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LMP1 Regulates Periodontal Ligament Progenitor Cell Proliferation and Differentiation

Zhao Lin¹, Valeria Pontelli Navarro¹, Kathryn M. Kempeinen¹, Lea M. Franco¹, Qiming Jin¹, James V. Sugai¹, and William V. Giannobile^{1,2}

¹Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor

²Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor

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 and gene regulatory mechanisms of *LMP1* in MSCs at the molecular level. 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 and gene regulatory mechanisms of *LMP1* in PDL cells at the molecular level. 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. Further, we found that in PDL cells, *LMP1* is a downstream target gene of TGF- β 1 that is an early signal critical in preosteoblast proliferation and differentiation. TGF- β 1 stimulates PDL cell proliferation, however, this effect is compromised when *LMP1* is knocked down. We further identified that the activation of TAK1-JNK/p38 kinase cascade is involved in the *LMP1* gene regulation by TGF- β 1. We conclude that *LMP1* is a downstream gene of TGF- β 1, involved in PDL cell proliferation. Our findings advance the understanding of the physiological function of *LMP1*, and define a regulatory mechanism of *LMP1* in PDL progenitor cells and other MSCs.

Keywords

LMP1; periodontal diseases; tissue engineering; signal transduction; TGF- β 1

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

Address correspondence to: William V. Giannobile, Department of Periodontics and Oral Medicine, University of Michigan, 1011 N. University Avenue – Room 3397, Ann Arbor, Michigan 48109-1078, USA, Tel: (734) 764-1562, Fax: (734) 763-5503, william.giannobile@umich.edu.

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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].

TGF- β 1 is one of the most abundantly deposited growth factors sequestered in bone matrix [10]. It has multiple functions in osteogenesis, regulating osteoblast precursor proliferation, differentiation and migration [10-13]. It is strongly expressed in proliferating osteoblasts during intramembranous ossification, and is strongly expressed in proliferating chondrocytes during chondrogenesis and endochondral ossification [14]. TGF- β 1 knock-out mice display a 30% decrease in tibial length and a reduction in bone mineral content [15]. Recombinant TGF- β 1 administration increases bone formation and promotes fracture healing *in vivo* [10]. TGF- β 1 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 activating target gene transcription [16,17]. Besides the Smad dependent pathway, other signaling pathways are used by TGF- β 1 including the Erk, JNK and p38 MAPK kinase pathways. [16].

Periodontal ligament (PDL) cells are a unique mesenchymal stem cell population that can differentiate into multiple cell types, such as osteoblasts, adipocytes, and neurons [18,19]. The PDL cell is a promising cell source for periodontal hard and soft tissue regeneration [20,21]. This study sought to determine the physiological function and gene regulatory mechanisms 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. We also demonstrate that *LMP1* is regulated by TGF- β 1 in PDL cells, and *LMP1* knockdown inhibits the proliferation effect mediated by TGF- β 1. We further identified that the activation of TAK1-JNK/p38 kinase cascade is involved in the *LMP1* gene regulation by TGF- β 1. Our findings may aid in the better understanding of the role of *LMP1* in PDL cells proliferation and differentiation, and for the first time, define a regulatory mechanism of *LMP1* at molecular level.

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 [18]. PDL cells from 5 patients (Age range 20-50 years) were pooled and used at passages 2 to 6.

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- β 1 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- β 1 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'-gtttgagttgtgtgaagtt-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 instructions. Briefly, PDL cells were transfected by retrovirus twice over 36 h, with a 12 h interval between infections. Next, puromycin (1 μ g/ml) was added for 3 d. 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 (Clontech, 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 d.

[methyl-³H]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 (counts per minute) [methyl-³H]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-³H]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 μ g/ml propidium iodide and RNase A (10 μ g/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 obtained from Abcam and monoclonal antibody for alpha-tubulin (1:1000) was purchased 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- β 1. 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 instructions. 1 μ g RNA was subjected to reverse transcription in a 50 μ l 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, CAAACCGCAGAAGGCCCTCCGCCCC; *Smad4*, Hs00232068_m1, GGCTTCCACAAGTCAGCCTGCCAGT; *Smad2*: Hs00183425_m1, TGGACACAGGCTCTCCAGCAGAACT.

