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Function of SLPI on the collagen synthesis and mineralization of mouse periodontal ligament fibroblasts cultured on titanium surface for development of periodontio-integrated implant

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치주인대접목 임플란트 개발을 위한 티타늄 표면에서 생쥐 치주인대 섬유모세포의 아교질 합성과 광화에 대한 SLPI의 기능

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초 록

치주인대접목 임플란트 개발을 위한 티타늄 표면에서 생쥐 치주인대 섬유모세포의 아교질 합성과 광화에 대한 SLPI의 기능

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분비백혈구단백분해효소억제제(secretory leukocyte protease inhibitor, SLPI)는 상처 치유와 세포증식을 촉진하고, 치주염을 포함한 염증조직에서 이틀뼈 흡수를 억제시킨다. SLPI는 상아질 기질 형성 관련 단백질의 신호 조절에 관여하며, 티타늄 표면에서 뼈모세포의 부착과 세포 생존율을 높이며 분화와 광화를 촉진하는 것으로 알려져 있다. SLPI는 티타늄 표면에서 치주인대 섬유모세포(periodontal ligament fibroblast, PDLF)의 부착을 촉진하고 염증을 억제했다. 따라서, 본 연구에서는 티타늄 표면에 부착된 치주인대 섬유모세포의 증식, 아교섬유 및 석회화에 관련된 분자들의 발현과 이와 관련된 신호전달과정에서 SLPI의 기능을 규명하고자 했다.

본 연구에서, 분화 배지를 사용하여 4일부터 10일까지 티타늄 표면에서 배양된 치주인대 섬유모세포에서 SLPI를 처리한 군(SLPI/PDLF)의 세포증식은 대조군(PDLF)에 비해 유의적인 수준으로 증가하였다. 분화 배지에서 배양된 치주인대 섬유모세포에서 Ras, pERK1/2, pElk-1, c-fos, Runx-2 및 Osx 발현은 시간 의존적으로 증가하였으며, SLPI/PDLF에서 이들 단백질 발현은 PDLF에 비하여 유의적으로 높았다. SLPI를 처리한 치주인대 섬유모세포(SLPI/PDLF)에서 Ras, c-fos, Runx-2, Osx 단백질의 발현 및 ERK1/2와 Elk-1의 인산화는 대조군에 비해 증가하였으며, MEK 억제제 PD98059를 처리한 치주인대 섬유모세포(PD/PDLF)에서 Ras를 제외한 나머지 단백질의 발현 및 인산화 수준은 SLPI/PDLF에 비하여 감소하였다. 치주인대 섬유모세포는 분화 배지에서

시간 경과에 따라 Picro-Sirius red, ALP 발현 및 Alizarin red S 등이 증가하였으며, SLPI에 의해 이들의 발현은 더욱 증가되었다. 분화 배지에서 배양된 치주인대 섬유모세포에서 ALP, BSP, DSPP, DMP-1, OCN, ON 및 Col I 발현은 시간 의존적으로 증가하였으며, SLPI/PDLF에서 이들 mRNA 발현은 PDLF에 비하여 유의적으로 높았다.

따라서, 본 결과들은 SLPI가 티타늄 표면의 치주인대 섬유모세포에서 Ras를 통해 ERK1/2, Elk-1, c-fos를 조절하여 분화와 1형 교원질 합성을 촉진하며, Runx-2와 Osx를 조절하여 뼈기질의 형성과 광화를 촉진시키는 신호 분자임을 보여준다.

이러한 결과로서 SLPI는 치주인대 섬유모세포(치주인대접목임플란트)를 이용할 경우 임플란트 식립 후 뼈모세포에 의한 뼈유착이 형성되기 전에 교원질의 분비와 광화의 촉진으로 티타늄 표면과 뼈 사이에 치주인대 섬유를 포함한 인공적인 치아주위조직의 형성을 촉진시킬 수 있는 중요한 조절 인자 중 하나로서 제시할 수 있다.

ABSTRACT

Function of SLPI on the collagen synthesis and mineralization of mouse periodontal ligament fibroblasts cultured on titanium surface for development of periodontio-integrated implant

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Secretory leukocyte protease inhibitor (SLPI) promotes wound healing and cell proliferation and inhibits bone resorption in inflammatory tissues including periodontitis. SLPI is known to be involved in the signal regulation of proteins related to dentin matrix formation, and to promote differentiation and mineralization while increasing the adhesion and cell viability of osteoblasts on titanium (Ti) surface. SLPI promoted the adhesion of periodontal ligament fibroblasts (PDLFs) on the Ti surface and suppressed inflammation. Therefore in this study, the purpose was to investigate the function of SLPI in the expression of molecules related to proliferation, collagen fibers and calcification, and in the related signal transduction process in PDLF attached to the Ti surface.

In this study, cell proliferation of the SLPI-treated group (SLPI/PDLF) in PDLFs cultured on Ti surfaces from 4 to 10 days in a differentiation medium was significantly increased compared to the control group (PDLF). Expressions of Ras, pERK1/2, pElk-1, c-fos,

Runx-2 and Osx in PDLF cultured in the differentiation medium increased in a time-dependent manner and the expression of these proteins in SLPI/PDLF was significantly higher than that in PDLF. In SLPI/PDLF, expression of Ras, c-fos, Runx-2 and Osx protein and phosphorylation of ERK1/2 and Elk-1 were increased compared to the control group. In MEK inhibitor, PD98059-treated PDLFs (PD/PDLF), expression and phosphorylation levels of other proteins except Ras were decreased compared to SLPI/PDLF. In PDLF, expression of Picro-Sirius red, ALP, and Alizarin red S increased over time in the differentiation medium and their expression was further increased by SLPI. Expression of ALP, BSP, DSPP, DMP-1, OCN, ON and Col I in PDLF cultured in the differentiation medium increased in a time-dependent manner and expression of these mRNAs in SLPI/PDLF was significantly higher than that in PDLF.

Therefore these results show that SLPI is a signal molecule that promotes differentiation and Col I protein synthesis by controlling ERK1/2, Elk-1 and c-fos through Ras in PDLF on the Ti surface and promotes the formation and mineralization of bone matrix by controlling Runx-2 and Osx.

As a result, when using PDLFs (periodontio-integrated impant) SLPI will be presented as one of the important control molecules that can promote the formation of artificial periodontium including periodontal ligament fibers between Ti surface and bone by promoting the secretion and mineralization of collagen before osseointegration is formed after implant placement.

INTRODUCTION

Dental implants, which are osseointegrated implants, lack of periodontal ligament (PDL), causing various biomechanical problems due to reduced stress absorption and concentration phenomenon and the host defense mechanism against external harmful stimuli such as bacterial infection is reduced and then periodontal tissue destruction proceeds at a faster rate than natural teeth (Schou et al., 1993; Gulati et al., 2014). Therefore, in order to compensate for the disadvantages of these implants, studies on cells related to periodontal tissue regeneration and osseointegration, and the periodontio-integrated implant (ligaplant), which attempts to regenerate periodontal tissue by implanting PDL, on the surface of the implant in a tissue engineering method are being actively conducted (Gault et al., 2010; Choi et al., 2015; Jeong et al., 2015a; Lee et al., 2017; Washio et al., 2018).

The PDL is a highly differentiated cellular connective tissue that is located between the cementum and the alveolar bone to attach and support the teeth to the alveolar bones, resist various mechanical stimuli such as masticatory forces, and perform sensory functions (Cho and Gagant, 2000). Periodontal ligament fibroblasts (PDLFs) are the most abundant cells in the PDL and are known to be responsible for tissue homeostasis and regeneration through the synthesis and decomposition of extracellular matrix including collagen in the PDL (Kook et al., 2009). In addition, it has been reported that PDLF exhibits high alkaline phosphatase (ALP) activity and characteristics similar to osteoblasts, such as secretion of bone matrix protein and formation of calcified nodules (Cho et al., 1992; Basdra and Komposch, 1997).

Molecules of signaling pathways involved in bone formation interact with each other and play an important role in the proliferation and differentiation of osteoblasts. Extracellular signal-regulated kinase (ERK), one of the mitogen-activated protein kinase (MAPK) family molecules, is widely expressed in cells and is involved in gene expression in the regulation of various cellular processes such as proliferation, differentiation, migration, and survival (Shen et al., 2018). Most of the exogenous

growth factors involved in bone formation, such as bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor (FGF), bind to the surface receptors of osteoblasts and activate the intracellular ERK signaling pathway to stimulate proliferation and differentiation of osteoblasts (Chau et al., 2009). Ras-ERK signal pathway activated by growth factor stimulation promotes cell proliferation and survival by phosphorylating ternary complex factor (TCF) Elk-1 to form ternary complex and increase *c-fos* gene activity (Treisman, 1994). ERK regulates osteoblast differentiation by inducing phosphorylation and stabilization of runt-related transcription factor 2 (Runx-2) and osterix (Osx), which are osteoblast specific transcription factors (Choi et al., 2011a; Artigas et al., 2014). In addition, it has been reported that Elk-1 increases the Runx-2 promoter activity in the late stage of osteoblast differentiation (Zhang et al., 2009).

C-fos, an immediate early gene expressed in response to extracellular stimuli such as growth factors and cytokines, which is dimerize with c-jun to form an activator protein-1 (AP-1) transcription complex, and regulates a variety of cellular processes including cell growth, differentiation, transformation, and apoptosis (Tu et al., 2013). In particular, c-fos is known to be essential for bone development by affecting the proliferation and differentiation of osteoblasts and osteoclasts. Also, c-fos was expressed in osteoblasts in the growth region of fetal bone during embryonic development and specifically in osteoblasts in ossifying calluses during fracture healing (Dony and Gruss, 1987; Oyama et al., 1998). Also, osteopetrosis occurred in c-fos deficient mice due to blockage of the differentiation of osteoclast but osteosarcoma and chondrosarcoma were formed in c-fos overexpressed transgenic mice (Wang et al., 1991; Grigoriadis et al., 1994).

Runx-2 and Osx are transcription factors essential for osteoblast differentiation and bone formation and Runx-2 is important for osteoprogenitor formation from mesenchymal stem cells and Osx is involved in the differentiation of Runx-2 expressed progenitor cells into mature and functional osteoblasts (Komori, 2006). Fetuses from Runx-2 knockout mice die during development because neither endochondral ossification nor intramembranous ossification occurs. In Osx deficient mice, osteoblast differentiation is inhibited and bone formation is not achieved.

