodontium development and regeneration [25-27]. The fact that LMP1 is regulated by TGF-β1 and PDGF-BB, but not other BMPs, also supports our hypothesis that LMP1 is a mitogenic player in PDL cells.

Our studies demonstrate a signaling pathway in which TGF-β1 regulates *LMP1* gene expression. It appears that canonical Smad signaling is not involved in TGF-β1-induced LMP1 expression. Instead, TAK1-JNK/p38 cascade mediates the TGF-β1 effect in PDL cells. Here, our data suggest a possible model of TGF-β1-induced *LMP1* gene regulation (Figure 5-7). TGF-β1 ligand binding activates type II and I receptors, and then stimulates TAK1 activation which further phosphorylates JNK and p38 MAPK kinases. The phosphorylation of JNK and p38 kinase will activate *LMP1* gene transcription through AP-1 or ATF2 activation. TGF-β1-induced *LMP1* expression is independent of Ras-Erk signaling and Smad signaling pathways. Bioinformatics studies also support this model since there is an AP-1 binding site in the *LMP1* promoter, whereas no Smad binding site is found [24]. In this model, TRAP6 may be the player mediating the type I TGF-β receptors and TAK1 because recently it has been shown that TRAP6 is specifically required for the Smad-independent activation of JNK and p38 via the physical interaction between its carboxyl TRAF homology domain with type I TGF-β receptors [28, 29]. Of note, other TGF-β1 downstream genes have been shown to be regulated by Smad independent, but JNK and/or p38 dependent, pathways. For example, TGF-β1 induces fibronectin synthesis through JNK dependent, but a Smad4 independent pathway [30];

p38 signaling is used by TGF-β1 to induce connexin43 gene expression in normal murine mammary gland epithelial cells and these effects are Smad-independent [31]. Of course, it is still possible that other signaling pathways, such as RhoA and PP2A, are involved in the *LMP1* gene regulation.

In summary, we define a regulatory mechanism of *LMP1* gene expression in PDL progenitor cells and other MSCs. This study will help better understanding the possible function LMP1 during PDL cell proliferation and differentiation.

5.5 Materials and methods

Cell culture

The isolation of human periodontal ligament (PDL) cells and human bone marrow stroma cells (BMSC) was approved by the University of Michigan Health Sciences Institutional Review Board. PDL cells were obtained from extracted third molar or premolar teeth of healthy patients and cultured in 100 mm tissue culture dishes in a DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin [19]. PDL cells from 5 patients (Age range 20-50 years) were pooled together and were used at passages 2 to 6. Human BMSC cells were extracted from iliac crest and expanded in a bioreactor. MG63 osteosarcoma cells were maintained in DMEM with 10% FBS.

Growth factor and kinase inhibitor treatment

Confluent cultures of the above cells were brought to a stage of quiescence by

rinsing the monolayers with phosphate-buffered saline (PBS) and maintained in serum-free DMEM medium for 24 h prior to treatment. Recombinant human TGF-β1 was reconstituted and used according to the manufacturer's directions (R&D, Minneapolis, MN)). For kinase inhibition experiments, different kinase inhibitors were suspended in DMSO and added to cells 1 h before TGF-β1 treatment. Cycloheximide and SB-431542 were purchased from Sigma-Aldrich (St Louis, MO), while PD98059, SB203580, and SP600125 were acquired from A.G. Scientific Inc. (San Diego, CA).

Short Hairpin RNAs (shRNA) and Retroviral Infection and Constructs

Retrovirus-based shRNA knockdown system (pSIREN-RetroQ vector, from Clontech (Mountain view, CA) was utilized to stably knock down endogenous *LMP1* expression. Target sequences were selected with software available on the Dharmacon web sites. Oligonucleotides synthesized by Invitrogen (Carlsbad, CA) were annealed and subcloned into retroviral vectors at EcoRI and BamHI sites. The two target sequences to *LMP1* identified were: si1: 5'-gtttgagtttgctgtgaagtt-3' and si2: 5'-gcaagagccgagataaagcca-3'. Non-target scramble shRNA sequence is: 5'-aaaaccgacggctatctct-3'. shRNA expression vectors were delivered into PDL cells using retroviral transduction according to the manufacturer's directions. Briefly, PDL cells were transfected by retrovirus twice over 36 hours, with a 12 hour interval between infections. Next, puromycin (1μg/ml) was added for 3 days. Resistant clones were pooled together for subsequent experiments. At least 6 independent transfections had been performed and the efficiency and specificity of suppression by shRNAs were evaluated with analyses of protein and/or RNA levels as indicated.

Cell Lysates and Immunoblotting

Cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). SDS-PAGE gels were run and transferred to PVDF membranes (Bio-Rad, Richmond, CA). After blotting, the membranes were incubated overnight with primary antibodies and appropriate secondary antibodies (anti mouse IgG or anti rabbit IgG, Amersham, Buckinghamshire, UK) for 1 h. The membranes were washed and visualized by an ECL chemiluminescence detection kit (Amersham). Monoclonal antibody for LMP1 (1:1000) was from Abcam and monoclonal antibody for alpha-tubulin (1:1000) was from Sigma-Aldrich.

