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Original Article

Er:YAG laser promotes proliferation and wound healing capacity of human periodontal ligament fibroblasts through Galectin-7 induction

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Background/purpose: Among various dental lasers, the erbium-doped yttrium-aluminum-garnet (Er:YAG) laser has great potential for periodontal treatment including soft and hard tissue ablation with minimal thermal side effects under suitable energy densities and it has multiple effects on tissues for wound-healing benefits. In the present study, we sought to reveal the molecular mechanism underlying the impact of Er:YAG laser on PDL fibroblasts.
Methods: Cells were irradiated by a Er:YAG laser with various energy densities (3.6–6.3 J/cm^2). MTT assay was used for cell proliferation, and the transwell system was employed for migration and invasion abilities. The wound healing capacity was evaluated by a scratch assay. After confirming these effects, qRT-PCR and western blotting analysis was applied to identify the differentially galectin-7 expression in the irradiated cells. Knockdown experiments were conducted to reveal the functional role of galectin-7 in the modulation of Er:YAG laser-mediated effects.
Results: 4.2 J/cm^2 was the lowest energy density to induce the optimal cell proliferation, migration and invasion abilities. In the group of upregulated genes, galectin-7 was selected for further examination and its elevation after Er:YAG laser treatment was validated by RT-

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PCR and Western blot. We demonstrated that silence of galectin-7 abrogated the effects of Er:YAG laser on cell proliferation, migration ad invasion, suggesting the Er:YAG laser promoted these effects through induction of galectin-7.
Conclusion: These findings indicated that Er:YAG laser may accelerate the regeneration process in periodontal tissues through enhancement of their proliferative and mobile activities. Additionally, the significance of galectin-7 in the Er:YAG laser-elicited benefits was demonstrated.

Introduction

The main goals of periotonal treatment are the elimination of infection and resolution of chronic inflammation.[1] Therefore, oral hygiene instructions (OHI) and the initial nonsurgical therapy of periodontitis by means of scaling and root planing (SRP) is necessary to remove bacterial plaque and deposits of calculus for controlling subgingival microflora.[2] Moreover, the use of systemic or local antibiotics are thought to be required for elimination of pathogenic bacteria.[3] Recently, Er:YAG laser has been considered as an useful adjunctive instrument for periodontal therapy as it has high absorbability in both water and hydroxyapatite, so it can effevtively ablate soft and hard tissues as well as calculus with minimal heated-related side effects, simultaneously, Er:YAG laser also exhibited highly bactericidal effect to diminish the periodontopathic bacteria[4,5] following root debridement by Er:YAG laser irradiation (Akiyama F, Aoki A et al., Laser Medical Sciences 2011). It has been shown that Er:YAG laser enabled a more efficacious removal of subgingival calculus with homogeneous and smoother root surface preservation compared to SRP. Several systematic review and meta-analysis also concluded that Er:YAG laser was suitable to serve as an alternative or adjuvant for SRP in the treatment of chronic periodontitis[6–8] compared with conventional treatments.

Aside from the favorable results for calculus removal and bactericidal effect, various studies have reported that low level Er:YAG laser irradiation promoted faster adhesion and growth of human gingival fibroblasts (HGFs)[9] and periodontal ligament fibroblasts (PDL fibroblasts).[10] It has been shown that Er:YAG laser generated rough topographies on the root surfaces, which was proportional to the energy set for the laser application. A lower energy (60 mJ/pulse) presented a homogenous roughness with enhanced cell growth and attachment, while a higher energy (100 mJ/pulse) or root planing created disproportionate and sharp edges that hindered the attachment of cells.[9] Also, short-pulse laser setup (laser I) often served as the first choice when considering the periodontal regeneration since it yielded promising results in terms to proliferation, attachment, and orientation of PDL fibroblasts[11], which is essential for osteogenesis and cementogenesis during development and remodeling.[12,13] These studies showed that Er:YAG laser irradiation affected the surface topography and made it more biocompatible to human PDL fibroblasts. Recently, the biological effects of low level Er:YAG laser on primary HGFs have been investigated and proven to stimulate the cell proliferation.[14–16] Genes that were associated with heat-related responses and ER-related degradation, such as heat shock protein (HSP) 70 family have been found to be upregulated following Er:YAG laser irradiation on HGFs.[16] Besides, a pronounced increase in the protein expression of galectin-7 in the HGFs has also been reported.[15] Nevertheless, studies to investigate the effect of Er:YAG laser on the cell proliferation of PDL fibroblasts are still limited, let alone the possible molecular mechanisms underlying its effects.

