Palmitoyl-RGD promotes the expression of dermal-epidermal junction components in HaCaT cells

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Abstract. With age, the dermal-epidermal junction (DEJ) becomes thinner and production of its protein components decreases; this may be associated with increased fragility and wrinkling of skin. Topical treatment with palmitoyl-Arg-Gly-Asp (PAL-RGD) improves facial wrinkles, skin elasticity and dermal density in humans. In the present study, the effect of PAL-RGD on expression of DEJ components, such as laminin and collagen, was assessed. Human HaCaT keratinocytes were treated with PAL-RGD. The protein expression levels of laminin-332, collagen IV and collagen XVII were examined by western blotting. Reverse transcription-quantitative PCR was used to analyze laminin subunit (*LAM*)*A3*, *LAMB3*, *LAMC2*, collagen type IV α 1 chain (*COL4A1*) and *COL17A1* mRNA expression levels. Western blot analysis showed that the expression levels of proteins comprising the DEJ, including laminin α3, β3 and γ2 and collagen IV and XVII demonstrated a significant dose-dependent increase following PAL-RGD treatment. Furthermore, PAL-RGD treatment significantly enhanced *LAMA3*, *LAMB3*, *LAMC2*, *COL4A1* and *COL17A1* mRNA expression levels. PAL-RGD may enhance the DEJ by inducing the expression of laminin-332, collagen IV and collagen XVII.

Introduction

The dermal-epidermal junction (DEJ), located between the epidermis and dermis, provides a specific niche that mediates numerous signals such as MAPK in addition to providing structural support to keratinocytes (1,2). The DEJ serves an important role in skin cohesion, resistance to mechanical stress and exchange of the signals between the dermis and epidermis (3). As skin ages, the wavelike structure of the DEJ becomes thinner and appears flattened with a loss of rete ridges (4-6). Furthermore, previous studies have reported that production of protein components of the DEJ, including collagen IV, VII and XVII, nidogen, integrin β4 and laminin-332, decreases with age (6-9). Laminins are the most abundant glycoproteins of the basement membrane (BM) extracellular matrix (ECM) and are key for supporting tissue architecture and stability (10). Laminins are involved in skin reepithelization and wound healing via regulation of adhesion, proliferation, migration, apoptosis and differentiation (11). Collagen IV is a primary component of anchoring fibrils, which provide mechanical support for keratinocytes (12). Collagen XVII, another structural component of anchoring fibrils, serves important roles in assembly and function of the cell-matrix adhesion structure, signal transduction and keratinocyte differentiation (13). Based on the key roles of the DEJ in skin homeostasis, modulation of DEJ components has been suggested as a potential strategy to mitigate skin aging (14-16).

Our previous study demonstrated that palmitoyl-Arg-Gly-Asp (PAL-RGD) decreases the appearance of human facial wrinkles, skin elasticity and dermal density via stimulation of procollagen synthesis and inhibition of matrix metalloproteinase (MMP)-1 expression in human dermal fibroblasts (17). Although collagen degradation within the dermis is considered the primary cause of wrinkles, biological changes in the epidermis and DEJ, such as decreased collagen IV, collagen VII, collagen XVII, integrin β4, and laminin-332, are also reported to affect wrinkle formation (6). In the present study, the effects of PAL-RGD on expression of DEJ components, including laminin-332, collagen IV and collagen XVII in HaCaT keratinocytes was investigated.

Materials and methods

Reagents. PAL-RGD was purchased from Celltrion Chemical Research Institute. MTT was purchased from Sigma-Aldrich (Merck KGaA). Dulbecco's Modified Eagle's Medium

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(DMEM), penicillin, streptomycin, PBS, fetal bovine serum (FBS) and trypsin/EDTA were purchased from Gibco (Thermo Fisher Scientific, Inc.).

Cell culture. The human keratinocyte HaCaT cell line, kindly gifted by Dr Norbert Fusenig of the German Cancer Research Center (Heidelberg, Germany), was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. Cell viability was determined using MTT assay. Briefly, HaCaT keratinocytes were seeded into a 24-well plate at $5x10^4$ cells/well and incubated at 37˚C for 24 h. Following serum starvation at 37˚C for 24 h, cells were treated with PAL-RGD (0, 1, 5, 10, 15 and 20 μg/ml) for 48 h in fresh serum-free DMEM at 37˚C. MTT was added and cells were incubated at 37˚C for 4 h to allow reduction of the MTT reagent to formazan dissolved with DMSO. The absorbance was measured at 570 nm using a VersaMax microplate reader (Molecular Devices, LLC).