RNAi

For PDL cells or hBMSCs RNAi experiments, cells were seeded in 6-well-plates at $2x10^5$ cells per well, and transfected with 100 nM siRNA for 72 h in serum-free and antibiotic-free DMEM. Next, media were changed and cells were stimulated with or without TGF-β1. siGENOME SMARTpool siRNA targeting *Smad2, Smad4 and TAK1 ALK5*, and scramble control siRNA were purchased from Dharmacon.

Quantitative RT-PCR

Total RNA samples were extracted with RNAeasy Mini kit (Qiagen, Maryland) according to the manufacture's instruction. 1 ug RNA was subjected to reverse transcription in a 50μl RT reaction using TaqMan Reverse transcription reagents (Applied Biosystems, Foster city, CA). cDNA was generated using random hexamer primers and oligo-T primers with 2:1 ratio). For quantitative real-time PCR, the generated cDNA was

analyzed, in triplicate, with the Master Mix (Applied Biosystems) in the ABI7500 Sequence Detection System. The results were normalized with 18s transcript. The primers and probes were ordered from Applied Biosystem. The probe sequences were: 18S: Hs99999901_s1, TCCATTGGAGGGCAAGTCTGGTGCC; *LMP1*: Hs01103928_g1, CAAACCGCAGAAGGCCTCCGCCCCC; *Smad4*, Hs00232068_m1, GGCTTCCACAAGTCAGCCTGCCAGT; *Smad2*: Hs00183425_m1, TGGACACAGGCTCTCCAGCAGAACT.
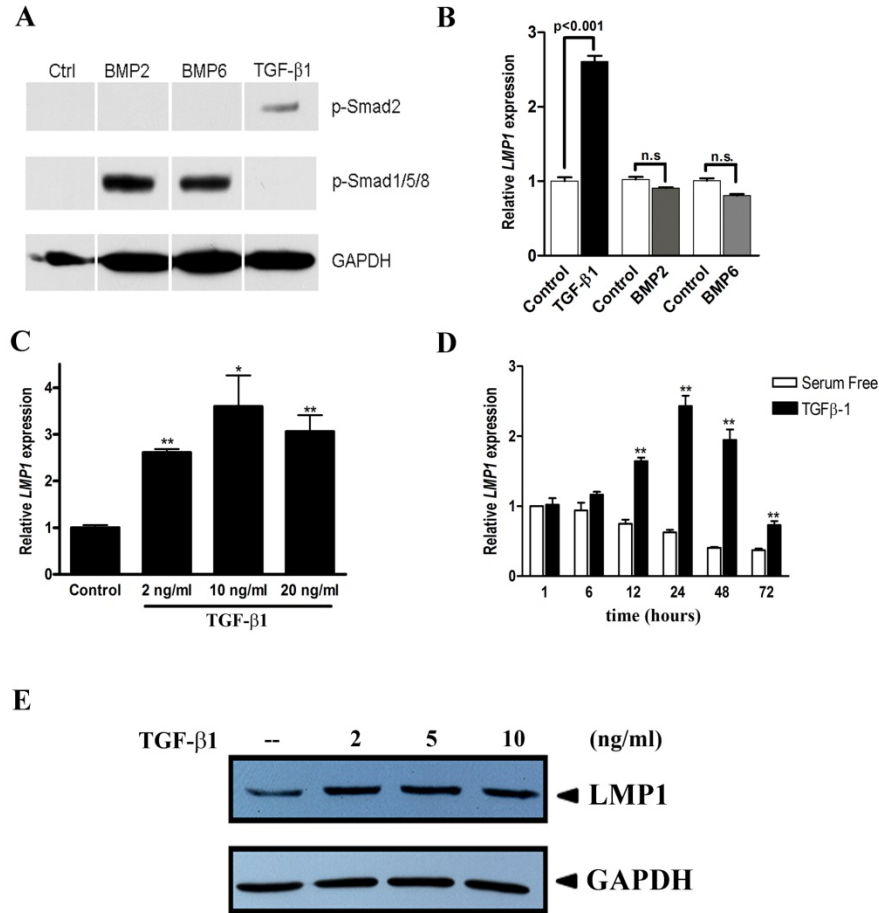
Determination of cell number by crystal violet staining

$3x10^3/cm^2$ PDL cells were seeded in 12-well plates in triplicate with osteogenic induction media. Media were changed every 3 days. Two weeks later, the cells were fixed with ice-cold methanol for 10 minutes. After PBS washing, 0.5% crystal violet solution was added for 10 minutes. Crystal violet was removed and the plates were washed carefully with water 5 times. Photographs were taken using a Nikon digital camera. For crystal violet quantification, Sorenson's buffer (0.1 M sodium citrate, 50% ethanol, 50% H_2O) was used to extract the dye and further measured using a spectrometer (Beckman Coulter, Mason, MI) at A540. The optical density readout is positive correlated to cell numbers.

Statistical analysis

All data are presented as the mean ± SD. The significance of the differences was determined by using the two-tailed Student's t-test and one-way ANOVA. In each figure, representative results from 2-3 repeated independent experiments were shown.

5.6 Figures

A

Ctrl   BMP2   BMP6   TGF-β1

p-Smad2

p-Smad1/5/8

GAPDH

B

p<0.001

Relative *LMP1* expression

n.s.   n.s.