As such, we sought to examine the direct effect of Er:YAG laser on the cell viability and wound healing capacity of PDL fibroblasts. Furthermore, we assessed whether these benefits were mediated by the Er:YAG laser-induced galectin-7 in order to provide an insight into the potential wound-healing effect of Er:YAG laser.

Materials and methods

Isolation and culture of periodontal ligament cells

All procedures were conducted in accordance with the approved guidelines from the Institutional Review Board of Chung Shan Medical University Hospital (IRB approval number:CSMUH No:cs18196). PDLs were isolated from patients with premolars extracted for orthodontic reasons after acquiring informed consents. PDLs were maintained with culture medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin).

Laser irradiation

An Er:YAG laser (Erwin AdvErl™, wavelength 2,940 nm, pulse width 250 μs, J. Morita Mfg, Kyoto, Japan) device with an emitting wavelength of 2,940 nm was used in this study. Prior to laser irradiation, the medium of PDL fibroblasts was removed in order to expose the monolayers to laser treatment. Laser irradiation was performed perpendicularly to the culture dish at a distance of 20 cm without cover sleeve nor contact tip for the hand piece. The energy densities were set at 3.6, 4.2 and 6.3 J/cm^2 according to a previous study.[16]

Cell proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used for examination of cell proliferation. After irradiation, MTT solution was added into each well and incubated for 3 h. Subsequently, the MTT formazan was dissolved in DMSO and evaluated spectrophotometrically at 570 nm. Optical density values of each group was expressed as a percentage of the control.[17]

Migration and invasion assays

A 24-well plate with transwell® polycarbonate membrane inserts of 8-μm pore size transwell (Corning, Acton, MA, USA) were used. Cell solution was placed on top of the filter membrane in a transwell insert with serum free medium and medium containing 10% FBS served as chemo-attractant liquid in the bottom well. For invasion assay, the top filter membrane was coated with a layer of Matrigel before loading the cells. After 48 h of incubation, the migrated/invaded cells that attached to the other side of the membrane were stained with crystal violet followed by quantification using an inverted microscope and image J.[18]

Wound healing assay

PDL fibroblasts were allowed to grow to a monolayer in a 6-well plate around 80% confluence. A scratch was induced in the monolayer with a sterile 200 μL pipette tip to generate a wound gap. PDL fibroblasts that moved toward the center of the wound area was photographed at 0 and 48 h under a microscope.[18] The evaluation of wound closure areas was quantified by ImageJ software (Image J, NIH, Bethesda, MD, USA).

Quantitative RT-PCR

Total RNA was isolated using a Trizol RNA extraction reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The first-strand cDNA synthesis reaction was catalyzed using SuperScript® III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) from total RNA according to the manufacturer's instruction. qRT-PCR reactions on the resulting cDNAs were conducted on the ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Carlsbad, CA, USA). Primer sequences were listed LGALS7: 5′-GGGGGACGTTCTGCAAGAGT-3′ and 5′-CCCATCTGTTTCCC TGTCTGT-3′.[17]

Western blot

The whole-cell extracts were prepared by resuspending cells in lysis buffer. Samples were boiled and loaded onto a 10% SDS-PAGE gel, and proteins were electrophoretically transferred from the gel to polyvinyl difluoride membranes (Amersham, Arlington Heights, IL, USA). The primary antibody against LGALS7 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The corresponding secondary antibody was added to the membrane after primary antibody incubation. GAPDH was used as a protein loading control. The immunoreactive bands were detected using an ECL-plus chemiluminescence substrate (Perkin—Elmer, Waltham, MA, USA) and captured by LAS-1000 plus Luminescent Image Analyzer (GE Healthcare, Piscataway, NJ, USA).[17]