Determination of cell number by crystal violet staining

$3 \times 10^3/\text{cm}^2$ PDL cells were seeded in 12-well plates in triplicate with osteogenic induction media. Media were changed every 3 d. 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% H₂O) was used to extract the dye and further measured using a spectrometer (Beckman Coulter, Mason, MI) at A540. The optical density readout is positively 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 $3 \times 10^3/\text{cm}^2$. In order to induce PDL cells to mineralize, 50 μ g/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 [22]. Matrix mineralization was evaluated by alizarin red staining and von kossa staining.

Statistical analysis

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

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 [18], 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 Fig 1A, *LMP1* expression is upregulated at 3 d and decreases at later time points. The same pattern was seen at protein level as well (Fig. 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. *LMP1* expression was verified at mRNA (Fig. 2A) and protein (Suppl. Fig. 1A) 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 (Fig. 2B). At 10 d, cells were fixed and stained with crystal violet staining and less staining was found in knockdown cells (Fig. 2 C, D). Consistent with this observation, *LMP1* knockdown in PDL cells inhibited DNA synthesis which was shown by ³H methyl thymidine incorporation assay (Fig. 2E). Since this effect can also be explained by the increase of apoptotic cells while *LMP1* was knocked down, we further tested the expression of an apoptosis marker Caspase-3. Caspase-3 is a critical driver of both intrinsic and extrinsic apoptosis, as it is responsible for the proteolytic cleavage of many key factors involved in apoptosis [23]. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated p17 and p12 fragments [23]. There is no significant increase of cleaved caspase-3, which indicates that the *LMP1* knockdown effect may be related to impaired proliferation and not apoptosis (Suppl. Fig. 1B). Further, by RT-qPCR, we confirmed that *LMP1* knockdown resulted in less expression of Cyclin D1 and Cyclin B1 compared to controls (Fig. 2F). FACS analysis further showed that a greater degree of *LMP1* knockdown of cells were blocked at the G1 phase compared to scramble control cells (Table 1). Taken together, knockdown of *LMP1* expression in PDL cells appears to impair 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 (Suppl. Fig. 2). However, less ALP positive cells were seen when *LMP1* was stably knocked down by shRNA, and the ALP activity was lower in knockdown cells (Fig. 3A). Consistent with this finding, less mineralized nodules were observed in *LMP1* knockdown PDL cells at the late stage of PDL osteogenic differentiation, which was shown by Alizarin Red staining and extracellular measurement (Fig. 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* (Fig. 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 domains (Fig. 4A and Suppl. Fig. 3). The antibody we used can recognize the N-terminus of LMP1, which made it possible to detect both forms in Western blot (Fig. 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. Significant cell death was not observed during cell culture. Consistent with this finding, there was no significant increase in the cleaved active fragments of caspase-3, for both control and LMP-t PDL cells (Suppl. Fig. 4). By ³H methyl thymidine incorporation assay we further showed that DNA synthesis is upregulated in LMP1 overexpression PDL cells (Fig. 4D). Therefore, our result suggests that PDZ and ww-interacting domains are not sufficient to stimulate the mitotic effect. This finding is consistent with the work of Durick *et al* reporting that LMP1 mediates the mitogenic signaling in mouse fibroblasts [24].