Control TGF-β1 Control BMP2 Control BMP6

C

Relative *LMP1* expression

*   **

Control   2 ng/ml   10 ng/ml   20 ng/ml

TGF-β1

D

Relative *LMP1* expression

☐ Serum Free
■ TGFβ-1

**   **   **   **

1   6   12   24   48   72

time (hours)

E

TGF-β1   --   2   5   10   (ng/ml)

◄ LMP1

◄ GAPDH

Figure 5-1 *LMP1* gene and protein expression is regulated by TGF-β1 in PDL cells.
(A) PDL cells were stimulated with TGF-β1 (2ng/ml), BMP2 (100ng/ml), or BMP6 (100ng/ml). Phosphorylated-Smad1/5/8 and phosphorylated-Smad2 was examined by Western blot. (B) RNA was extracted after 24 h and RT-qPCR was used to evaluate *LMP1* gene expression. *LMP1* mRNA expression values was normalized to 18s RNA relative to that of serum-free controls. (C) After incubation in serum-free medium for 24 hours, human PDL cells were stimulated with TGF-β1 (2 ng/ml, 10 ng/ml, and 20ng/ml). 24 hours later, RNA was extracted and RT-qPCR was use to measure the expression level of *LMP1*. **: $P<0.01$ compared to serum free control; *: $P<0.05$ compared to serum free control. (D) PDL cells were treated by TGF-β1 at 2 ng/ml. At various time points, *LMP1* mRNA expression was measured by RT-qPCR. Open bar: PDL cells in serum free condition without TGF-β1. Closed bar: with TGF-β1. **: $p<0.01$ compared to serum-free control at the same time point. (E) PDL cells were treated with TGF-β1 for 24 hours. Western blot was performed to evaluate the LMP1 expression.
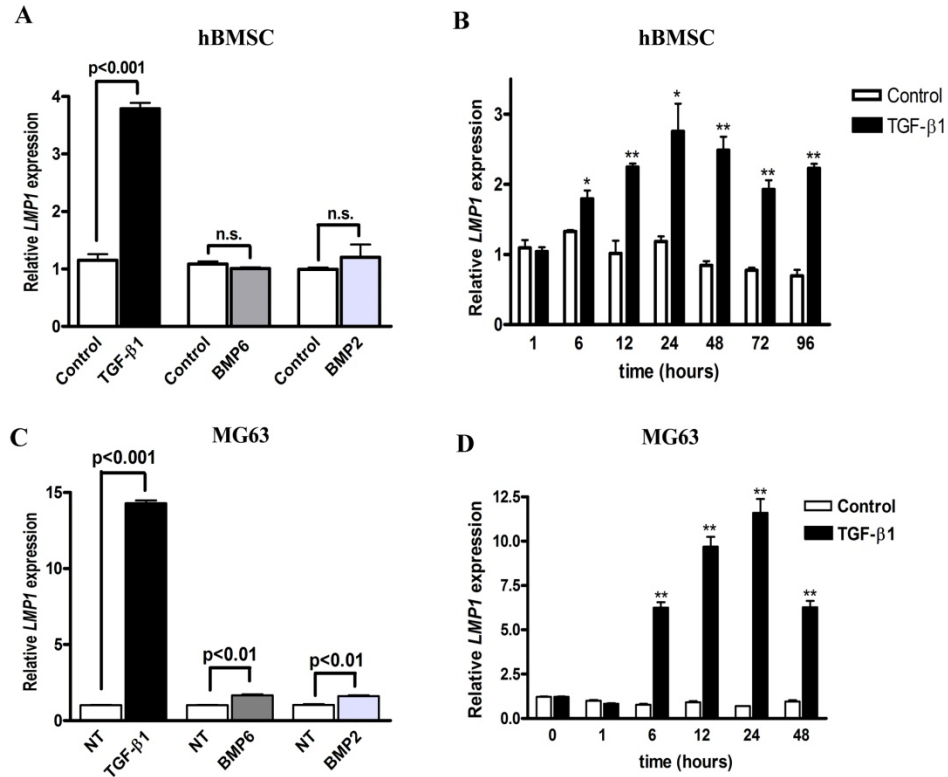
Figure 5-2 *LMP1* gene expression is regulated by TGF-β1 in BMSCs and MG63 cells.
Human BMSCs (A) and osteosarcoma cell line MG63 (C) were stimulated with TGF-β1