Moreover, Runx-2 was normally expressed in mesenchymal cells of Osx null mice, but Osx expression was not observed in Runx-2 deficient mice (Nakashima et al., 2002; Komori, 2006). Runx-2 and Osx induce differentiation of osteoblasts by regulating the expression of bone forming genes including type I collagen (Col I), ALP, osteonectin (ON), osteocalcin (OCN) and bone sialoprotein (BSP) (Komori, 2006; Zhang, 2010). In addition, it was reported that Runx-2 and Osx play an important role in tooth formation by stimulating the activation of dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) genes required for the differentiation of odontoblasts and cementoblasts (Chen et al., 2005; Cao et al., 2012).

Bone formation is initiated from osteoblasts formed through differentiation stage from mesenchymal stem cells, and differentiation of osteoblasts is divided into proliferation, extracellular matrix maturation and calcification, and each stage is accompanied by the expression of specific genes. Osteoblasts produce growth and extracellular matrix related genes c-fos and Col I during proliferation, and express genes related to bone matrix formation such as ALP, OCN, and BSP in the early stages of differentiation including extracellular matrix maturation. And OCN and ON genes are increased in osteoblasts in the late stage of differentiation where calcification of the extracellular matrix occurs, and calcium is deposited in the secreted extracellular matrix to form calcified nodules (Miron and Zhang, 2012; Shupp et al., 2018).

Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor such as neutrophil elastase, which promotes wound healing and cell proliferation, and inhibits the expression of pro-inflammatory cytokines and bone resorption in various inflamed tissues and periodontitis tissues (Song et al., 1999; Ashcroft et al., 2000; Angelov et al., 2004; Lee et al., 2016). SLPI induces cancer cell metastasis by increasing cell proliferation, migration, and invasion in oral squamous cell carcinoma and as a signaling molecule, in gastric cancer cells, it promotes cell migration and metastasis by increasing the expression of MMP-2, -9 (Matrixmetalloproteinase-2, -9) through Elk-1 phosphorylation (Choi et al., 2011b; Wang et al., 2011). SLPI is involved in the signal regulation of dentin matrix formation related proteins during

the differentiation and mineralization of odontoblasts (Jeong et al., 2015b), and induces the expression of MMP-2 and MMP-9 through the PKC-δ/ERK/Elk-1 signaling pathway to promote the migration of odontoblasts (unpublished data). In addition, it is known that SLPI promotes the adhesion of osteoblasts on the titanium (Ti) surface to increase cell viability, and promotes differentiation and mineralization of osteoblasts by increasing the expression of genes related to bone matrix formation (Jeong et al., 2015a; Choi et al., 2016). And, it has been reported that SLPI promotes the expression and mineralization of bone forming genes such as Runx-2, ALP, and Col I in human periodontal ligament cells of force-induced tooth movement (Lee et al., 2021).

In our previous study, SLPI promoted the adhesion of PDLF to the Ti surface and effectively inhibited the inflammatory response (unpublished data). From these results, it has been suggested that SLPI may regulate the proliferation, differentiation and mineralization after attachment of PDLF. PDLF not only synthesizes and secretes PDL fibers in the healing and regeneration process of periodontal tissue, but also secretes bone matrix proteins and proteins related to mineralization, which is thought to play an important role in the healing and regeneration of alveolar bone and cementum.

For the reason of this characteristics, it is in a necessary state to study the applicability of the periodontio-integrated implant using PDLF and the discovery of biological molecules that can control the functions of this cell. Therefore, in this study, the function and mechanism of SLPI in the process of differentiation and mineralization of PDLF attached to the Ti surface was to investigated.

MATERIALS AND METHODS

1. Titanium

Three types of Ti discs, 15 mm, 20 mm and 48 mm in diameter and 2 mm in thickness, were used. The polished commercially pure titanium (Cp-Ti) discs were prepared using a previously described method (Jeong et al., 2015a).

2. Cell culture and differentiation

The mouse periodontal ligament fibroblasts (PDLFs) were cultured in Dulbecco's modified eagle's medium (DMEM) (WelGENE, KOR) containing 10% fetal bovine serum (FBS; WelGENE) and 1% antibiotic antimycotic solution (WelGENE). The cells were transferred to a Ti surface and changed to a differentiation medium (DMEM supplemented with 5% FBS, 10 mM β-glycerol phosphate, 50 ㎍/㎖ ascorbic acid and 1% antibiotic antimycotic solution) with or without 1 ㎍/㎖ recombinant human (rh)SLPI (R&D systems, USA) after 24 h. The cells were placed into a humidified chamber maintained in an atmosphere containing 5% CO_2 at 37℃.

3. Treatment of inhibitor and SLPI

The PDLFs (1×10^6 cells/㎖) were transferred onto Ti surface and then serum starvation was carried out for 16 h. After serum starvation, 1 ㎍/㎖ of SLPI was treated to the PDLFs (SLPI/PDLF) for 10 min. A 5 μM MEK inhibitor PD98059 (Sigma-Aldrich, USA) was treated to the PDLFs (PD/PDLF) for 1 h. In addition, PD98059 was pretreated to the PDLFs for 1 h before the SLPI treatment (SLPI/PD/PDLF).

4. Cell proliferation assay

The PDLFs plated on the Ti discs (5×10^4 cells/㎖) were incubated for 4, 7 and 10 days in differentiation medium with or without 1 ㎍/㎖ of the SLPI. A 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was performed to examine the cell viability, as described previously (Jeong et al., 2015a).

5. Western blotting

The protein was extracted using NP-40 lysis buffer. Protein concentration was determined by DC protein assay kit (Bio-Rad Laboratories, USA). A 25 ㎍ of total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, GER). The membrane was blocked with 5% bovine serum albumin (BSA; Bioshop, USA) or 5% skim milk (Bioshop) for 1 h at room temperature and blotted with the primary antibodies for 16 h at 4℃, such as 1:1,000 of anti-rabbit SLPI (Takara, Japan), 1:1,000 of anti-mouse Ras (Upstate, USA), 1:2,500 of anti-rabbit phospho-ERK1/2 (pERK1/2, Cell signaling technology, USA), 1:2,500 of anti-rabbit ERK1/2 (Upstate), 1:1,000 of anti-mouse phospho-Elk-1 (pElk-1, Cell signaling technology), 1:1,000 of anti-mouse c-fos (Proteintech, USA), 1:1,000 of anti-rabbit Runx-2 (abcam, UK), 1:1,000 of anti-rabbit osterix (abcam), and 1:2,500 of anti-mouse β-actin (Santa Cruz Biotechnology, USA). β-actin was used as the internal control. After washing, the membrane was blotted with 1:5,000 of either horseradish peroxidase (HRP)-conjugated goat anti-rabbit or mouse-IgG (Enzo Life Sciences, USA) for 1 h at room temperature. The blots were detected with enhanced chemiluminescence reagents (Merck Millipore) and the band densities were quantified with Science Lab Image Gauge (Fuji Film, Japan).

6. ALP staining, collagen secretion and extracellular matrix mineralization

The PDLF plated on the Ti discs (1×10^4 cells/㎖) were incubated for 4, 7 and 10 days in differentiation medium with or without 1 ㎍/㎖ of the SLPI. The cells were washed with phosphate buffered saline (PBS) followed by fixation in 4% paraformaldehyde for 10 min. ALP staining was performed with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) ALP substrate solution for 1 h at 37℃ in a dark room. The samples were washed thoroughly with distilled water (DW) to acquire the images. For the quantitative analysis, the stain

was dissolved in 10% cetylpyridinum chloride (CPC) in 10 mM sodium phosphate, and the optical density at 410 nm was measured using a microplate reader (BioTek, USA). Collagen secretion was stained by Picro Sirius Red (0.1% in saturated picric acid) for 1 h. The unbound stain was removed in 0.5% acetic acid, and then images were captured using a stereo microscope (Carl Zeiss, GER). To quantitatively assess the collagen secretion, the stain on the samples was eluted in destain solution (0.1 M NaOH) and the absorbance was measured at 550 nm. Extracellular matrix (ECM) mineralization was stained with 2% Alizarin Red S (pH 4.2) for 15 min. After thorough washing with DW, the images were taken by stereo microscope (Carl Zeiss). To quantify mineralization, bound dye was extracted in 10% CPC in 10 mM sodium phosphate and quantified by spectrophotometric absorbance at 562 nm.

7. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells with RiboEX^{TM} reagent (GeneAll, KOR) according to the manufacturer' s instructions. Total RNA was quantified using an nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Total RNA (1 μg) was used to synthesize the complementary DNA (cDNA) with RT Premix (GeNet Bio, KOR). The PCR reaction was conducted in a thermocycler (Takara, Japan) by using a PCR premix (Bioneer, KOR) and 1 $\mu\ell$ cDNA. The mouse gene specific primers were designed using the nucleotide sequences of alkaline phosphatase (ALP), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein 1 (DMP-1), osteocalcin (OCN), osteonectin (ON), collagen type I (Col I) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The following primers were used for PCR amplification: ALP forward, 5′-AAG ACG TGG CGG TCT TTG C-3′ and reverse, 5′-GGG AAT CTG TGC AGT CTG TG-3′; BSP forward, 5′-ACC GGC CAC GCT ACT TTC TTT AT-3′ and reverse, 5′-TCC TCG TCG CTT TCC TTC ACT TT-3′; DSPP forward, 5′-CGA CCC TTG TCC AGG A-3′ and reverse, 5′-CAT GGA CTC GTC ATC GAA-3′; DMP-1 forward, 5′-CGA GTC TCA GGA GGA CA-3′ and reverse, 5′-CTG TCC TCC TCA CTG GA-3′; OCN forward 5′-TGA GGA CCC TCT CTC TGC TC-3′

and reverse 5′-GAG CTC ACA CAC CTC CCT GT-3′; ON forward 5′-ATT TGA GGA CGG TGC AGA GG-3′ and reverse 5′-TCT CGT CCA GCT CAC ACA CCT-3′; Col I forward, 5′-ATT CGG AGC TCA AGA TGT AA-3′ and reverse, 5′-CAG TCA AGT CCT AGC CAA AC-3′; GAPDH forward, 5′-CCA TGG AGA AGG CTG GG-3′ and reverse, 5′-CAA AGT TGT CAT GGA TGA CC-3′. All primers were synthesized by Bioneer (Daejeon, KOR). The annealing temperature for each primer and number of cycles were as follows: ALP 62 ° C and 30 cycles; BSP, 60 ° C and 30 cycles; DSPP, 56 ° C and 35 cycles; DMP-1, 55 ° C and 35 cycles; OCN, 53 ° C and 30 cycles; ON, 50 ° C and 35 cycles; Col I, 50 ° C and 35 cycles; and GAPDH, 60 ° C and 30 cycles. GAPDH was used as the internal control. The density of the expressed bands was measured using Science Lab Image Gauge (Fuji Film).