Silencing LGALS7 expresssion with lentiviral system in PDLs

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence was described in the manufacturer's protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets LGALS7 was synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. The target sequences for are: Sh-LGALS7-1: 5′-AAAAGCCTTGGCTGGCAAATAAATTGGATC CAATTTATTTGCCAGCCAAGGC-3′; The target sequences for are: Sh-LGALS7-2: 5′-AAAAGGCTGGCAAATAAAGCGTTTT GGATCCAAAACGCTTTATTTGCCAGCC -3′.[17]

Statistical analysis

Three replicates of each experiment were performed. All results were shown as the mean ± SD. One-way ANOVA was used for multiple comparison and Student's *t*-test was used for comparisons between two grups. $p < 0.05$ was considered statistically significant.

Results

Assessment of cell proliferation

MTT assay was performed to evaluate the PDL fibroblasts survival in a fluence range of 3.6—6.3 J J/cm^2 according to a previous report[16], which suggested that the Er:YAG laser-induced cell death at higher energy levels. The proliferative activity of PDL fibroblasts was significantly enhanced at all tested energy levels (3.6—6.3 J/cm^2) after the application of Er:YAG laser (Fig. 1). In particular, laser irradiation

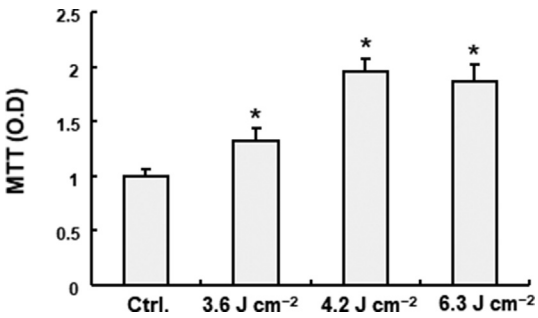

Figure 1 The effect of Er:YAG laser on the cell proliferation of PDLfibroblasts. Cell proliferation was examined after irradiation using the MTT assay. All results were displayed as arbitrary units relative to the control group (without irradiation), and Er:YAG laser irradiation caused an increase in proliferative activity at the total energy density of 3.6—6.3 J/cm^2. Data are shown as mean ± SD, *$p < 0.05$.

at 4.2 J/cm^2 significantly promoted cell proliferation ~ 2-fold compared to the control group. This result showed that treatment with Er:YAG laser amplified the cell proliferation and viability of PDL fibroblasts.

Examination of cell motility

The transwell migration and invasion assays were employed to analyze the ability of PDL fibroblasts to change their position within tissues in the vertical direction or penetrate tissue barriers. As shown in Fig. 2A, the ability of migration in PDL fibroblast was elevated in response to laser irradiation. Similarly, the laser treatments at all tested energy levels dramatically increased their invasion capacity (Fig. 2B).

The effect of Er:YAG laser on the horizontal migration of these cells was examined by wound healing assay. We showed that the collective migration of PDL fibroblasts was increased following low-level Er: YAG laser irradiation (Fig. 3), which was consistent with the transwell assay. Collectively, these results suggested that Er: YAG laser was able to enhance the cell motility and may accelerate the regeneration process.

Expression of LGALS7

According to the abovementioned results, the optimal energy setting for the induction of PDL fibroblast proliferation and cell motility was the value of 4.2 J/cm^2.

To investigate the potential molecular mechanism, we chose galectin 7 for further examination as it is critical to epithelial homeostasis and tissue repair[19] and was found upregulated in the Er:YAG laser-irradiated HGFs.[15] To verify the effect of Er: YAG laser on the expression of LGALS7 (galectin 7) in PDL fibroblasts, qRT-PCR was carried out and showed that the gene expression of LGALS7 upregulated following various energy densities of Er: YAG laser (Fig. 4B). Likewise, we showed that the protein expression of galectin-7 in PDL fibroblasts was elevated following laser irradiation (Fig. 4C).