(2ng/ml), BMP2 (100ng/ml), or BMP6 (100ng/ml). RNA was extracted after 24 h and
RT-qPCR was used to evaluate *LMP1* gene expression. hBMSCs (B) and MG63 cells
(D)were treated by TGF-β1 at 2 ng/ml. At various time points, *LMP1* mRNA expression
was measured by RT-qPCR. Open bar: PDL cells in serum free condition without TGF-β1.
Closed bar: with TGF-β1. **: $p < 0.01$ compared to serum-free control at the same time
point.
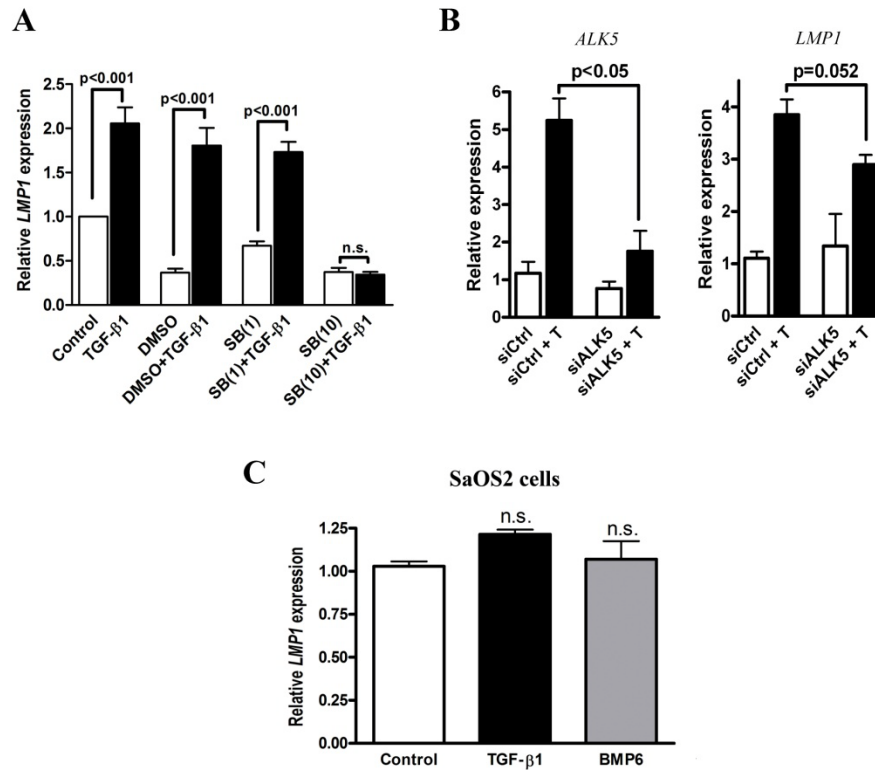
Figure 5-3 TGF-β1 induction of *LMP1* is specifically mediated by TGF-βI receptors.
(A) Confluent PDL cells were free for serum for 24 h. Prior to adding TGF-β1 (2 ng/ml), PDL cells were pretreated by SB431542 for 1 h. 24 h after TGF-β1 treatment, *LMP1* gene expression was measured by RT-qPCR, normalized to 18s mRNA, and given relative to that of serum-free control. Control: serum-free. SB(1): SB431532 in DMSO, 1μM. SB(10): SB431532 in DMSO, 10μM. n.s.: no significant difference. (B) ALK5 siRNA was transfected into PDL cells for 72 h in serum-free media, followed by TGF-β1 stimulation. At 24 hours, RNA was extracted and qRT-PCR was used to examine the expression of *ALK5* and *LMP1*. ALK5 knockdown compromised the effect of TGF-β1 on *LMP1* gene expression. (C) Human steosarcoma cells (SaOS2) was stimulated with TGF-β1. RNA was extracted after 24 h and RT-qPCR was used to evaluate *LMP1* gene expression.
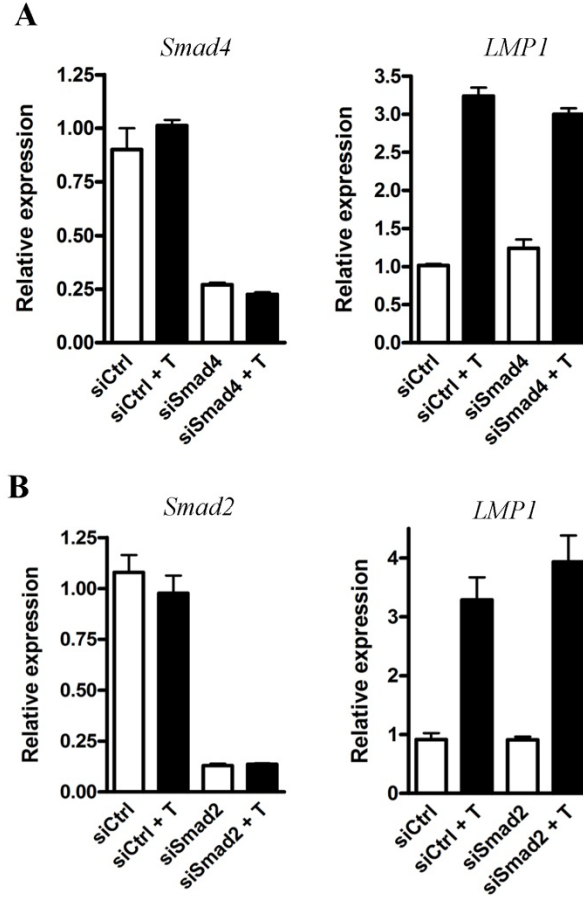
Figure 5-4 TGF-β1 induction of *LMP1* was Smad4 and Smad2 independent. (A):
PDL cells were transiently transfected with 100nM siRNA (targeting Smad4, Smad2, or
non-targeting control) for 72 h, in serum-free and antibiotics-free DMEM. After treatment,