Western blotting. HaCaT cells were seeded into a 6-well plate at $1x10^6$ cells/well and incubated at 37˚C for 24 h. Following serum starvation at 37˚C for 24 h, the cells were treated with PAL-RGD (0.0, 5.0, 7.5, 10.0, 12.5 and 15.0 μg/ml) at 37˚C for 48 h in fresh serum-free DMEM. Cell lysates were prepared using PRO-PREP™ Protein Extraction Solution (Intron Biotechnology, Inc.) and conditioned medium was collected. Protein concentration was determined with the BCA method (Pierce Biotechnology). The 20 μg protein from cell lysate and conditioned media were separated on 4-15% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories, Inc.) and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skimmed milk in Tris-buffered saline for 1 h at room temperature and incubated with primary antibodies overnight at 4˚C against laminin α3 (1:500; cat. no. ab151715; Abcam), laminin β3 (1:500; cat. no. sc-133178; Santa Cruz Biotechnology, Inc.), laminin γ2 (1:500; cat. no. MAB19562; MilliporeSigma), collagen type IV (COL4; 1:500; cat. no. AB769; MilliporeSigma), COL17 (1:500; cat. no. ab184996; Abcam) and β-tubulin (1:1,000; cat. no. SC-9104; Santa Cruz Biotechnology, Inc.). After rinsing with TBS + 0.05% Tween-20, the membranes were incubated at room temperature for 2 h with horseradish peroxidase-conjugated anti-goat (cat. no. #605-4302), anti-mouse (cat. no. #610-4302) and anti-rabbit antibodies (all 1:5,000, #611-1302, all Rockland Immunochemicals, Inc.). Membranes were developed with ECL solution (SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, Inc.). Signals were visualized using the ChemiDoc XRS system (Bio-Rad Laboratories, Inc.) and analyzed with Image Lab 5.0 (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Cells were incubated with PAL-RGD (0.0, 5.0, 7.5, 10.0, 12.5 and 15.0 μg/ml) for 2, 4, 8 and 24 h. Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's protocol. First-strand complementary DNA (cDNA) synthesis using 1 μg total RNA was performed using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR Green and StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The final reaction mixture contained 10 ng cDNA, 100 nmol each primer, 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and RNase-free water to a final volume of 20 μl. PCR was performed an initial denaturation step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Dissociation curve was generated to verify the specificity of each reaction. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method and expressed as percent change in gene expression relative to *36B4* (18). The primer sequences are presented in Table I.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Sequence, 5'-3'
LAMA3	F: CAATTCTGAAGACCCCAGGA
	R: TCCAAGACCCAAAGATCAGG
LAMB3	F: CAGATTGGGTTGGAATGCTT
	R: CCCAGCTTCCTTGACTTGAG
LAMC2	F: GGCTGGTCTTACTGGAGCAG
	R: TATGGCAGCTTCACTGTTGC
Col4A1	F: CTGGTCCAAGAGGATTTCCA
	R: TCATTGCCTTGCACGTAGAG
COL17A1	F: TCTGCCTACAGCAACGTGAC
	R: CTCACGGCTTGACAGCAATA
36B4	F: TCGACAATGGCAGCATCTAC
	R: TGATGCAACAGTTGGGTAGC

LAM, laminin subunit; COL4A1, collagen type IV α 1 chain; COL17A1, collagen type XVII α 1 chain; F, forward; R, reverse.

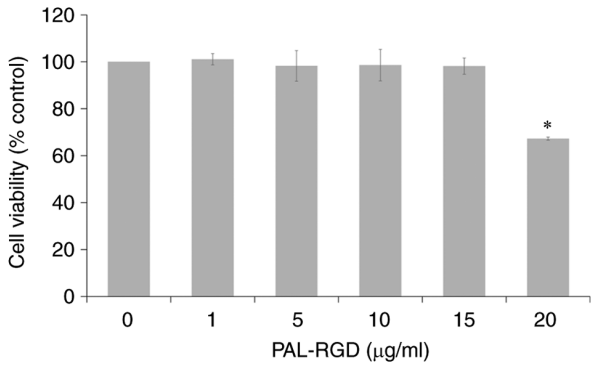

Figure 1. Effect of PAL-RGD on viability of HaCaT keratinocytes. Cells were incubated with PAL-RGD for 48 h. Data are presented as the mean ± SD of three independent experiments. *P<0.05 vs. 0. PAL-RGD, palmitoyl-Arg-Gly-Asp.