8. Statistical Analysis

All experiments were carried out in triplicate. All data is reported the mean and standard deviation determined using SPSS 25.0 (SPSS, USA). The significant differences ($*p < 0.05$) were determined using an independent samples t-test.

RESULTS

1. Proliferation on the SLPI-treated PDLF during differentiation

The proliferation of differentiation-induced periodontal ligament fibroblasts (PDLFs) on the titanium surface increased with time from 4 to 10 days in PDLF and SLPI-treated periodontal ligament fibroblasts (SLPI/PDLF). And compared with PDLF, SLPI/PDLF showed significance on days 7 and 10, and increased approximately 1.2 times (Fig. 1).

2. Effect of SLPI on the protein expression on the proliferation and differentiation in PDLF and MEK inhibitor treated PDLF during differentiation

The expression of SLPI, Ras, pERK1/2, pElk-1, c-fos and osterix proteins showed a gradual increase from day 4 to 10 in PDLF and SLPI/PDLF, and that expression was significantly higher in SLPI/PDLF than in PDLF at all time points. SLPI protein expression was 1.9, 1.8, and 1.5 times higher in SLPI/PDLF at day 4, 7, and 10, respectively, compared to PDLF. The expression of Ras was 1.2, 1.3, and 1.1 times higher in SLPI/PDLF than in PDLF on days 4, 7, and 10, respectively. Phosphorylation of ERK1/2 was 1.3, 1.4, and 1.2 times higher than that of PDLF on days 4, 7, and 10 of SLPI/PDLF, respectively. pElk-1 protein expression was 1.2, 1.3, and 1.1 times higher in SLPI/PDLF than in PDLF at 4, 7, and 10 days, respectively. In addition, c-fos protein expression was 1.3 times higher at 4 and 7 days and 1.2 times higher at 10 days in SLPI/PDLF compared to PDLF. The expression of osterix protein was 1.2, 1.4, and 1.1 times higher in SLPI/PDLF than in PDLF at 4, 7, and 10 days, respectively. Runx-2 protein expression increased up to 7 days in PDLF and decreased slightly at 10 days, but gradually increased up to 10 days in SLPI/PDLF. Also, Runx-2 was expressed significantly higher in SLPI/PDLF than in PDLF from day 4 to day 10 (Fig. 2A and 2B).

The expression of Ras, pERK1/2, pElk-1, c-fos, Runx-2 and osterix proteins in SLPI/PDLF was significantly increased compared to that of PDLF, and the

expression of these proteins except Ras was effectively suppressed in PD98059-treated periodontal ligament fibroblasts (PD/PDLF). The expression of these proteins in PD/PDLF was lower than that of SLPI/PDLF, and in periodontal ligament fibroblasts treated with SLPI after PD98059 pre-treatment (SLPI/PD/PDLF) increased compared to PD/PDLF, but decreased compared to SLPI/PDLF.

The protein expression of Ras was 1.1 times higher in SLPI/PDLF and 1.0 times lower in PD/PDLF than in PDLF. The protein expression of Ras in PD/PDLF was 1.1 times lower than that of SLPI/PDLF, and in SLPI/PD/PDLF, it was 1.1 times higher than that of PD/PDLF and 1.0 times lower than that of SLPI/PDLF. Phosphorylation of ERK1/2 was 1.3 times higher in SLPI/PDLF and 1.3 times lower in PD/PDLF than in PDLF. In PD/PDLF, phosphorylation of ERK1/2 was 1.7 times lower than that of SLPI/PDLF, and in SLPI/PD/PDLF, it was 1.2 times higher than that of PD/PDLF, and 1.5 times lower than that of SLPI/PDLF. Elk-1 phosphorylation was 1.5 times higher in SLPI/PDLF and 1.6 times lower in PD/PDLF than in PDLF. In PD/PDLF, phosphorylation of Elk-1 was 2.4 times lower than that of SLPI/PDLF, and in SLPI/PD/PDLF, it was 1.1 times higher than that of PD/PDLF and 2.1 times lower than that of SLPI/PDLF. The protein expression of c-fos, Runx-2 and osterix was increased 1.2 times in SLPI/PDLF compared to PDLF, and decreased by 1.8, 1.7, and 1.6 times in PD/PDLF, respectively. In PD/PDLF, expression of these proteins was decreased by 2.2, 2.1, and 1.9 times compared to SLPI/PDLF, respectively, and in SLPI/PD/PDLF, it increased by 1.2 times compared to PD/PDLF and decreased by 1.8, 1.7, and 1.7 times compared with SLPI/PDLF, respectively (Fig. 3A and B).

3. ALP expression, collagen secretion and nodule formation in SLPI-treated PDLF during differentiation

ALP expression, collagen secretion, and calcification nodule formation were similarly increased from day 4 to day 10 in both PDLF and SLPI/PDLF groups after differentiation induction, and SLPI/PDLF increased significantly from day 4 to 10 compared to PDLF (Figs. 4, 5 and 6).

ALP expression in SLPI/PDLF was 1.4, 1.3, and 1.5 times higher, respectively,

compared to that of PDLF on days 4, 7, and 10 (Fig. 4A and B). From 4 days after differentiation, the degree of Picro-Sirius Red staining increased in both PDLF and SLPI/PDLF, and from 4 to 10 days, collagen secretion in SLPI/PDLF was 1.1 times higher than that of PDLF (Fig. 5A and B). In Alizarin Red S staining, the number and size of nodules scattered in PDLF and SLPI/PDLF on the 4th day after differentiation showed an increasing pattern after 7 days. Calcification nodule formation was 1.3, 1.2, and 1.2 times higher in SLPI/PDLF days 4, 7, and 10, respectively, compared to PDLF (Fig. 6A and B).

4. Expression of non-collagenous and Col I gene in SLPI-treated PDLF during differentiation

mRNA expression of ALP, BSP, DSPP, DMP-1, OCN, ON and Col I was significantly increased in SLPI/PDLF compared to PDLF at all time points. ALP and Col I mRNA expression increased 1.1 times in SLPI/PDLF compared to PDLF at all time points, and BSP mRNA expression increased 1.3, 1.2, and 1.1 times from day 4 to 10. In SLPI/PDLF, DSPP mRNA expression increased 1.3, 1.1, and 1.1 times on days 4, 7, and 10, respectively, compared to PDLF, and DMP-1 mRNA expression increased by 1.1, 1.2, and 1.1 times. In SLPI/PDLF, OCN mRNA expression increased 1.2, 1.1, and 1.1 times compared with PDLF from day 4 to 10 and ON mRNA expression increased by 1.2, 1.1, and 1.1 times (Fig. 7A and B).

5. Illustration of SLPI signaling transduction pathway in differentiation and mineralization of PDLF on the titanium surface.

Based on these results, Figure 8 presents SLPI promotes differentiation and collagen synthesis by ERK1/2, Elk-1, Runx-2 and c-fos through Ras signaling pathway and promotes the formation and mineralization of bone matrix by Runx-2 and Osx in PDLF on the Ti surface.

DISCUSSION

Dental implants are prosthetics, an artificial implant that replaces the function of defective teeth by placing directly in alveolar bones. In addition, close bone adhesion between bone and implant interfaces is required to achieve long-term successful clinical results as a treatment method widely used to restore function and aesthetics, which are the ultimate goals of prosthetic treatment. Recently, as the society enters an aging society, the demand for dental implant treatment of the elderly and patients with systemic diseases is rapidly increasing. In particular, in the case of systemic diseases, the ability to heal wounds is poor, so alveolar bone regeneration is not well performed, and it takes a long time to complete after implant placement. Therefore, since there is a limit to simple mechanical and chemical surface treatment of the implant surface, and research on dental implants is being actively conducted by fusing bioactive substances such as bone morphogenetic proteins (BMPs) and growth factors that can improve bone quality overall through rapid osteoconduction and osteoinduction (Pugdee et al., 2007; Chen et al., 2013; Lee et al., 2014; Teng et al., 2016). Growth factors including recombinant human BMP-2 (rhBMP-2) are easily decomposed by enzymes in tissues, so the duration of function is limited when applied to the human body, and the high concentration and high dose applied during treatment are known to cause various complications and toxicity (Rosa-Mar¡ a et al., 2015). Therefore, there is a need for additional research on the safety of treatment and new physiologically active substances that can replace them. Recently, the development of implant products using various peptides has been studied, but it takes a lot of time and effort to commercialize them as they are still in their early stages.

Dental implants are one of the best treatments considered first in repairing defective teeth, but they have some fundamental limitations due to the absence of periodontal ligament (PDL). Implants do not have a regenerative function, and destruction of the surrounding alveolar bone may occur due to excessive occlusal

force. In addition, the implant is directly attached to the bone, making it impossible for physiological and corrective movement of teeth, and are vulnerable to infection (Beertsen et al., 1997). Recently, research on a periodontio-integrated implant that can be physiologically and functionally maintained by grafting a PDL to an implant to compensate for the drawbacks of such osseointegrated implants has been conducted. The results of various studies on periodontio-integrated implants suggest a new treatment method that can extend the life of implants by overcoming the disadvantages of osseointegrated implants and improving PDL regeneration and biological functions.