LMP1 gene and protein expression is regulated by TGF- β 1—It has not been clear about how *LMP1* gene expression is regulated at 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- β 1, BMP2, BMP6, and PDGF-BB. To test our hypothesis, PDL cells were treated by different growth factors and we first confirmed that all the growth factors can activate downstream signaling molecules, for instance, smad1/5/8 and smad2 (Fig. 5A). Consequently, we found that TGF- β 1 but not BMP-2/6/ stimulated *LMP1* gene expression (Fig. 5B). PDGF-BB also stimulated *LMP1* expression, however, the effect is very limited compared to TGF- β 1 (data not shown). We further confirmed that TGF- β 1-induced *LMP1* expression occurs in other osteoblast progenitor cells as well, such as hBMSCs and MG63 cells, even with much stronger effect (Suppl. Fig. 5). Of the doses that we tested, 2 ng/ml TGF- β 1 induced consistent high level *LMP1* gene expression (Fig. 5C). We next examined the temporal profile of *LMP1* in response to TGF- β 1 in PDL cells. *LMP1* expression was induced by 6 h following TGF- β 1 treatment, peaking at 24 h, and slowly returned to basal levels by 72 h (Fig. 5D). Using Western blotting, we further found that LMP1 protein was increased at 24 h post-treatment in PDL cells (data not shown). These results indicate that LMP1 is regulated by TGF- β 1 in PDL cells.

LMP1 knockdown attenuates the TGF- β 1 effect on PDL cell proliferation—The effect of TGF- β 1 on PDL cell proliferation appears to depend on TGF- β 1 dose and cellular context. In the PDL cells we used, TGF- β 1 stimulus significantly induced cell proliferation (Fig. 5E) and DNA synthesis (data not shown). Next, we evaluated whether LMP1 is involved in the TGF- β 1 effect on PDL cells proliferation. LMP1 knockdown of expression by shRNA in PDL cell resulted in a blockage of TGF- β 1 dependent proliferation (Fig. 5F). This further suggests that LMP1 may be involved in the proliferative effect of TGF- β 1 in the early stage of PDL differentiation.

TGF- β 1 induction of LMP1 is specifically mediated by TGF- β 1 receptors—TGF β 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 kinases (ALKs), have been divided into two categories: ALK-4, -5, and -7, corresponding to the TGF β /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- β 1 induced *LMP1* expression in PDL (Fig. 6A). These results suggest that TGF- β 1 induces *LMP1* expression through TGF- β RI activation. In order to rule out the non-specific effect of SB-431245, we used siRNA to knockdown ALK5 expression in PDL cells. Consistent with this was ALK5 knockdown compromised the *LMP1* expression stimulated by TGF- β 1 (Fig. 6B), although this effect couldn't be completely abolished. This result may be due to the redundancy of other TGF- β RI such as ALK4 and ALK7. It has been reported that SaOS2 cells possess type I but no type II TGF β receptors on the cell surface [25]. This led to the very limited effects by TGF- β 1 on cell proliferation and proteoglycan synthesis of SaOS2 cells. We found that *LMP1* is not induced by TGF- β 1 in SaOS2 cells that further supports that the TGF- β 1 effect on *LMP1* expression is specifically through TGF- β RI receptors.

TAK1-JNK/p38 cascade is involved in TGF- β 1 induction of *LMP1*—To further identify the signaling pathway that TGF- β 1 uses to stimulate *LMP1* gene expression, we first knocked down the canonical Smad signaling by siRNA. Unexpectedly, both Smad4 and Smad2 knockdown did not affect the up-regulation of *LMP1* after TGF- β 1 stimulation in PDL cells (Suppl. Fig. 6). It is known that activated TGF β receptors also trigger a Smad independent signaling pathway such as the mitogen-activated protein kinase (MAPK) signaling cascade [16, 26-28]. 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- β 1-induced *LMP1* gene expression (Suppl. Fig. 7). Consistent with it, another Ras-Erk signaling inhibitor PD98059 had very limited effect on TGF- β 1-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- β 1 was significantly blocked (Fig. 6C). 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- β RI is activated, TAK1 phosphorylates JNK and p38, but not Erk1/2 [29]. After siRNA transfection, the expression of *TAK1* was successfully knocked down ~90%. The gene knockdown of TAK1 inhibits the *LMP1* gene expression ~50% (Fig. 6D). Taken together, non-canonical pathways, particularly TAK1-JNK/p38 cascade, play an important role in TGF- β 1-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 identified that there is an AP-1 binding site in the *LMP1* promoter, which suggested that TGF- β 1 induction of *LMP1* requires *de novo* protein synthesis [30]. To test this, we utilized Cycloheximide (CHX) to inhibit protein synthesis. Pre-treatment with CHX 1 h prior to addition of TGF- β 1 effectively blocked *LMP1* mRNA induction in PDL cells (Fig. 6E). This is consistent with the observation that *LMP1* is not an early response gene of TGF- β 1, and *LMP1* mRNA begins to increase several hours after TGF- β 1 treatment. Taken together, the activation of TAK1-JNK/p38 kinase cascade is used by TGF- β 1 to regulate *LMP1* gene expression.