The functional role of LGALS7 in cell motility

In an effort to investigate the significance of LGALS7 in the Er: YAG laser-induced cell motility, small hairpin RNAs (shRNAs) were utilized to silence LGALS7 in PDL fibroblasts. The knockdown efficiency of the gene or protein expression was confirmed in two lines of cells (Fig. 5A and B). Our results showed that the transwell migration and invasion capacities were not increased in PDL fibroblasts with sh-LGALS7 following low-level Er: YAG laser irradiation (Fig. 5C and D). Also, knockdown of LGALS7 mitigated the elevation of wound healing ability after treatment with Er: YAG laser in sh-LGALS7-PDL fibroblasts compared to sh-Luc group (Fig. 5E). These findings suggested that the effect of Er: YAG laser on the increased cell motility of PDL fibroblasts was mediated by the upregulation of LGALS7.

Discussion

After being introduced as an alternative to conventional debridement, the applications of Er:YAG laser in periodontics have been broadly evaluated over the past decades. Due to its property of thermo-mechanical ablation with minimal thermal side effects such as melting and

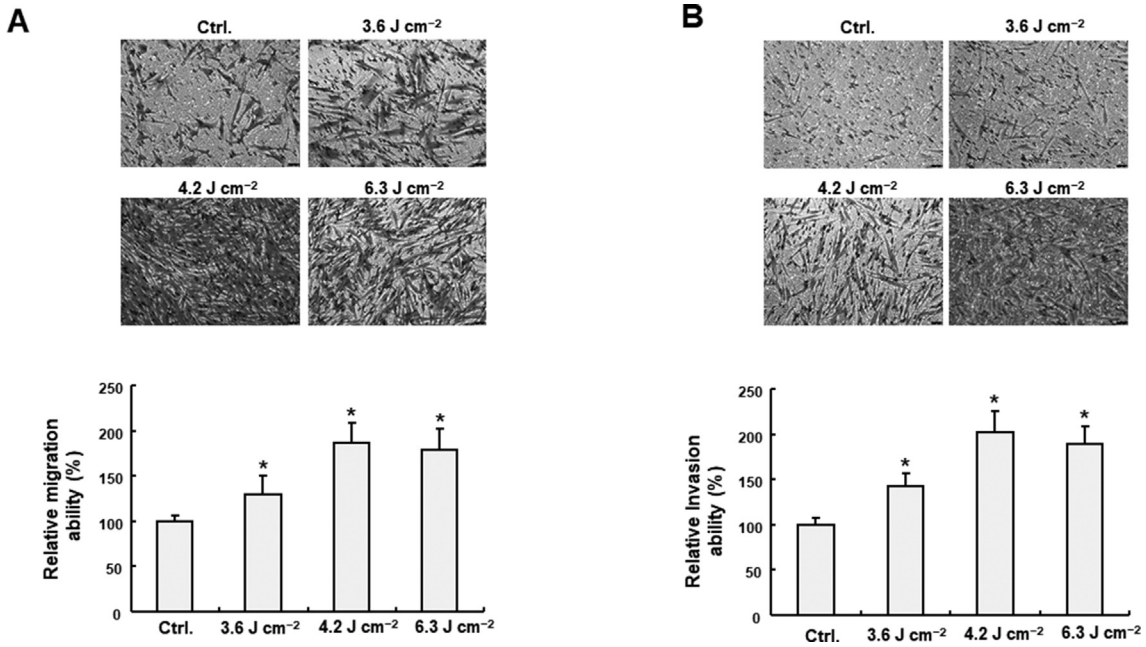

Figure 2 Migration and invasion activities after Er:YAG laser irradiation. The Transwell system was applied to examine the migration (A) and invasion (B) capacities of Er:YAG laser-irradiated PDL fibroblasts. All results were shown as the percentage of the control group. The experiments were repeated three times, and representative results were presented as means ± SD. *$p < 0.05$ as compared with the control group. Magnification, × 200.