PDL is a fibrous connective tissue that constitutes periodontal tissue and has nutrition, homeostasis, regeneration, sensation, and defense functions in addition to tooth support functions. The constituent cells of the PDL are derived from the ectomesenchyme of the dental organ and are composed of various cells such as fibroblasts, osteoblasts, osteoclasts, cementoblasts, epithelial cell rests of Malassez, and undifferentiated mesenchymal cells (Lekic and McCulloch, 1996). In particular, fibroblasts are a major cell type present in the PDL and are involved in the self-renewal of the PDL and the regeneration of periodontal tissue through the production and degradation of collagen and extracellular matrix components (Kook et al., 2009). Periodontal ligament fibroblasts (PDLFs) have similar characteristics to osteoblast and cementobalst, such as expressing bone matrix proteins such as alkaline phosphatase (ALP), osteocalcin (OCN), bone sialoprotein (BSP), and osteopontin (OPN), forming mineralized nodules *in vitro*, and forming cementum-like tissues *in vivo* (Cho et al., 1992; Ivanovski et al., 2001; Marchesan et al., 2011). In addition, PDLFs mainly produce osteoprotegerin (OPG), a osteoclast formation inhibitor, but when physiological balance is disturbed by bacterial infection or mechanical stimulation, osteoclast formation stimulation molecules such as macrophage-colony stimulating factor (M-CSF), tumor necrosis factor-α (TNF-α), and receptor activator of nuclear factor-κB ligand (RANKL) are synthesized to increase osteoclast differentiation (Sokos et al., 2015). As described above, PDLFs are known to play an important role not only in the formation and maintenance of PDL, but also in the restoration and regeneration of adjacent alveolar bone and

cementum. Osteoblasts or mesenchymal stem cells were mainly used for research on implant surfaces and physiologically active substances, which are important elements of protein expression related to cell adhesion, proliferation, differentiation, and calcification in periodontal tissue, and research related to periodontal ligament cells (PDLCs) is insufficient.

Secretory leukocyte protease inhibitor (SLPI) is known to inhibit excessive inflammatory reactions by controlling the expression of inflammation related factors, and to promote the adhesion, differentiation, and mineralization of osteoblast on the titanium (Ti) surface (Jeong et al., 2015a; Choi et al., 2016; Lee et al., 2016). In our previous study, SLPI promoted the adhesion of PDLFs on Ti surface and showed anti-inflammatory effects (unpublished data). From these results, the possibility has been raised that SLPI will be able to regulate the biological process post-adhesion of PDLFs. Therefore, this study analyzed the expression and regulation of the molecules involved in the proliferation, differentiation, collagen fiber secretion and calcification of PDLF attached to the Ti surface by SLPI, and attempted to identify the function of SLPI and the biological location of each molecule in the signal transduction process.

Cell proliferation and differentiation compensate for cell and tissue loss and are essential for wound healing, tissue regeneration, and pathological tissue repair processes. Insulin-like growth factor 1 (IGF-1) and K-carrageenan, a natural polysaccharide extracted from red algae, enhanced the proliferation and osteogenic differentiation during bone formation of human dental pulp stem cells (DPSCs) and MC3T3-E1 cells (Feng et al., 2014; Cao et al., 2021). BMP-9 and neural epidermal growth factor like protein-1 (NELL-1) promoted osteogenic differentiation during bone formation in human periodontal ligament stem cells (PDLSCs) (Chen et al., 2012; Ye et al., 2014), but suppressed pyrophosphate (PPi) and the homeobox gene HOXA10 (Liang et al., 2021; Wang et al., 2021). Silver nanoparticles (AgNps) promoted osteogenic differentiation of human PDLFs (hPDLFs) during bone formation (Xu et al., 2019). In addition, in hPDLF stimulated by TNF-α, proanthocyanidins (PA) promoted osteogenic differentiation during bone formation (Huang et al., 2020).

In studies of cells cultured on Ti surface, zinc increased proliferation and osteogenic differentiation during bone formation of DPSCs, and enamel matrix derivatives (EMD) promoted the viability and proliferation of PDLSCs (Yusa et al., 2016; Li et al., 2017). In addition, fibronectin and thymosin β4 (Tβ4) also promoted the proliferation and differentiation of MC3T3-E1 cells on the Ti surface (Ku et al., 2005; Choi et al., 2015; Jeong and Jeong, 2016). miR-148b and NELL-1 inhibited the proliferation and promoted the differentiation of rat mesenchymal stem cells and MC3T3-E1 cells on the Ti surface (Song et al., 2013; Shen et al., 2018). SLPI increased the proliferation of normal human oral keratinocytes (NHOKs), and promoted the proliferation and differentiation of bone marrow hematopoietic progenitor cells and acute myelocytic leukaemia cell line (NB4) cells (Wang et al., 2011; Klimenkova et al., 2014). In addition, on the Ti surface, SLPI promoted the proliferation and differentiation of MC3T3-E1 cells (Jeong et al., 2015a; Choi et al., 2016), and the adhesion and proliferation of PDLFs (unpublished data). In this study, SLPI significantly increased the proliferation of PDLFs (SLPI/PDLF) cultured on Ti surfaces from day 4 to day 10 using a differentiation medium compared to the control group (PDLF). Therefore, compared with other studies, these results suggest that SLPI is one of the signaling substances that can induce or promote the proliferation and differentiation of PDLFs on Ti surfaces.

Ras proteins are small GTPases that play an essential role in controlling the activity of multiple signaling pathways, including pathways that regulate proliferation and differentiation. Ras activated by the Grb2 protein bound to receptor tyrosin kinase (RTK) stimulates the phosphorylation of the downstream molecule ERK1/2. The ERK1/2 signaling pathway plays an important role in bone formation and homeostasis maintenance by regulating the proliferation and differentiation of osteoblasts and bone marrow stromal cells (BMSC) during bone formation. In addition, ERK1/2 promotes the differentiation of osteoblasts by stimulating the phosphorylation and activation of runt-related transcription factor 2 (Runx-2) and osterix (Osx), which are osteoblast specific transcription factors, and inducing the expression of bone matrix proteins (Chau et al., 2009; Artigas et al., 2014). Galectin-7 promoted the proliferation and wound healing ability of hPDLF by

activating ERK1/2 (Huang et al., 2022b). Tβ4 increased the viability and proliferation of endothelial progenitor cells (EPCs) through the promotion of ERK phosphorylation (Zhao et al., 2011). Puerarin induced phosphorylation and activation of ERK1/2 to promote osteogenic differentiation of rat BMSC during bone formation (Yang et al., 2018). The expression of OCN, c-fos and RANKL, and the number of osteoclasts were decreased in bone development of ERK1/2 knockout mice, and chondrodysplasia was induced in chondrogenesis (Matsushita et al., 2009).

In this study, SLPI significantly increased the expression of Ras and pERK1/2 in PDLF cultured in the differentiation medium, indicating that SLPI is a factor that can promote proliferation and differentiation by activating ERK1/2 through the Ras signaling system.

Elk-1 is a transcription factor that is one of the members of the ternary complex factor (TCF) family and plays an important role in regulating cell proliferation and differentiation. The Elk-En protein fused to the Drosophila Engrailed (En) inhibitory domain of Elk-1 suppressed c-fos protein expression and cell proliferation in human EcR 293 cells, and induced cell cycle arrest and apoptosis (Vickers et al., 2004). Elk-1 activated the Runx-2 P1 promoter to promote differentiation of MC3T3-E1 cells (Zhang et al., 2009), and overexpression of inactive Elk-1 promoted differentiation of rat skeletal muscle cell line L6E9 cells (Khurana and Dey, 2002). The c-fos protein is one of the activator protein-1 (AP-1) transcription factors of the Fos, Jun and ATF families and is involved in the proliferation and differentiation of various cells. c-fos accelerated proliferation by stimulating the activities of cyclin A and cyclin-dependent kinase 2 (CDK2) in MC3T3-E1 cells (Sunters et al., 2004). In the chondrogenic cell line ATDC5 cells in which c-fos was overexpressed, chondrocyte differentiation was suppressed (Thomas et al., 2000). c-fos activates nuclear factor of activated T-cells c1 (NFATc1), a transcription factor related to osteoclast differentiation, in LPS-injected rat alveolar bone tissue and RANKL-treated RAW 264.7 cells, resulting in alveolar bone resorption and osteoclastogenesis was promoted (Kim et al., 2019). Mice deficient in c-fos (c-fos -/-) developed severe osteopetrosis with shortened long bones, ossification of the medullary cavity, and lack of tooth

eruption (Wagner, 2002). Mechanical stimulation increased the expression of type I collagen (Col I) protein by stimulating the activation of c-fos and c-jun transcription factors in hPDLF (Kook et al., 2009). Therefore, c-fos is an important transcription factor regulating the proliferation and differentiation of chondrocytes, osteoblasts and osteoclasts. Phosphorylated ERK1/2 binds to a serum response element (SRE) present in the c-fos promoter region and phosphorylates Elk-1, a transcription factor that forms part of the serum response factor (SRF) regulating the expression of c-fos.

In this study, SLPI/PDLF cultured in the differentiation medium significantly increased the expression of Ras and pERK1/2 compared to PDLF, as well as the expression of Elk-1 phosphorylation and c-fos. Therefore, comparing this results with previous results, this suggests that SLPI can promote PDLF differentiation and Col I protein synthesis by activating ERK1/2, Elk-1, and c-fos through Ras signaling.

Runx-2 and Osx are transcription factors essential for the differentiation process of osteoblast and play an important role in bone formation and tooth development. Runx-2 is a transcription factor necessary to differentiate mesenchymal stem cells into osteoblast, and plays a role as a regulator in the early stages of osteoblast differentiation and plays an important role in skeletal morphology, tooth development, cartilage formation, and blood vessel formation. Osx is a osteoblast specific transcription factor that differentiates immature osteoblast into mature osteoblast in the late stage of osteoblast differentiation, and is involved in the expression control and calcification of bone matrix proteins (Komori, 2006). Remifentanil elevated the expression of Runx-2 and Osx in osteoblast, and melatonin promoted differentiation by increasing the activity and stabilization of Osx (Han et al., 2017; Yoon et al., 2019). Luteolin promoted osteogenic differentiation during bone formation by increasing the expression of Runx-2 and Osx in PDLCs, and NELL-1 promoted differentiation by increasing the expression of Runx-2 and Osx in osteoblast on the Ti surface (Shen et al., 2018; Quan et al., 2019).