media were changed (serum and antibiotics-free), and TGF-β1 was added. After 24 h,
RT-qPCR was performed to measure gene expression. Smad4 and LMP1 mRNA
expression were normalized to 18S mRNA. Left: The mRNA expression of Smad4 was
examined by RT-qPCR. Right: LMP1 gene expression after Smad4 knockdown, w/wo
TGF-β1 treatment. (C) Left: The mRNA expression of Smad2 was examined by
RT-qPCR. Right: LMP1 gene expression after Smad2 knockdown, w/wo TGF-β1
treatment. Results showed that Smad2 knockdown did affect TGF-β1-induced LMP1
expression. siCtrl: scramble siRNA; siCtrl + T: scramble siRNA and TGF-β1 treatment;
siSmad4: Smad4 siRNA; siSmad4 + T: Smad4 siRNA and TGF-β1 treatment; siSmad2:
Smad2 siRNA; siSmad2 + T: Smad2 siRNA and TGF-β1 treatment.
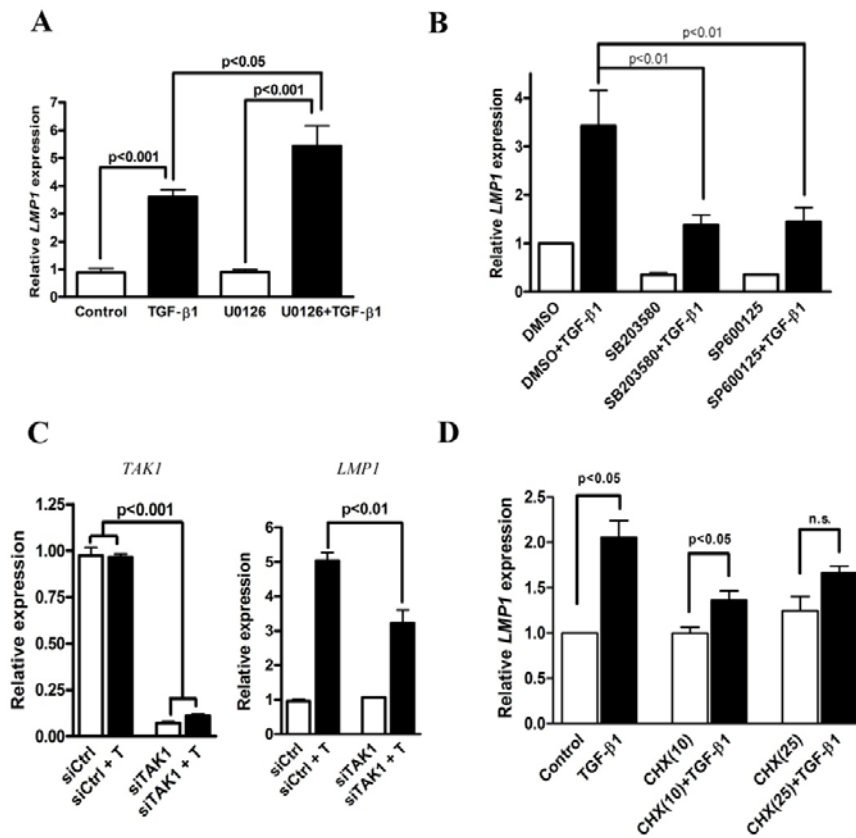
Figure 5-5 TAK1-JNK/p38 cascade is involved in TGF-β1 induction of LMP1.
Confluent PDL cells were serum starved for 24 hours. Before adding TGF-β1 (2 ng/ml), PDL cells were pretreated by MAPK kinase inhibitors for 2 h. 24 h after TGF-β1 treatment, *LMP1* gene expression was measured by RT-qPCR. DMSO was used as the solvent for all the inhibitors. (A) The effect of Erk kinase inhibitor U0126 (10μM). (B) The effect of SB203580: p38 inhibitor, 25μM. SP600125: JNK inhibitor, 25μM. (C) PDL cells were transiently transfected with 100nM siRNA (targeting TAK1 or scramble control) for 72 h, in serum-free and antibiotics-free DMEM. After treatment, media were changed (serum and antibiotics-free), and TGF-β1 was added. After 24 h, RT-qPCR was performed to measure gene expression. Left panel: TAK1 gene expression. Right panel: LMP1 gene