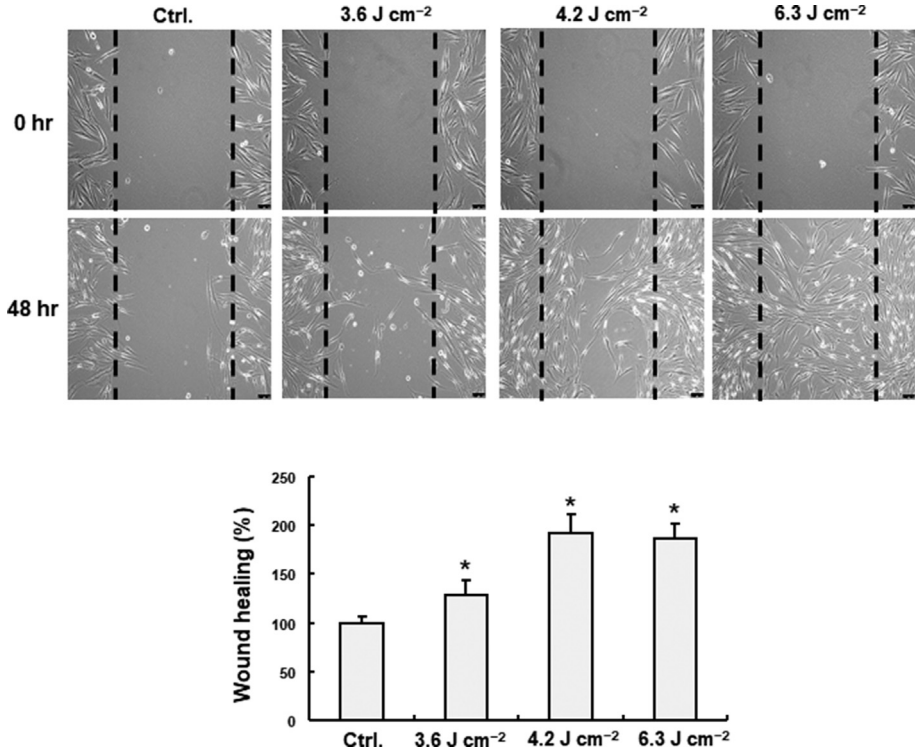

Figure 3 The effect of Er:YAG laser irradiation on the wound healing ability. The wound healing capacity of PDL fibroblasts treated with various energy densities of Er:YAG laser. All results were presented as the percentage of the control group. The experiments were repeated three times, and representative results were shown as means ± SD. *$p < 0.05$ as compared with the control group. Magnification, × 100.

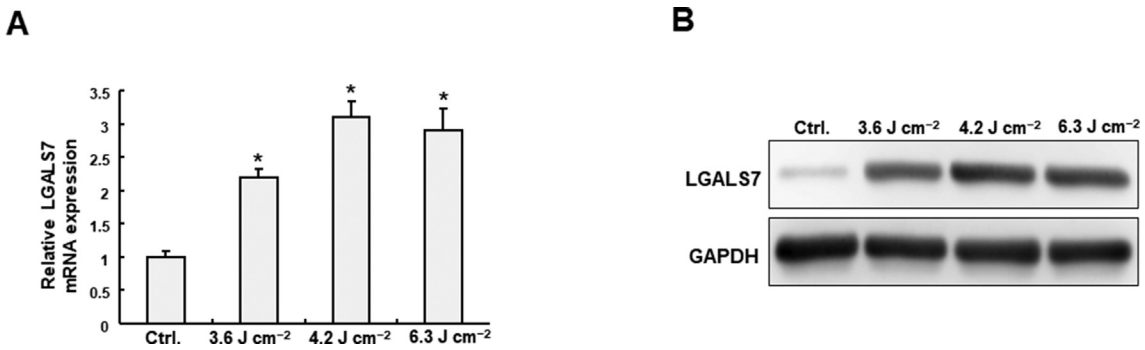

Figure 4 Upregulation of LGALS7 following Er:YAG laser irradiation. (A) mRNA and (B) protein expression of LGALS7 in PDL fibroblasts in response to various densities of Er:YAG laser. The results were shown as means ± SD. *$p < 0.05$ as compared with the non-arctigenin-treated group.