In this study, PDLF not only increased the expression of Runx-2 and Osx in

differentiation media, but also further increased in SLPI/PDLF. Therefore, this result shows that PDLF expresses bone matrix-specific proteins, and SLPI can promote the expression of bone matrix proteins and calcification of substrates by increasing the expression of Runx-2 and Osx in PDLF.

SLPI is a serine protease inhibitor that increases cell proliferation and migration during wound healing in the skin and oral mucosa, suppresses inflammatory responses, and promotes matrix remodeling (Ashcroft et al., 2000; Angelov et al., 2004). In the process of dentin formation, SLPI is involved in regulation of signal for dentin matrix formation related proteins during the differentiation and calcification of odontoblasts (Jeong et al., 2015b). It is known that SLPI increases the adhesion and proliferation of osteoblasts on Ti surface and promotes the differentiation and calcification of osteoblasts by increasing the expression of bone matrix proteins (Jeong et al., 2015a; Choi et al., 2016). In addition, SLPI increased the adhesion and proliferation of PDLFs on Ti surface (unpublished data). SLPI promoted proliferation of endometrial epithelial cells through Ras/MAPK activation, and increased cell viability by inducing ERK1/2 activation in ovarian cancer cells (Zhang et al., 2002; Rasool et al., 2010). SLPI promoted the metastasis process including cell migration and invasion by increasing MMP-2 and MMP-9 expression through Elk-1 phosphorylation in SNU638 gastric cancer cells (Choi et al., 2011b), and increased phosphorylation of ERK1/2 and Elk-1 in odontoblasts to promote the migration of odontoblasts (unpublished data). In this study, expressions of Ras, pERK1/2, pElk-1, c-fos, Runx-2, and Osx increased time-dependent in differentiation induced PDLF, and these protein expressions were significantly higher in SLPI/PDLF than in PDLF. Therefore, taking these results together, SLPI promotes proliferation and differentiation of PDLF on the Ti surface by activating ERK1/2, Elk-1, and c-fos signals through Ras, and the increase expression of Runx-2 and Osx shows that expression of bone matrix proteins including Col I and calcification of matrix can be promoted.

These results showed that SLPI induced an increase in Ras, pERK1/2, pElk-1, c-fos, Runx-2 and Osx in PDLF, but it remains questionable whether the induction of the transcription factors pElk-1, c-fos, Runx-2 and Osx acts as downstream

molecules of the ERK1/2 signaling pathway through Ras. Therefore, expression of these transcription factors was confirmed using an ERK1/2 inhibitor.

The mitogen-activated protein kinase (MAPK) pathway is a cellular protein chain that transmits signals from receptors on the cell surface to DNA in the nucleus and consists of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. The MAPK pathway transduces, amplifies and integrates signals from various stimuli such as growth factors and cytokines in mammalian cells to induce appropriate physiological responses including cell proliferation, differentiation, development, inflammatory response and apoptosis. In general, ERK is activated in response to growth factor and mitogen stimulation, whereas JNK and p38 MAPK are mainly activated by inflammatory cytokines and cellular and environmental stresses. Activation of ERK is initiated by receptor tyrosine kinase (RTK) by binding of growth factors and mitogens to cell membrane receptors, and activation of the small GTPase Ras is induced. Activated Ras activates Raf, a MAPKKK, and Raf subsequently induces phosphorylation and activation of MEK1/2, a MAPKK. ERK1/2, a MAPK activated by MEK1/2, phosphorylates ribosomal S6 kinase (RSK) in the cytoplasm, and both RSK and ERK are translocated to the nucleus to activate several transcription factors such as c-fos, Elk-1, c-Myc, and CREB (cAMP response element-binding protein) to induce cell proliferation and differentiation (Wortzel and Seger, 2011).

MAPK is an important signaling pathway related to osteoblast differentiation during bone formation, but the role of MAPK in regulating differentiation varied according to cell type and stimulus. In mice lacking MEK1, TAK1, and MKK3/6, developed bone formation defects due to clavicle hypoplasia, calvarium hypomineralization, and low bone mass. And ALP activity and expression of bone matrix genes including Runx-2, Osx, Col Ⅰ, and OCN were decreased in osteoblasts of transgenic mouse (Ge et al., 2007; Greenblatt et al., 2013). In addition, ERK1/2 inhibition by MEK1 mutation and MEK1 inhibitor (PD98059) induced a decrease in Runx-2 phosphorylation and OCN protein expression in MC3T3-E1 cells (Xiao et al., 2000). In MC3T3-E1 cells and C2C12 cells treated with MEK1 inhibitor (PD98059) after BMP-2 treatment, ALP activity and Col I

expression were enhanced, and extracellular matrix calcification was promoted (Higuchi et al., 2002). In mesenchymal progenitor cells (MPC) in which the activation of the MAPK pathway was inhibited using specific inhibitors (ERK1/2 inhibitor, PD98059; p38 inhibitor, SB203580) or siRNA (ERK1/2 siRNA, p38 siRNA), inhibition of p38 activation suppressed the expression of bone matrix formation genes such as Runx-2, ALP, and OCN, which were increased by BMP-9 induction, whereas inhibition of ERK1/2 activation further enhanced their expression (Zhao et al., 2012).

In epithelial ovarian cancer overexpressed SLPI, activation of the ERK1/2 cascade was induced (Rasool et al., 2010). SLPI promoted the proliferation of endometrial epithelial cells through Ras/MAPK activation (Zhang et al., 2002), and increased the adhesion and proliferation of osteoblasts and PDLF through FAK/Grb2/Ras/pERK1/2 signal transduction pathway on Ti surfaces (Jeong et al., 2015a; unpublished data). MEK1 inhibitor (PD98059) and Ras siRNA inhibited Runx-2 activated by strontium in mesenchymal stem cell-like C3H10T1/2 cells (Peng et al., 2009). ERK1/2 inhibitor (U0126) and Ras siRNA completely blocked mechanical stimulation-induced Runx-2 activation in MC3T3-E1 cells and rat cranial osteoblasts, whereas p38 inhibitor (SB203580) did not affect Runx-2 activation. In addition, Ras siRNA inhibited ERK1/2 activation induced by mechanical stimulation in osteoblasts, but not p38 MAPK activity (Kanno et al., 2007). In this study, phosphorylation of ERK1/2 in PDLF was increased by SLPI and significantly decreased when treated with MEK1 inhibitor PD98059. In the SLPI and PD98059 treatment group (SLPI/PD/PDLF), it was slightly increased compared to the PD98059 treatment group (PD/PDLF). Therefore, this result shows that the signaling of SLPI can regulate downstream molecules through the Ras/ERK1/2 pathway.

Phosphorylation of ERK1/2 induces activation of Elk-1, which promotes Elk-1 mediated ternary complex formation and transcriptional activity. SLPI promoted metastasis process involving cell migration and invasion by increasing the expression of MMP-2 and MMP-9 through Elk-1 phosphorylation in SNU638 gastric cancer cells (Choi et al., 2011b), and promoted the migration of odontoblasts

by inducing the expression of MMP-2 and MMP-9 through the PKC-δ/ERK/Elk-1 signaling pathway (unpublished data). Gastrin binds to the cholecystokinin B (CCK-B) receptor in AR42J, a rat pancreatic adenocarcinoma cell line, and induces activation of protein kinase C (PKC), ERK and c-fos and activated ERK increases the activity of the downstream transcription factor, Elk-1. PKC inhibitor (GF-109203X) significantly inhibited the gastrin induced activation of c-fos and ERK2, and an ERK inhibitor (PD98059) blocked the proliferation of AR42J cells promoted by gastrin (Stepan et al., 1999). In the early stage of response to mechanical stimulation in hPDLFs, ERK1/2 enhanced the activation of AP-1 transcription factors involved in the proliferation of osteoblasts and the regulation of bone forming matrix genes by inducing a rapid and continuous increase in c-fos and c-jun proteins (Kook et al., 2009). Farnesyltransferase (FTase) inhibitor, lonafarnib (Lon) suppressed osteoclasts formation and bone resorption through inhibition of ERK1/2 and c-fos activities in Ti particle induced osteolytic tissue and RANKL induced osteoclast differentiation (Huang et al., 2022a). This study also showed that pElk-1 and c-fos were increased by SLPI and significantly decreased when treated with PD98059. In the SLPI/PD/PDLF, it was increased compared to the PD/PDLF. Therefore, it is thought that SLPI can promote the synthesis of Col I protein by regulating c-fos as well as promoting cell migration through the ERK1/2/Elk-1 signaling pathway in PDLF.

ERK1/2 increases the phosphorylation and transcriptional activity of Runx-2 and Osx in the nucleus, thereby inducing the expression of bone specific genes to regulate the differentiation of osteoblasts. In PDLSC, p38 inhibitor (SB203580) reduced BMP-9 induced ALP activity, expression of Runx-2, OPN, OCN, and matrix calcification, but ERK1/2 inhibitor (PD98059) increased their expression (Ye et al., 2014). In PDLSCs, the ERK inhibitor (U-126) increases the expression of Osx and dentin matrix protein-1 (DMP-1), which were decreased by treatment with pyrophosphate, a calcification inhibitor. The p38 inhibitor (SP202190) promoted the osteogenic differentiation of PDLSCs during bone formation by increasing the expression of Runx-2 (Liang et al., 2021).

Diosmetin, flavonoid derivative increased phosphorylation of Runx-2, Elk-1, and

ERK1/2 in osteoblasts (MG-63, hFOB, MC3T3-E1) and phosphorylation of Runx-2 was decreased by ERK1/2 siRNA treatment. In addition, diosmetin induced activation of PKCδ, and PKCδ siRNA and PKCδ specific inhibitor (rotterlin) decreased diosmetin induced expression of ERK1/2 and Runx-2 (Hsu and Kuo, 2008). Puerarin and panax notoginseng saponin (PNS) induced osteogenic differentiation of rat BMSCs during bone formation by increasing the expression of Runx-2 through the ERK1/2 and p38 MAPK signal transduction pathways (Li et al., 2011; Yang et al., 2018). During the differentiation process of C2C12 cells, ERK1/2 activated by overexpression of MEK enhanced mRNA, protein expression and transcriptional activity of Osx and the ERK1/2 inhibitor (U0126) suppressed the protein level and transcriptional activity of Osx (Choi et al., 2011a). In rat cranial osteoblasts and MC3T3-E1 cells, ERK inhibitor (U0126) or p38 inhibitor (SB203580) reduced Runx-2 expression and ALP activity which were increased by myokine irisin and also inhibited cell proliferation and differentiation (Qiao et al., 2016). NELL-1 increased the expression of Runx-2 and Osx in MC3T3-E1 cells on the Ti surface, and their expression levels were significantly decreased which ERK1/2 inhibitor (U0126) and NELL-1 were co-treated (Shen et al., 2018).