expression. siCtrl: scramble siRNA; siCtrl + T: scramble siRNA and TGF-β1 treatment; siTAK1: TAK1 siRNA; siTAK1 + T: TAK1 siRNA and TGF-β1 treatment. (D) Confluent PDL cells were cultured in serum-free media for 24 h. Before adding TGF-β1 (2 ng/ml), PDL cells were pretreated by cycloheximide (CHX) for 2 h. 24 h after TGF-β1 treatment, *LMP1* gene expression was measured by RT-qPCR, normalized to 18S mRNA, and given relative to that of serum free control. CHX(10): cycloheximide 10μM; CHX(25): cycloheximide 25μM.
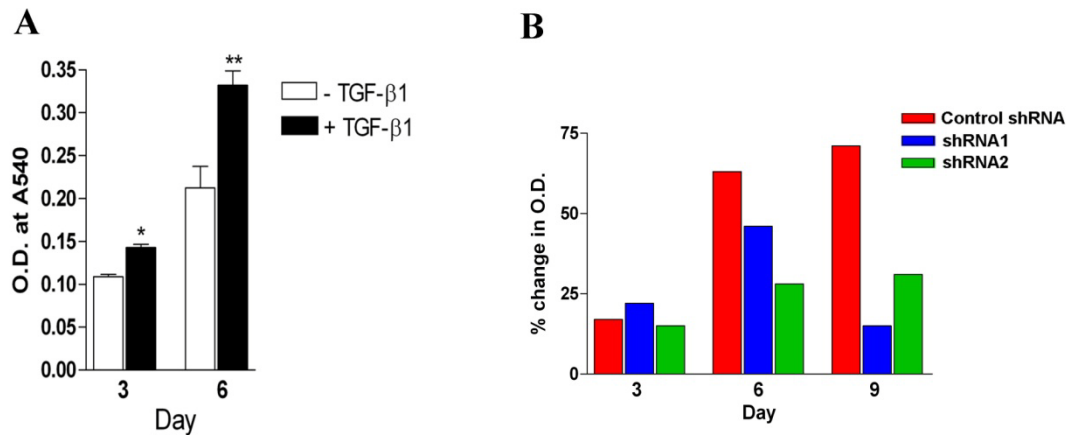
Figure 5-6 LMP1 knockdown attenuates the TGF-β1 effect on PDL cells proliferation. (A) PDL cells were cultured in DMEM with low serum concentration (2% FBS), and some cells were treated by TGF-β1. At day 3 and day 6, cells were fixed and stained with crystal violet. The optical density readout which correlates to cell numbers are shown, n=3. *: $p<0.05$, **: $p<0.01$. (B) LMP1 knockdown PDL cells and control cells were cultured in 6-well-plates in 2% FBS, with/without TGF-β1. At day 3, 6, 9, cells were fixed and stained with crystal violet. The data showed the percentage change of crystal violet measurement between no TGF-β1 and TGF-β1 treatment (n=3 per group).
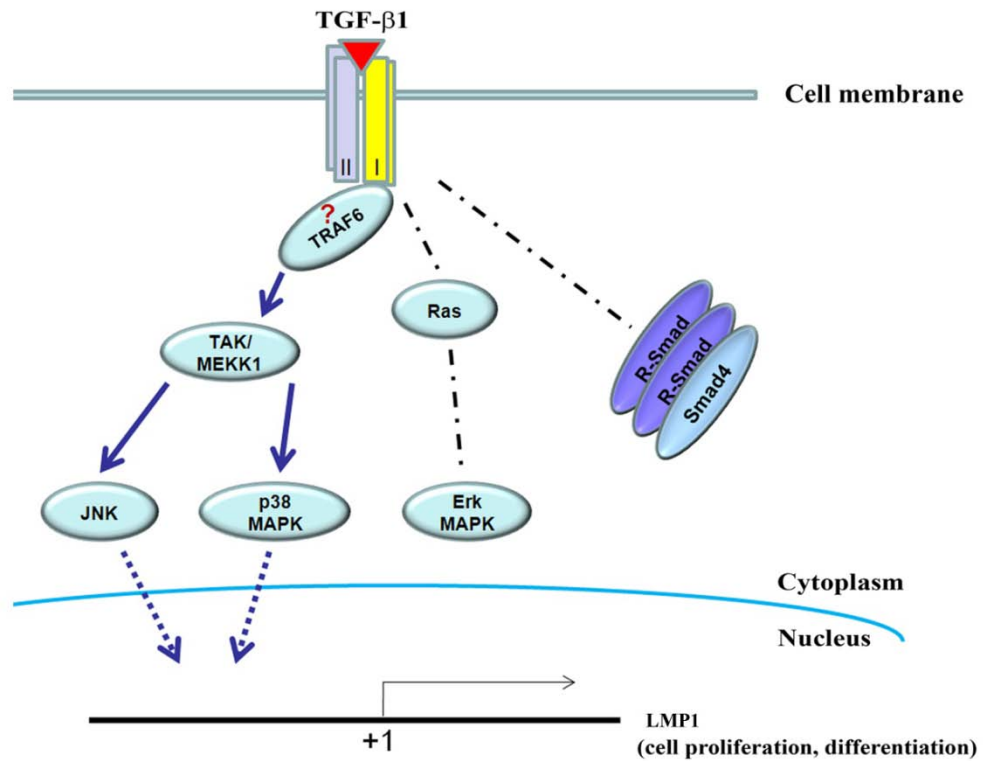
Figure 5-7 Schematic overview of the regulation of LMP1 gene expression by TGF-β1. TGFβ-1 ligand binding activates type II and I receptors, and then stimulates TAK1-JNK/p38 cascade, therefore activates downstream gene transcription and translation, ultimately activates LMP1 gene expression. TGFβ-1-induced Enigma expression is independent of Ras-Erk signaling and Smad signaling pathways.