carbonaization, Er:YAG laser was suitable for the removal of dental hard tissue and reduction of bacterial endotoxins from the root surface. In addition to bactericidal effect and less thermal damages, various studies have demonstrated that Er:YAG laser may accelerate the regeneration process of the periodontal tissues through the preservation of a more homogeneous root surface for HGFs and PDL fibroblasts to attach and proliferate.[11,12] Nertheless, the direct effect of Er:YAG laser on oral fibroblasts and the associated molecular mechanisms remained to be elucidated. In the last few years, a couple of studies have shown that Er:YAG laser irradiation stimulated the proliferation of osteoblasts and dental pulp cells. It has been shown that low-level Er:YAG laser with various laser settings (fluence 1.0—15.1 J/cm^2) resulted in a higher proliferation of osteoblasts through activation of MAPK/extracellular signal-regulated kinase (ERK) pathway.[20] Another study showed that Er:YAG laser enhanced the expression of matrix metalloproteinase (MMP)-2, which may further involve the activation of latent TGF-β1 and differentiation of odontoblasts from dental pulp cells.[21] As for oral fibroblasts, Er:YAG laser also has been shown to increase the proliferation of HGFs.[14—16] However, the setting of Er:YAG laser may need adjustments to achieve the best results as the

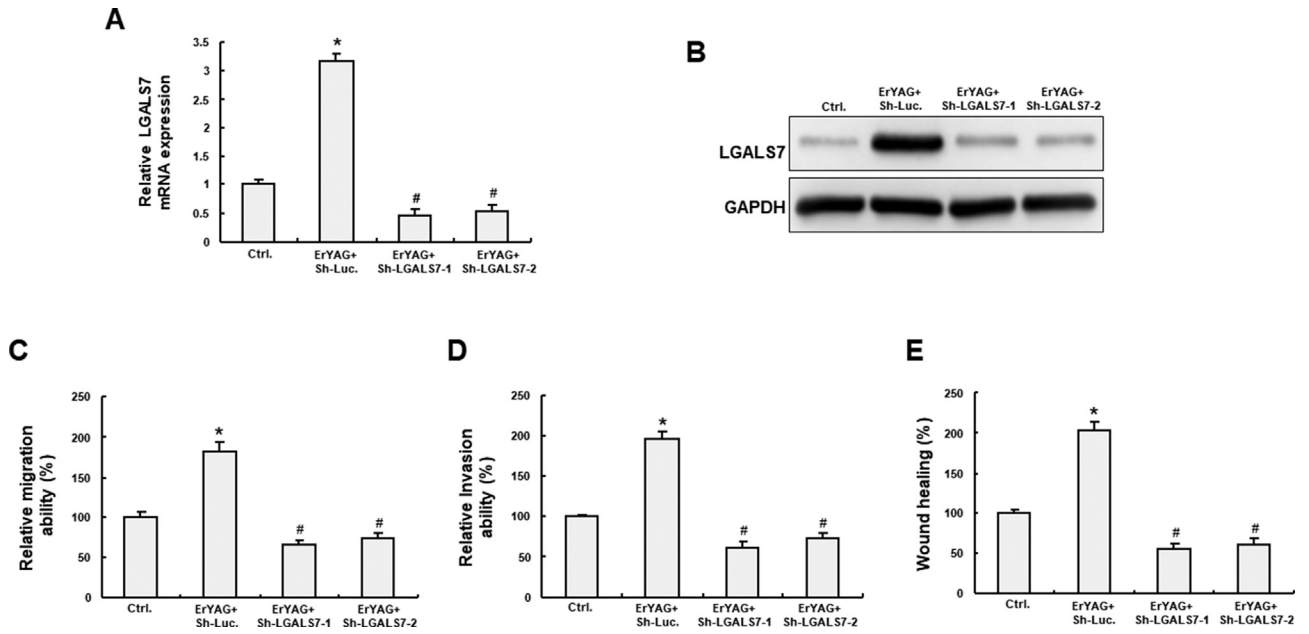

Figure 5 Galectin-7 mediates the Er:YAG laser-elicited effects in PDL fibroblasts. (A) Gene and (B) protein expression levels were evaluated to confirm the efficiency of Sh-LGALS7-1 and Sh-LGALS7-2; The Er:YAG laser-induced (C) migration, (D) invasion and (E) wound healing capacities were abolished in PDL fibroblasts with Sh-LGALS7s. All results were shown as the percentage of the control group. The experiments were repeated three times, and representative results were presented as means ± SD. $^*p < 0.05$ compared to the control group.

optimal energy density to enhance HGF proliferation varied in different research groups (3.37—6.3 J/cm^2).[14,16] In the present study, we observed that 4.2 J/cm^2 was the lowest density to yield the best results of cell viability and motility in PDL fibroblasts. In combination with the results from Aleksic et al.,[20] the favorable density to potentiate the repair of periodontal tissue in terms of a better fibrous connection with cementum and regeneration of alveolar bone may be around 4 J/cm^2