In this study, the expression of Runx-2 and Osx was higher in SLPI/PD/PDLF than that of PD/PDLF, which suggests that SLPI may induce cellular specificity of osteoblasts by regulating Runx-2 and Osx related to differentiation of osteoblasts through ERK1/2/Runx-2 or ERK1/2/Osx signal transduction pathways in PDLF.

Transduction of SLPI shRNA inhibited ERK1/2 phosphorylation during G-CSF (granulocyte colony stimulating factor) induced myeloid differentiation of hematopoietic cells and this reduction of ERK1/2 phosphorylation induced reduced phosphorylation of LEF-1 (lymphoid enhancer-binding factor-1) protein and reduced LEF-1 target gene c-Myc, survivin and cyclin D expression (Klimenkova et al. 2014). In hepatocellular carcinoma (HCC) cells overexpressing SLPI, expression of p-ERK1/2 was decreased and the expression of p-JNK and p-p38 was increased, whereas in SLPI knockdown HCC cells, expression of p-JNK and p-p38 was decreased and p-ERK1/2 expression increased (Sun et al., 2022).

In this study, expression of Ras, c-fos, Runx-2 and Osx proteins and

phosphorylation of ERK1/2 and Elk-1 in SLPI/PDLF were increased compared to the PDLF, and expression and phosphorylation levels of proteins other than Ras in PD/PDLF were decreased compared to SLPI/PDLF. These results show that signal transduction of SLPI can regulate the downstream transcription factors Elk-1, c-fos, Runx-2 and Osx through the Ras/ERK1/2 pathway.

Therefore it can be suggested that SLPI promotes proliferation and differentiation of PDLF by activating ERK1/2/Elk-1 signal through Ras on the Ti surface and promotes expression of bone matrix proteins including Col I and calcification of matrix through increased expression of c-fos, Runx-2 and Osx.

Col I is a major bone matrix protein that is highly expressed in the early proliferation phase of differentiation and plays a role as a template in the process of calcification of bone matrix by ALP expression, and the pattern and concentration of collagen can be examed through Picro-sirius red staining (Boonrungsiman et al., 2012). ALP is an early differentiation phenotypic marker of osteoblasts and its enzymatic activity increases as osteoblasts mature, acting as a regulator of inorganic phosphate transport, cell division and differentiation. Alizarin red S staining is used to confirm the degree of calcium deposition in the matrix formed by osteoblasts in the late stage of differentiation (Ma et al., 2014). Therefore an osteogenic staining assay was performed to investigate whether SLPI is involved in and promotes the expression of bone matrix proteins including Col I and the formation of calcification crystals in the PDLF of the Ti surface. PNS and IGF-1 increased ALP expression and matrix calcification during bone formation in rat BMSCs and human DPSCs (Li et al., 2011; Feng et al., 2014). BMP-9 and SAHA (suberoylanilide hydroxamic acid) promoted ALP expression and matrix calcification during differentiation in hPDLSCs compared to the control group whereas pyrophosphate inhibited their expression (Ye et al., 2014; Xuan et al., 2020; Liang et al., 2021). On the MAO (microarc oxidation) treated Ti surface, miR-148b, antimiR-138 and miR-29b increased ALP and collagen secretion and extracellular matrix calcified nodule formation in rat BMSCs (Song et al., 2013; Wu et al., 2013). Tβ4 and rosmarinic acid (RA) increased calcified nodule formation and ALP expression in differentiating MC3T3-E1 cells on Ti surface compared to the control

group (Jeong and Jeong, 2016; Jeong et al., 2021). SLPI increased the formation of calcified nodules in MDPC-23 cells and increased the deposition of calcified nodules in differentiating MC3T3-E1 cells on Ti surface (Jeong et al., 2015b; Choi et al., 2016). In this study, the expression of Picro-sirius red, ALP, and Alizarin red S increased in PDLF in a time-dependent manner in the differentiation medium, and their expression was further increased by SLPI. Therefore SLPI is thought to be involved in increase of collagen and bone matrix protein secreted by PDLF and the increase of calcification, promoting bone matrix deposition and mineralization on the Ti surface.

ALP induces calcification by locally increasing the concentration of inorganic phosphate in the early stage of osteoblast differentiation and depositing calcium phosphate in the extracellular matrix (Ma et al., 2014). BSP is expressed at the early stage of calcification of the substrate and acts as a nucleator role for early hydroxyapatite (HA) crystal formation and controls the direction of crystal growth to form ribbon-shaped apatite crystals (Hunter and Goldberg, 1994; Staines et al., 2012). Dentin sialophosphoprotein (DSPP), odontoblast specific protein is expressed not only in dentin but also in bone, cementum and non-mineralized tissues including lung and kidney and is degraded into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) proteins. DPP regulates the formation and growth of HA crystals by its high affinity to HA, calcium ions, and collagen, and DSP regulates the initiation of dentin calcification (Suzuki et al., 2009). DMP-1 is an important protein for mineralization of dentin and bone and promotes bone nodule formation and calcification (He et al., 2003). OCN is the most abundant non-collagenous protein in the bone matrix and is synthesized and secreted by mature osteoblasts in the late stages of osteoblast differentiation and binds to calcium and HA crystals with high affinity, promoting nucleator production and bone nodule growth (Carvalho et al., 2021). Osteonectin (ON) is mainly synthesized by osteoblasts but also by skin, tendon and PDLFs and binds to collagen and HA crystals to initiate calcification and promote the growth and proliferation of calcified crystals. In addition, ON is involved in collagen matrix assembly including collagen deposition and Col I is very important as a template for deposition of calcified

matrix (Trombetta and Bradshaw, 2010).

Irisin increased the expression of ALP, Col I, OCN and OPN in rat cranial osteoblasts and MC3T3-E1 cells and K-carrageenan increased the expression of OPN and DMP-1 in MC3T3-E1 cells promoting bone formation during osteogenic differentiation (Qiao et al., 2016; Cao et al., 2021). SAHA promoted differentiation by increasing the expression of OPN, OCN, Col I, ALP, BSP, DMP-1 and DSPP in PDLSC and MDPC-23 cells (Kwon et al., 2012; Xuan et al., 2020). Tβ4 and RA promoted osteogenic differentiation during bone formation by increasing the expression of ALP, BSP, DSPP, DMP-1, OCN, ON and Col I in MC3T3-E1 cells on the Ti surface (Jeong and Jeong, 2016; Jeong et al., 2021). SLPI was reported to increase the expression of BSP, OCN, ON and Col I, which are related to odontoblast differentiation and calcification in MDPC-23 cells (Jeong et al., 2015b). In addition, it has been reported that SLPI promotes differentiation and calcification of osteoblast by increasing the expression of ALP, BSP, DSPP, DMP-1, and Col I, which are genes related to bone matrix formation in MC3T3-E1 cells on Ti surface (Choi et al., 2016).

In this study, the expression of ALP, BSP, DSPP, DMP-1, OCN, ON and Col I increased in a time-dependent manner in PDLF cultured in differentiation medium and these mRNA expressions were significantly higher in SLPI/PDLF than in PDLF. Therefore, together with the results of Picro-sirius red, ALP, and Alizarin red S, these results show that SLPI can promote matrix calcification by increasing the expression of bone matrix proteins including Col I protein in PDLF on Ti surface.

This result clearly showed that SLPI induced an increase in bone matrix proteins in PDLF but the precise signaling pathways for these proteins were not well known until now. It is known that MAPKs including ERK1/2, p38 and JNK can directly regulate bone formation and differentiation through transcriptional regulation. It has also been reported that SLPI activates ERK1/2 signaling in osteoblasts and PDLF on Ti surface. Therefore, in future studies, it is necessary to identify the signal transduction system for each of these bone matrix proteins using MAPK (ERK1/2, p38) inhibitors and Smad 3 inhibitor in PDLF.

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FIGURE LEGENDS

Figure 1. Viability of the SLPI-treated PDLF during differentiation on Ti discs.

SLPI increased significantly the cell viability (day 7 and 10) of PDLF during differentiation compared to that of the untreated cells on Ti disc. C, undifferentiated PDLF without SLPI treatment on Ti discs (control). $*p < 0.05$.

Figure 2. The expression of mitosis and differentiation related signaling molecules in SLPI-treated PDLF during differentiation on Ti discs.

A) SLPI increased significantly the Ras, pERK1/2, pElk-1, c-fos, Runx-2 and osterix expression in PDLF compared to that of untreated cells during differentiation on Ti discs. B) Quantitative analysis of the expressed band density between the SLPI-treated and untreated PDLF on Ti discs. C, undifferentiated PDLF without SLPI treatment on Ti discs (control). $*p< 0.05$.

Figure 3. The mitosis and differentiation related signaling molecules expression through the ERK1/2 and Elk-1 signaling in SLPI-treated PDLF on Ti discs.

A) MEK inhibitor PD98059 antagonized the stimulatory effects of SLPI on ERK and Elk-1 phosphorylation in PDLF. B) Quantitative analysis of the expressed band density in PDLF between the SLPI-treated and untreated Ti discs. C, control. $*p< 0.05$.

Figure 4. The analysis of SLPI-treated PDLF Picro-Sirius Red staining on Ti discs.

A) Picro-Sirius Red staining showed that SLPI-treated PDLF increased collagen secretion compared to that of the untreated cells on Ti disc. B) Quantification of Picro-Sirius Red staining demonstrated a significant increase in SLPI-treated PDLF compared to that of the untreated cells.

scale bars, 1 mm; magnification, x8 or x40 as indicated. *$p<$ 0.05 .C, undifferentiated PDLF without SLPI treatment on Ti discs (control).