Statistical analysis. Data are presented as the mean ± SD of ≥3 independent experiments. Statistical analysis was performed using SAS 9.4 (SAS Institute, Inc.) software. Statistical significance was calculated using one-way ANOVA followed by

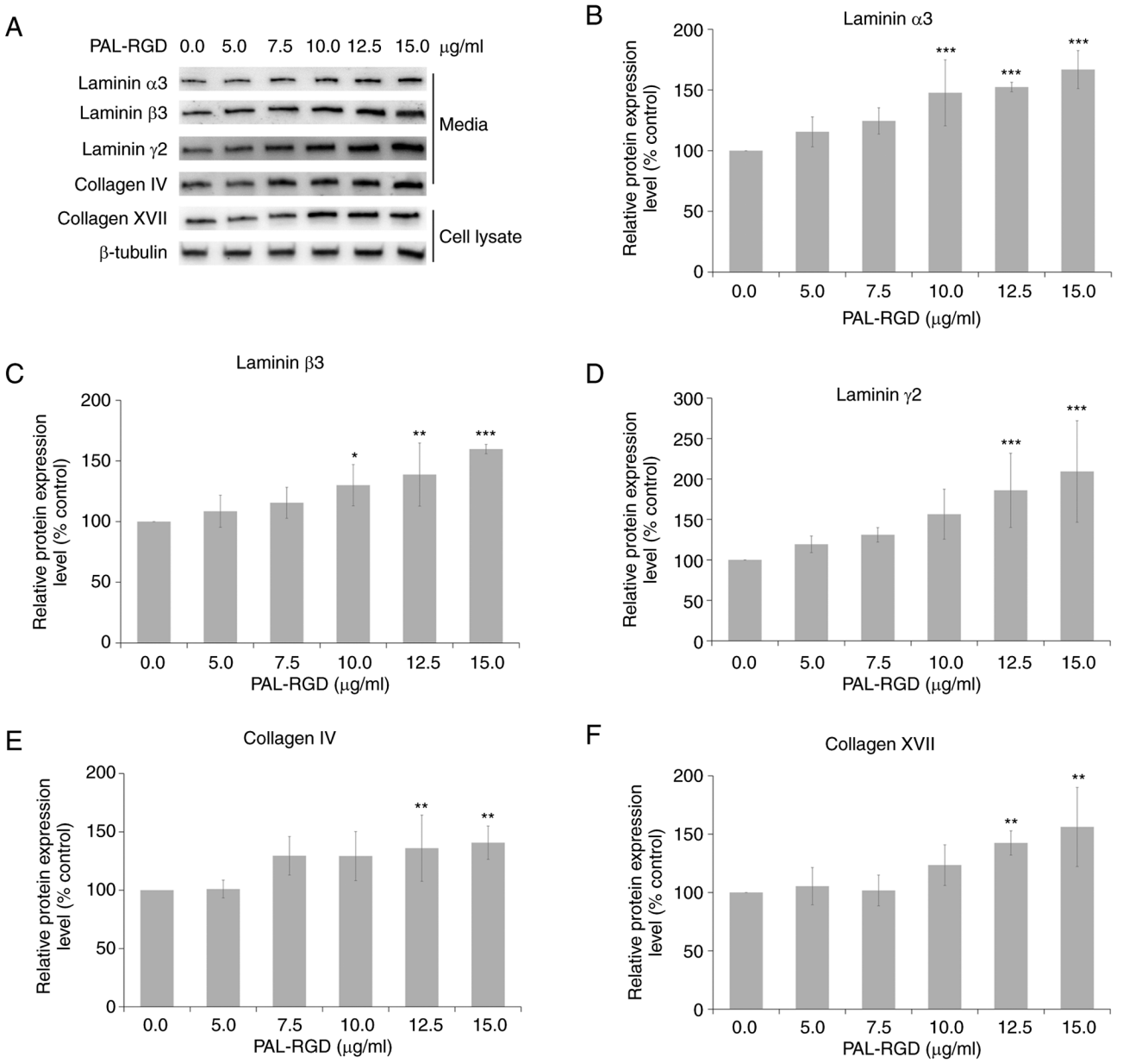

Figure 2. Effect of PAL-RGD on dermal-epidermal junction proteins in HaCaT keratinocytes. (A) Representative images of western blotting of cell lysate and conditioned media. Protein expression levels of (B) laminin α3, (C) laminin β3, (D) laminin γ2, (E) collagen IV and (F) collagen XVII following treatment with PAL-RGD. Protein expression levels were normalized β-tubulin. Data are presented as the mean ± SD of four independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. 0.0. PAL-RGD, palmitoyl-Arg-Gly-Asp.

two-tailed post hoc Dunnett's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of PAL-RGD on viability in HaCaT cells. MTT assay was performed to determine optimal PAL-RGD concentration for treatment of cells. PAL-RGD did not induce a significant effect on cell viability at concentrations <15 μg/ml. The cell viability significantly decreased to 67% only at 20 μg/ml Pal-RGD (Fig. 1). For subsequent experiments, the maximum concentration used was 15 μg/ml.