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CHAPTER SIX

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary of the thesis findings

There are four main aspects which have been addressed in this dissertation: 1) the gene expression profiles of *LMP1* in periodontium under physiological, pathological, and regeneration circumstances; 2) the function of *LMP1* in periodontal ligament cell (PDL) proliferation and differentiation; 3) the potential of AdLMP1 and AdLMP3 gene delivery in periodontal tissue regeneration; 4) the gene expression regulation mechanism of *LMP1* in periodontal ligament cell and other osteoblast precursors.

LMP1 is an intracellular scaffold protein that contains a PDZ domain and three LIM domains. LMP1 has multiple functions including regulating mesenchymal stem cell osteogenesis. Gene delivery of *LMP1* and *LMP3*, a truncated form variant, was reported to induce bone formation *in vivo* in heterotopic and orthotopic sites. It is largely unknown about the expression profile, function, and regulation of *LMP1* in the periodontal ligament cell, a multipotent cell which is able to differentiate to osteoblasts, adipocytes, chondrocytes, and neurons. The potential of *LMP1/3* gene therapy for periodontal regeneration is still not clear. The main contribution of **Chapter 2** was to identify the

gene expression pattern of *LMP1* in normal, diseased, and regenerating periodontium. Using laser capture microdissection, I was able to detect mRNA expression from decalcified cranial and dental histological sections. I first identified that LMP1 was more highly expressed in periodontal ligament tissue and gingival tissue than mature alveolar bone, which suggested that *LMP1* may be related to the homeostasis of the periodontium, in particular, the cell behavior of PDL cells. Next, I found that *LMP1* was modestly increased in the extraction socket during the normal healing process, whereas, it was gradually decreased over time in the healing of bony defect around a dental titanium implant. These distinct expression patterns might be related to the different wound healing dynamics between tooth extraction repair and bone healing around titanium. More immune cells were seen to infiltrate to the osteotomy area in response to titanium. Very interestingly, I found that LMP1 gene expression was significantly up-regulated in the inflamed gingival tissue in experimental periodontitis. This information sheds some light on the roles of LMP1 in wound healing and host modulation to pathogenic infection. Another contribution of **Chapter 2** was that, taking advantage of LCM, I generated the gene expression profiles of a panel of genes which may be related to periodontal wound healing and bone-implant osseointegration. These genes include growth factors, extracellular matrix protein, chemokines, and transcription factors. This data set will provide valuable information for the development of future periodontal regeneration therapies.

Most of the current research about *LMP1* focused on its potential as a gene therapy agent for bone regeneration, hence very little is known about the physiological function

of *LMP1*. So far, *LMP1* knockout mice still haven't been developed, and LMP1 knockdown in zebrafish is embryonical lethal. Therefore, gene knockdown experiments at the cell level are valuable for us to understand *LMP1* function. Using PDL cells as a model, in **Chapter 3**, I stably knocked down *LMP1* by shRNA and observed what basic cellular function was affected. I found stable gene knockdown of *LMP1* impaired PDL cell proliferation, which further led to decreased mineralization *in vitro*. *LMP1* knockdown resulted in decreased DNA synthesis without significant cell apoptosis, and more cells were arrested in the G0/G1 phase. I also found that overexpression of LMP1 increased cell proliferation. In the comparison of full length LMP1 and a truncated form which only contains PDZ and ww-interacting domains, we found that the PDZ and ww-interactiong domains are not enough to mediate this effect. I concluded that *LMP1 is* involved in PDL cell proliferation. Our findings will contribute to the understanding of the physiological function of *LMP1* in PDL cells and other osteoblast precursors.

In **Chapter 4**, I sought to explore whether adenoviral gene delivery of LMP1 and LMP3 can be used to enhance PDL cell mineralization *in vitro* and stimulate bone formation *in vivo*. Interestingly, we found that AdLMP3 treatment, but not AdLMP1, induced significant matrix mineralization in PDL cells and hBMSCs. The AdLMP3 effect might be related to the up-regulation of ALP and BSP gene expression. However, PDL cell transduced with AdLMP3 or AdLMP1 alone failed to induce ectopic bone formation *in vivo*. The same results were seen in BMSCs transduced with AdLMP3 or AdLMP1. Interestingly, when combined with AdBMP7, AdLMP1 treatment synergistically promoted ectopic bone formation in BMSCs. AdBMP7 and AdLMP1 combinatory gene

delivery tended to induce more bone formation, although the result is not statistically different due to our small sample size. Our studies are the first studies to investigate the potential of LMP gene therapy in periodontal regeneration. Although the results from single gene delivery experiment was unexpected, the striking *in vitro* effect suggests future study will be needed to characterize inhibitory factors preventing the osteogenic effect of LMP3 in PDL cells under *in vivo* circumstances, and optimize the gene delivery strategy. The synergistic effect between BMP7 and LMP1 is worthwhile to be investigated which will help to lower the BMP dose in bone regeneration.

The major contribution of the fourth portion of this dissertation was to determine the gene regulatory mechanisms of *LMP1* at the molecular level. In **Chapter 5**, I show that *LMP1* is a downstream target gene of TGF-β1 that is an early signal critical in preosteoblast proliferation and differentiation. TGF-β1 stimulates *LMP1* expression in human PDL cells and other pre-osteoblasts, both at mRNA level and protein level. However, neither BMP2 nor BMP6 could stimulate the *LMP1* mRNA expression. I further demonstrated that the activation of TAK1-JNK/p38 kinase cascade was involved in the *LMP1* gene regulation by TGF-β1. TGF-β1 stimulated PDL cell proliferation. However, this effect was compromised when *LMP1* is knocked down. Thus, the conclusion is the *LMP1* is regulated by TGF-β1 through TAK1-JNK/p38 kinase axis. This study, for the first time, defined a regulatory mechanism of *LMP1* in PDL cells and other mesenchymal stem cells.

There is a great clinical need for periodontal regeneration. Gene delivery of *LMP* appears to be a candidate therapeutic for periodontal regeneration. The work described in this dissertation contributes to our understanding of the function and regulation of LMP in PDL cells, and will help us to better design future therapeutics to repair bony defects in the oral and craniofacial complex due to disease, trauma or congenital abnormalities.

6.2 Future research directions

In this dissertation, we provided information about the regulation of LMP1 in periodontium under healthy and disease situation. Although the trends are very clear, some data showed relatively big variance. This is associated with our small sample size (n=4~6). It might be also related to the accuracy of area of interest we selected in LCM since we identified the borders of the extraction socket and the osteotomy based on our experience. Other methods such as bone labeling will help to identify the area of interest more precisely and easily.