Discussion

Although it has been reported that *LMP1* play a role in osteoblast differentiation [4,31,32], 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 explored 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 [33]. 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 [1,24]. On the other side, overexpression of a truncated form of LMP1 without LIM domains inhibited the mitogenic effect of Ret/ptc2 [24]. 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 complex process involving cell proliferation, 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 of Boden and co-workers [2]. Contrastingly, when LMP1 was stably overexpressed in PDL cells, it did not promote mineralization nodule formation *in vitro* and bone formation *in vivo* (data not shown). We found that constitutional expression of LMP1 tends to inhibit mineralization in PDL cells. Future studies will need to better determine the role of LMP1 in affecting PDL cell-mediated mineralization *in vivo*. One possible explanation is that LMP1-overexpressed PDL cells tend exist in a proliferative state, thus delaying the cell's transit into a differentiation state to allow subsequent mineralization of the matrix.

To date, the regulatory mechanism of *LMP1* remains “enigmatic”. Because of the up-regulated expression of LMP1 in early stages of MSC osteogenesis and the significant effect of LMP1 in PDL progenitor cell proliferation, we hypothesized that *LMP1* is regulated by some of mitogenic growth factors in the early signals of osteogenesis. It is widely accepted that TGF- β 1 stimulates MSC proliferation during endochondral ossification and the early phase of bone fracture healing [10]. In this study we identified that *LMP1* is a downstream gene of TGF- β 1 in human MSCs including PDL cells, bone marrow MSCs and the preosteoblast cell line, MG63 (Fig. 5, Suppl. Fig. 5). It is worth mentioning that TGF- β 1 induces ~10 fold increase of LMP1 expression in MG63, and it would be interesting to further investigate its biological mechanisms. Boden *et al.* reported that *LMP1* is regulated by BMP6 in rat calvarial osteoblasts [2], however, none of the BMPs that we tested were able to stimulate *LMP1* expression in PDL cells and hBMSCs (data not shown). Although *LMP1* may indeed respond to different TGF β superfamily members in different species, our data suggests that TGF- β 1, but not BMPs, is the main regulator of *LMP1* gene expression in human preosteoblastic cells. In addition to TGF- β 1, PDGF-BB is a mitogenic growth factor involved in periodontium development and regeneration [19,34,35]. The fact that LMP1 is regulated by TGF- β 1 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- β 1 regulates *LMP1* gene expression. We found that it appears that canonical Smad signaling is not involved in TGF- β 1-induced LMP1 expression, however, TAK1-JNK/p38 cascade mediates the TGF- β 1 effect. Here, our data suggest a possible model of TGF- β 1-induced *LMP1* gene regulation (Fig. 6F). TGF- β 1 ligand binding activates type II and I receptors, and then stimulates TAK1 activation that 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- β 1-

induced *LMP1* expression is independent of Ras-Erk signaling and Smad signaling pathways. Bioinformatics finding also supports this model since there is an AP-1 binding site in the *LMP1* promoter, whereas no Smad binding site is found [30]. 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 [36,37]. Of note, other TGF- β 1 downstream genes have been found to be regulated by Smad independent, JNK and/or p38 dependent, pathways. For example, TGF- β 1 induces fibronectin synthesis through JNK dependent but a Smad4 independent pathway [38]; p38 signaling is used by TGF- β 1 to induce connexin43 gene expression in normal murine mammary gland epithelial cells and these effects are Smad-independent [39]. However, it is still plausible that other signaling pathways, such as RhoA and PP2A, are involved in the *LMP1* gene regulation.