Previously, the gene expression levels of five DEGs associated with HSPs and ERK pathway (HSPA1A, HSPA1B, HSPA6, DUSP5, and DUSP6) were validated to be upregulated in HGFs after Er:YAG laser irradiation.[16] Moreover, their work proved that activation of transient receptor potential channels participated in the Er:YAG laser-induced cell proliferation.[16] Another study by Ogita et al. showed that the gene expression of galectin-7 in HGFs was significantly elevated and the administration of recombinant galectin-7 enhanced the cell proliferation.[15] In line with this finding, we also found that the gene and protein expression levels of galectin-7 were both upregulated in Er:YAG laser-irradiated PDL fibroblasts. Moreover, suppression of galectin-7 in Er:YAG laser-irradiated PDL fibroblasts impeded these laser-induced effects. Altogether, these results suggested that Er:YAG laser may accelerate reparative processes through galectin-7 induction.

Galectin-7, a 14-kDa member of the galectin family of β-galactoside-binding proteins, is primarily expressed in stratified epithelia, such as epidermis, cornea, and oral cavity.[22] It has been indicated that galectin-7 may be implicated in the tissue response to environmental stimuli.[23] In addition, galectin-7 has been shown to regulate the proliferation, migration and apoptosis in keratinocyte during ultraviolet radiation irradiation-induced skin repair[19], and it has been demonstrated to modulate the proliferation and differentiation of keratinocytes through JNK-miR-203-p63 axis.[24] In oral squamous cell carcinoma cells, the silence of galectin-7 did not alter the proliferation or apoptosis rates, but the cell migration and invasion capacities were decreased.[25] Their work demonstrated that overexpression of galectin-7 enhanced the cell migration/invasion through ERK and JNK-mediated upregulation of MMP-2 and MMP-9.[25] Since it was reported that Er:YAG laser activated ERK pathway in osteoblasts[20] and induced MMP2 expression in dental pulp cells[21], it is reasonable to hypothesize that Er:YAG laser promoted cell motility via galectin-7/ERK/MMP-2 signaling pathway in PDL fibroblasts as well. Further studies will be required to verify this assumption.

In summary, the present data demonstrated that Er:YAG laser irradiation caused a direct effect of promoting proliferation, migration, and invasion of PDL fibroblasts through the upregulation of galectin-7. These findings provided an insight into the molecular mechanism underlying the effect of Er:YAG laser on periodontal therapy.

Declaration of Competing Interest

The authors have no conflicts of interest relevant to this article.

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