Increasing evidence suggests that LMP1 may play an important role in joint/ligament homeostasis. A previous study reported that LMP1 is highly expressed in the future joint area in embryonic rats. Our data showed that LMP1 is highly expressed in PDL and gingival tissues, whereas at relative lower level in mature alveolar bone. When PDL cells were induced to osteogenic differentiation, LMP1 was upregulated at early stages and then decreases at the late stages. Furthermore, gene knockdown of LMP1 in PDL cells impaired its proliferation capability. Based on these findings, I propose that LMP1 is

important in periodontium homeostasis. A LMP1 knockout mouse model will be valuable to test this hypothesis *in vivo*. Since gene knockdown of LMP1 in zebrafish led to embryonic lethality, it is likely that LMP1 knockout mice will be embryonic lethal as well. Thus, inducible gene knockout or tissue specific gene knockout strategies can be considered in the future. Tet-on/Tet-off inducible gene knockout system will allow us to study the function of LMP1 in adult animals or in animals in certain development stages. LMP1 can be knocked out specifically in ligament tissues as well. In the past, several ligament/PDL specific markers have been identified, such as *Scleraxis* (Scx) and *Periostin*. Using the *Scleraxis* promoter or the *Periostin* promoter, LMP1 can be specifically knocked out in ligament tissue. The phenotype observed in these LMP1 gene knockout mice will be important to answer if LMP1 is important for ligament/periodontal ligament development and homeostasis.

In terms of the potential of LMP1 gene therapy, we found that gene delivery of LMP1 alone is insufficient to promote ectopic bone formation. However, LMP1 seems to have synergistic effect with BMP7. In the future, more studies should be done to identify the mechanism underlying this effect. One possible mechanism is that LMP1 binds to Smurf1 and subsequently prevents the Smad degradation. Thus, LMP1 prolongs the BMP signaling. If this is the case, it will be very important to identify the binding motifs which will be a potential pharmaceutical target for bone regeneration. Another possible mechanism is that LMP1 stimulates osteogenesis under high BMP7 level. It would be important to identify the fate of the transplanted cells with LMP1 and BMP7 co-transfection.

We have shown that AdLMP3 gene delivery promoted the matrix mineralization of PDL cells and BMSC *in vitro*. Although the *in vivo* results in ectopic bone formation model appeared to be elusive, in my opinion, it is still worthwhile to determine the mechanism behind these distinct differences. Some of the possibilities might be: 1) ectopic bone model is not an ideal approach to test the osteogenic potential of AdLMP3 *in vivo* because other factors are needed for the successful AdLMP3 therapy are missing, such as growth factors, hormone, and extracellular matrix associated with bone formation. Previous successful experiences using AdLMP3 to induce bone formation mostly happened in or very close to bone defect area [1-3]. Therefore, *in situ* wound healing model in either a long bone or alveolar bone should be considered in the future. 2) The scaffold carrier in our tissue engineering model should be optimized. We used PLGA polymer in our study because it was easy to fabricate and handle for surgical procedure, and also because of its relative rigid physical property compared to gel foam. However, it may not be ideal for ectopic bone formation, especially for a molecules less potent that BMP and Runx2. Osteoconductive or osteoinductive materials, such as hydroxyapatite, may be considered in the future.

We also determined the gene regulatory mechanism of LMP1 in this dissertation. We found that LMP1 is regulated by TGF-β1 in PDL cell and other osteoblast precursors. This finding raised an interesting question: what are the roles of LMP1 to affect TGF-β1 in MSCs. Although we have shown some preliminary data that LMP1 mediated the proliferation effect on PDL cells, other evidence also suggests that LMP1 may also be involved in the TGF-β1-induced cell migration. As we know, TGF-β1 is an important

chemoattactant for pre-osteoblasts. LMP1 has been reported to interact with several cytoskeleton related proteins, such as β-tropomyosin, tubulin, caldesmon, and meosin, and APS (Actin cytoskeleton organization) [4-7]. LMP1 can also bind to Smurf1, which plays critical effect on cell migration [7-10]. Taken together, it will be interesting to investigate if LMP1 is involved in TGF-β1-induced cell migration. It will be interesting as well to determine whether LMP1 has a role in the epithelial-mesenchymal transition (EMT).

This dissertation also demonstrated that LMP1 has multiple functions. Besides its roles in PDL cell proliferation and differentiation, increasing evidence suggest that LMP1 may also play a role in the host immune response and bone resorption: 1) We showed that LMP1 is upregulated during the early stages of periodontal diseases; 2) LMP1 is highly expressed in leukocytes; 3) Recently, it was reported that LMP1 overexpression suppresses the activation of NF-κB in pre-osteoclasts. 4) Our data showed that LMP1 is regulated by TGF-β1, which is an important inhibitor of immune system. In the future, it will be valuable to identify the function of LMP1 in the host modulation of periodontal diseases. It would be interesting to investigate if LMP1 gene delivery can prevent periodontal bone loss.

In summary, studies in this dissertation demonstrate that LMP1 is an important regulator in PDL cell proliferation and differentiation. Gene delivery of LMP1 synergistically promotes the osteogenic effect of BMP7. The studies also suggest that

LMP1 may play a role in the host modulation in periodontal diseases. These studies are important for the understanding of periodontal diseases and regeneration.

6.5 Bibliography

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