Figure 5. The analysis of SLPI-treated PDLF ALP staining on Ti discs.

A) ALP staining showed that SLPI-treated PDLF increased ALP production compared to that of the untreated cells on Ti disc. B) Quantification of ALP staining demonstrated a significant increase in SLPI-treated PDLF compared to that of the untreated cells. scale bars, 1 mm; magnification, x8 or x40 as indicated. *$p<$ 0.05. C, undifferentiated PDLF without SLPI treatment on Ti discs (control).

Figure 6. The analysis of SLPI-treated PDLF Alizarin Red S staining on Ti discs.

A) Alizarin Red S staining showed that SLPI-treated PDLF increased mineral nodules compared to that of the untreated cells on Ti disc. B) Quantification of Alizarin Red S staining demonstrated a significant increase in SLPI-treated PDLF compared to that of the untreated cells. scale bars, 1 mm; magnification, x8 or x40 as indicated. *$p<$ 0.05. C, undifferentiated PDLF without SLPI treatment on Ti discs (control).

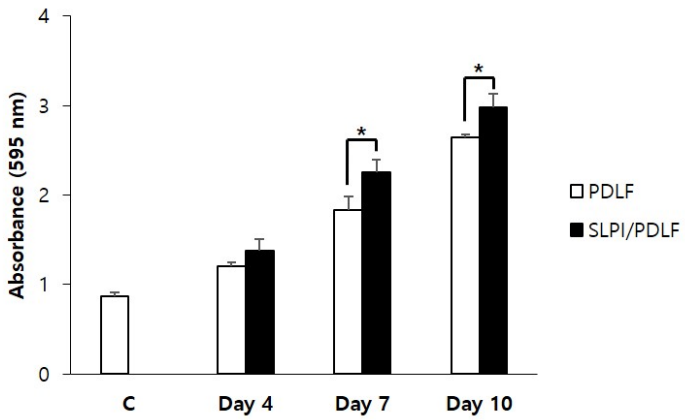
Figure 7. The expression of non-collagenous and collagenous genes in SLPI-treated PDLF during differentiation on Ti discs.

A) SLPI increased the ALP, BSP, DSPP, DMP-1, OCN, ON and Col I mRNA expression in PDLF compared to that of the untreated cells during differentiation on Ti discs. B) Quantification of the RT-PCR was calculated from result of A. M, size marker; C, undifferentiated PDLF without SLPI treatment on Ti discs (control). *$p<$ 0.05.

Figure 8. Illustration of SLPI signaling transduction pathway in differentiation and mineralization of PDLF on the titanium surface.

FIGURES

Figure 1.



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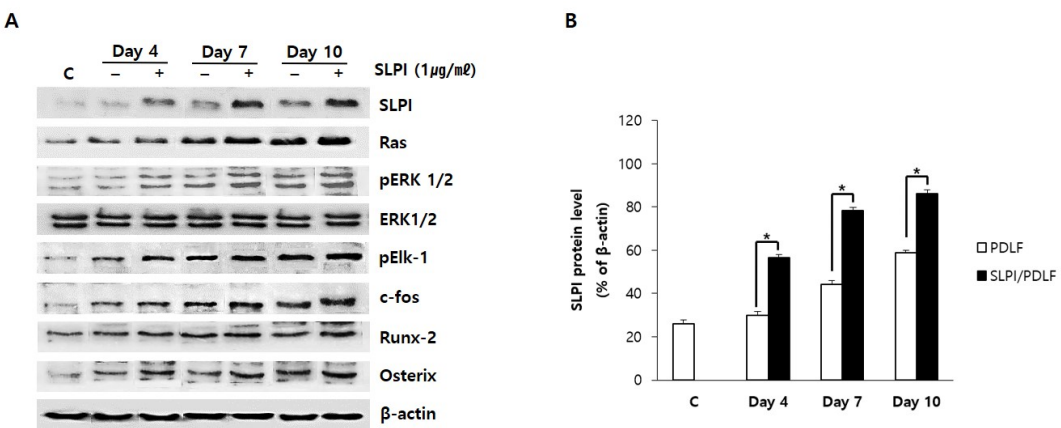

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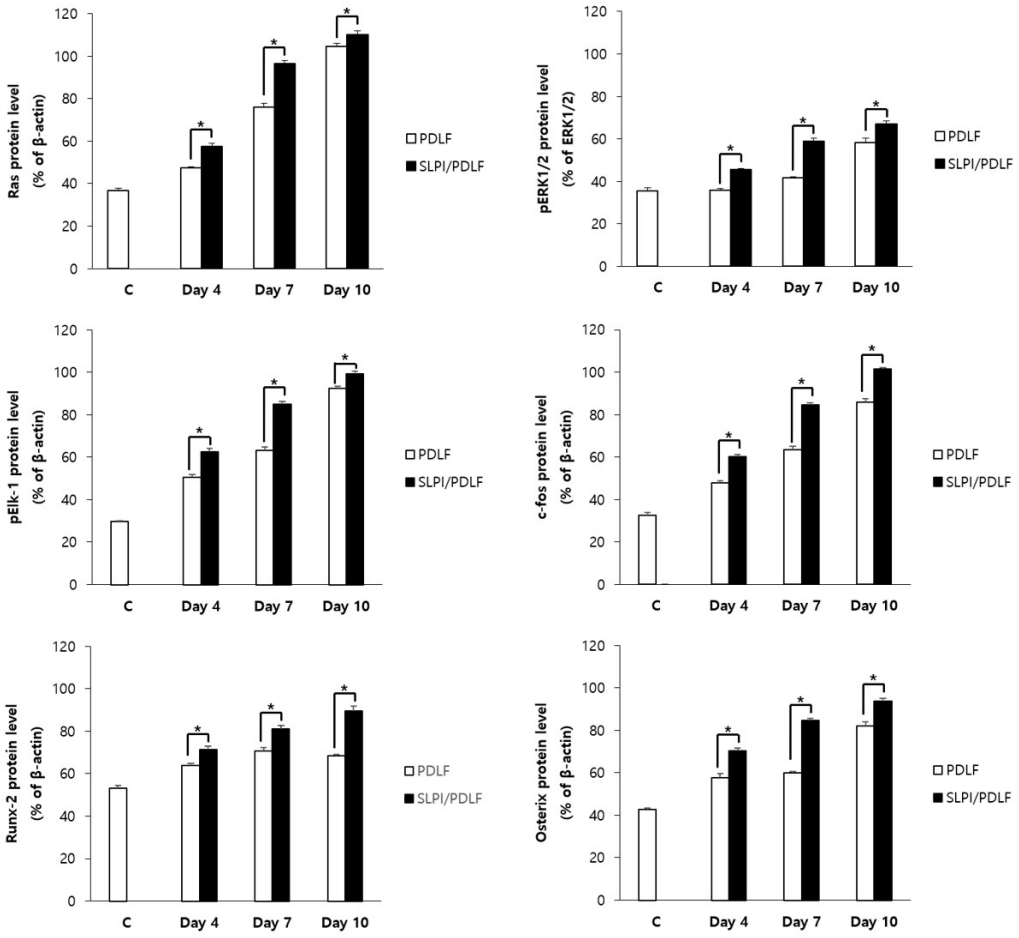

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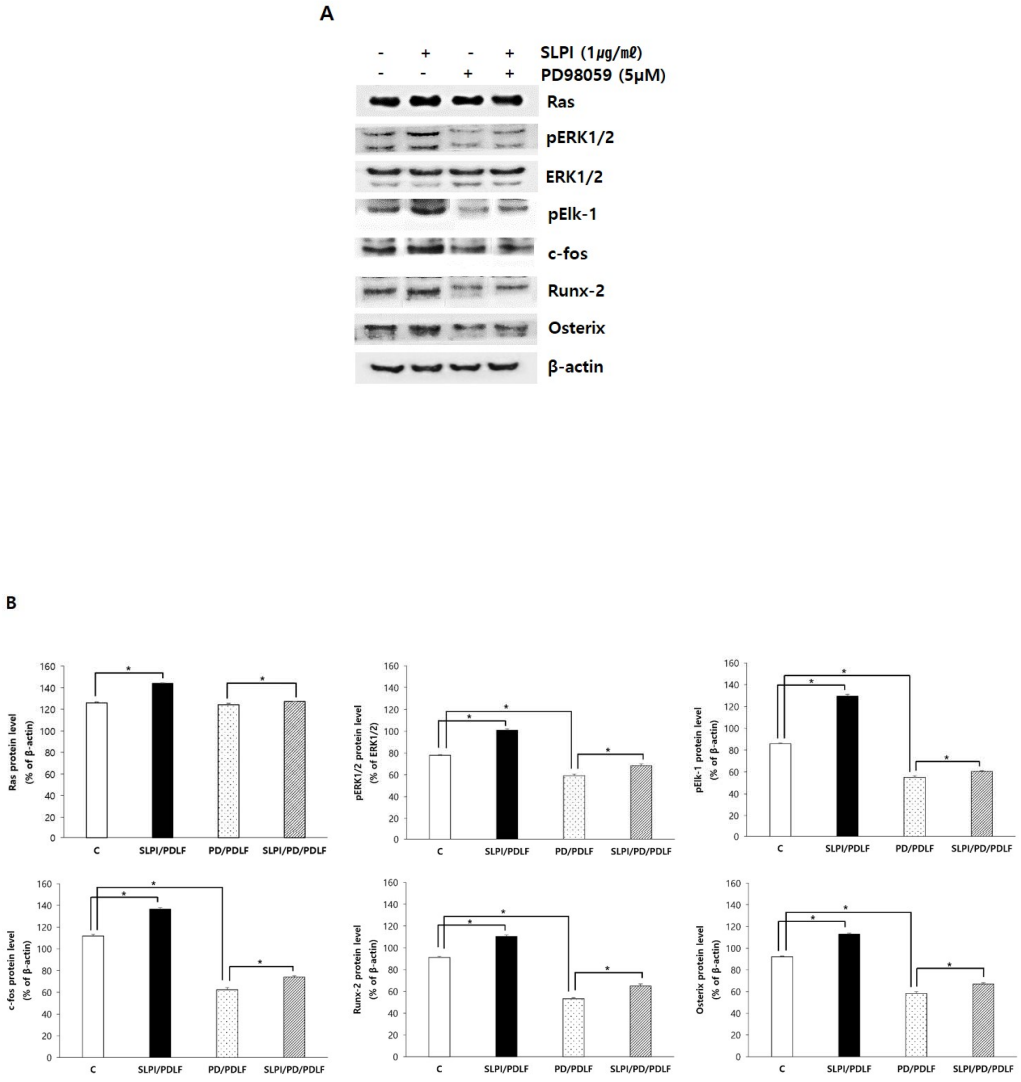