In addition to *LMP1*'s role in osteogenesis, it 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 with 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 adipogenesis.

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*, and define a regulatory mechanism of *LMP1* in PDL progenitor cells and other MSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TGF-β1	transforming growth factor β-1
PDGF	platelet-derived growth factor
PDL	periodontal ligament
hBMSC	human bone marrow stromal cell
siRNA	small interfering RNA
shRNA	small hairpin RNA
JNK	c-Jun N-terminal Kinase
ERK	extracellular signal-regulated kinase
MAPK	mitogen-activated protein kinase
Smad2	SMAD family member 2

Smad4	SMAD family member 4
CHX	Cycloheximide
ALP	alkaline phosphatase

Fig. 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. (n=3 per group) (B) The LMP1 protein expression is shown by Western Blot. Relative expression ratios after normalization to GAPDH are shown at the bottom.

Fig. 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. (A) *LMP1* mRNA expression was evaluated by RT-qPCR. (B) *LMP1* stably knocked down and control PDL cells were seeded in 6-well-plates at low density ($3 \times 10^3/\text{cm}^2$). 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. (C) At day 10, cells were fixed and stained with crystal violet. (D) Subsequently, the crystal violet staining was washed and quantified. The optical density readout which correlates to cell numbers are shown. (E) *LMP1* stably knocked down and control PDL cells were seeded in 12-well-plate at $3 \times 10^3/\text{cm}^2$ and cultured in osteogenic induction media. ³H methyl thymidine was added after overnight attachment. At 5 d, the DNA was harvested and the ³H methyl thymidine incorporation was measured by scintillation counter. (n=4 per group). (F) 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).

Fig. 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 ($3 \times 10^3/\text{cm}^2$). 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 (C). 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.

Fig. 4. PDZ and ww-interacting domains are not enough 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 ($3 \times 10^3/\text{cm}^2$). Cells were harvested by trypsin and counted using hemocytometry, $n=6$ per group. (D)

PDL cells were seeded in 6-well-plate at $3 \times 10^3/\text{cm}^2$ and ^3H methyl thymidine was added. At 5 d, the DNA was harvested and the ^3H methyl thymidine incorporation was measured by scintillation counter. (n=4 per group)

Fig. 5. LMP1 knockdown attenuates the TGF-β1 effect on PDL cells proliferation

(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. n=3 per group. (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- β 1. Closed bar: with TGF- β 1. **: $p < 0.01$ compared to serum-free control at the same time point. (E) PDL cells were cultured in DMEM with low serum concentration (2% FBS), and some cells were treated by TGF- β 1. 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, $n=3$ per group. *: $p < 0.05$, **: $p < 0.01$. (F) LMP1 knockdown PDL cells and control cells were cultured in 6-well-plates in 2% FBS, with/without TGF- β 1. 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- β 1 and TGF- β 1 treatment ($n=3$ per group).

Fig. 6. TAK1-JNK/p38 cascade is involved in TGF-β1 induction of LMP1

(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, 1 μM. SB(10): SB431532 in DMSO, 10 μM. 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) 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. SB203580: p38 inhibitor, 25 μ M. SP600125: JNK inhibitor, 25 μ M. (D) 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- β 1 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- β 1 treatment; siTAK1: TAK1 siRNA; siTAK1 + T: TAK1 siRNA and TGF- β 1 treatment. (E) Confluent PDL cells were cultured in serum-free media for 24 h. Before adding TGF- β 1 (2 ng/ml), PDL cells were pretreated by cycloheximide (CHX) for 2 h. 24 h after TGF- β 1 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 10 μ M; CHX(25): cycloheximide 25 μ M. (F) Schematic overview of the regulation of LMP1 gene expression by TGF- β 1. (n=3 per group in each experiment).

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

	G1	S	G2/M
Control shRNA	36.17 ± 1.41 ^{a,b}	45.28 ± 1.37 ^{a,b}	18.54 ± 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