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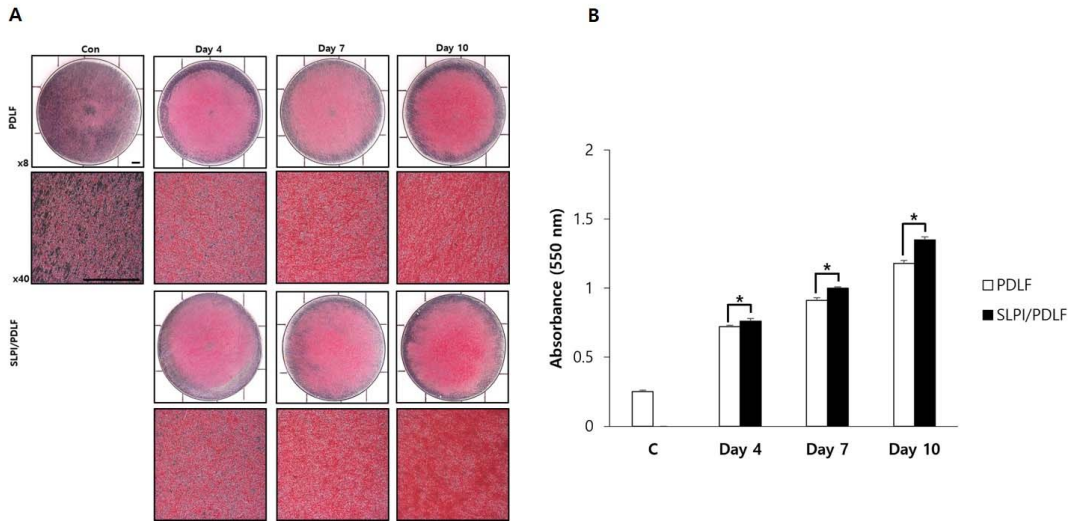

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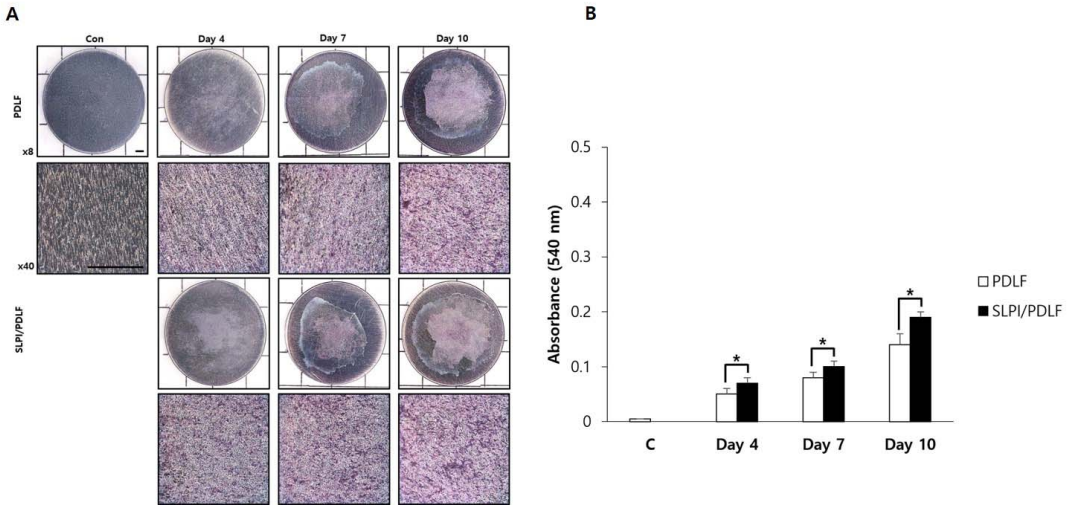

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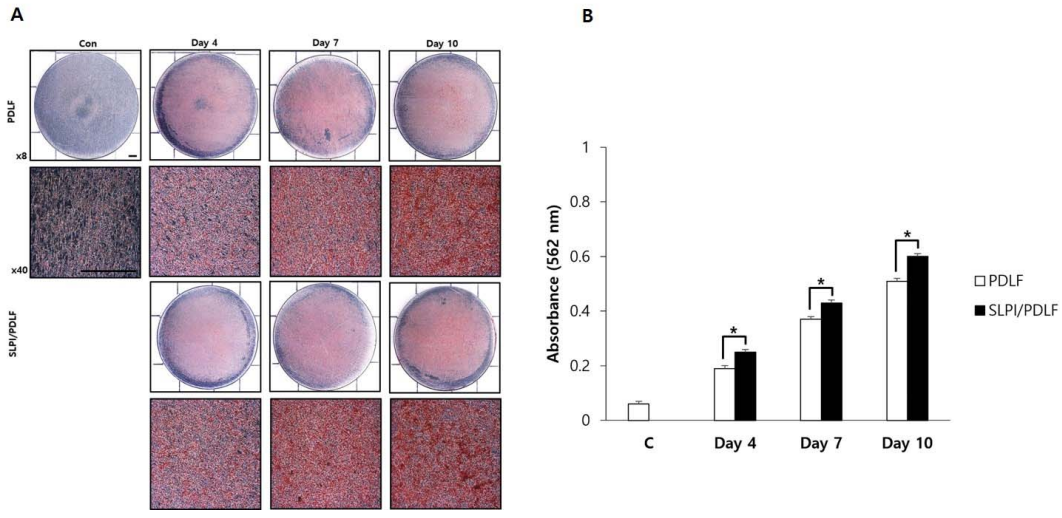

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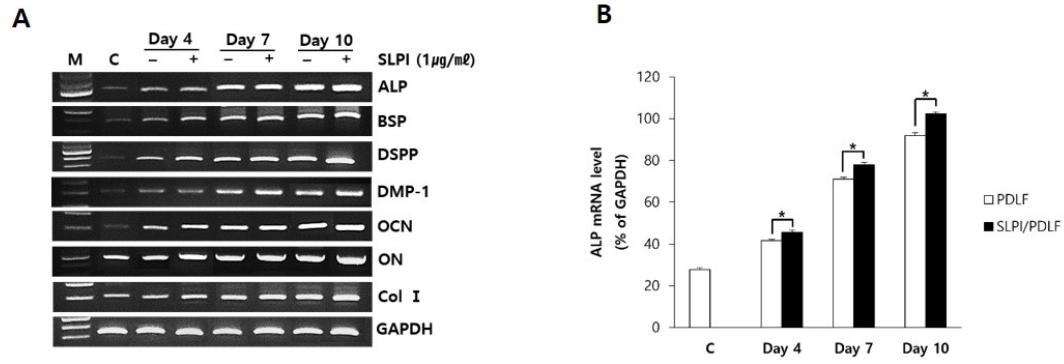

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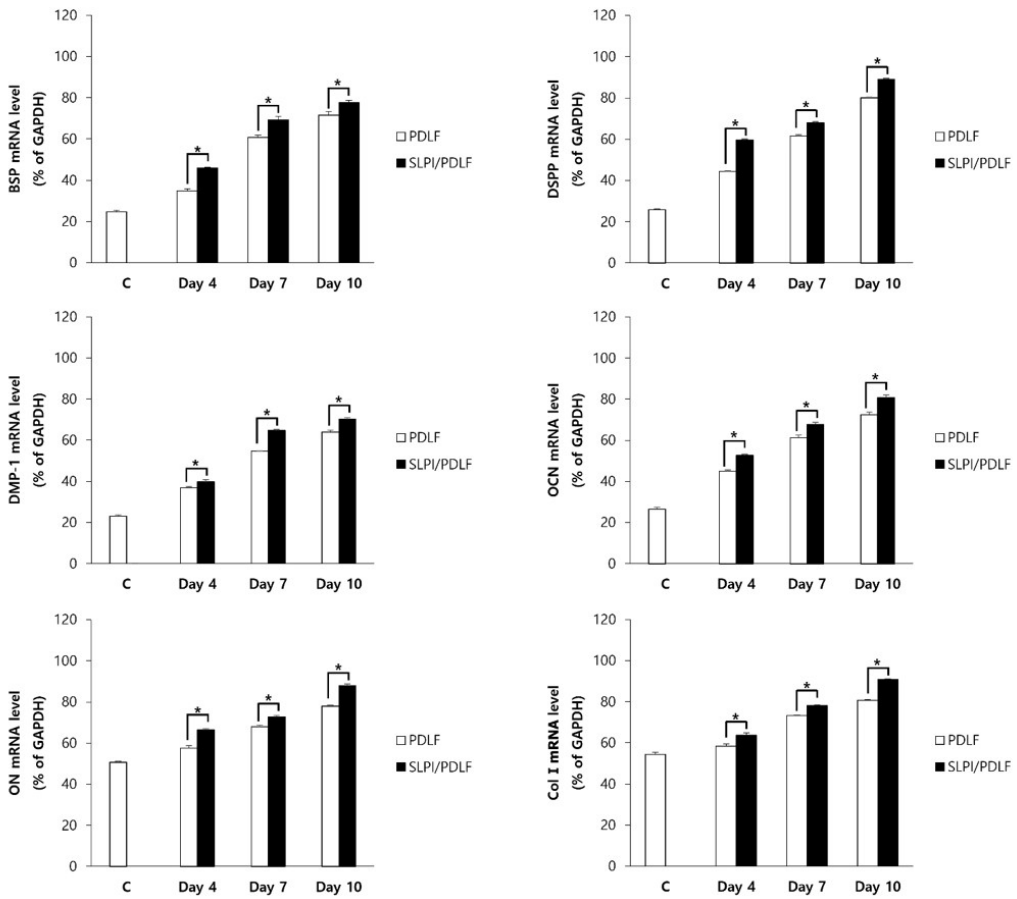

Figure 8.