Effect of PAL-RGD on DEJ protein expression in HaCaT cells. HaCaT cells were treated with PAL-RGD for 48 h. Total protein extracted from cell lysate and conditioned media was analyzed using western blotting. PAL-RGD dose-dependently increased the expression of DEJ proteins (Fig. 2). Treatment with 15 μg/ml PAL-RGD significantly increased protein expression of laminin α3 (167%), laminin β3 (160%), laminin γ2 (209%), collagen IV (141%) and collagen XVII (156%) compared with the control.

Effect of PAL-RGD on laminin subunit (LAM)A3, LAMB3 and LAMC2 mRNA expression in HaCaT cells. Treatment with PAL-RGD enhanced mRNA expression levels of *LAMA3*, *LAMB3* and *LAMC2* in HaCaT cells (Fig. 3). *LAMA3* mRNA expression increased in a dose-dependent manner at 2, 4 and 8 h, then decreased to the basal level at 24 h. *LAMA3* mRNA expression levels significantly increased

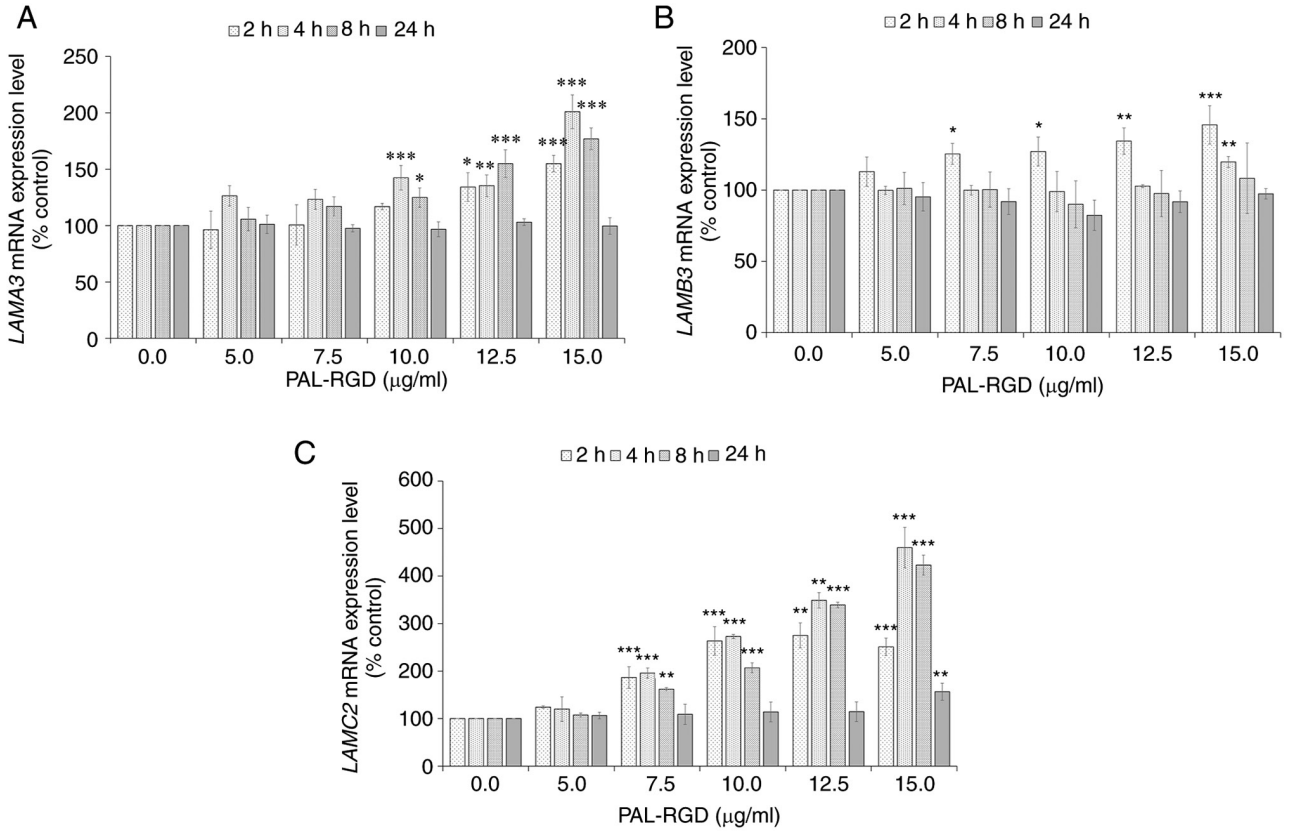

Figure 3. Effect of PAL-RGD on expression of genes encoding LAM-332 in HaCaT keratinocytes. The mRNA expression levels of (A) *LAMA3*, (B) *LAMB3* and (C) *LAMC2* following treatment with PAL-RGD. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. 0. PAL-RGD, palmitoyl-Arg-Gly-Asp; LAM, laminin subunit.

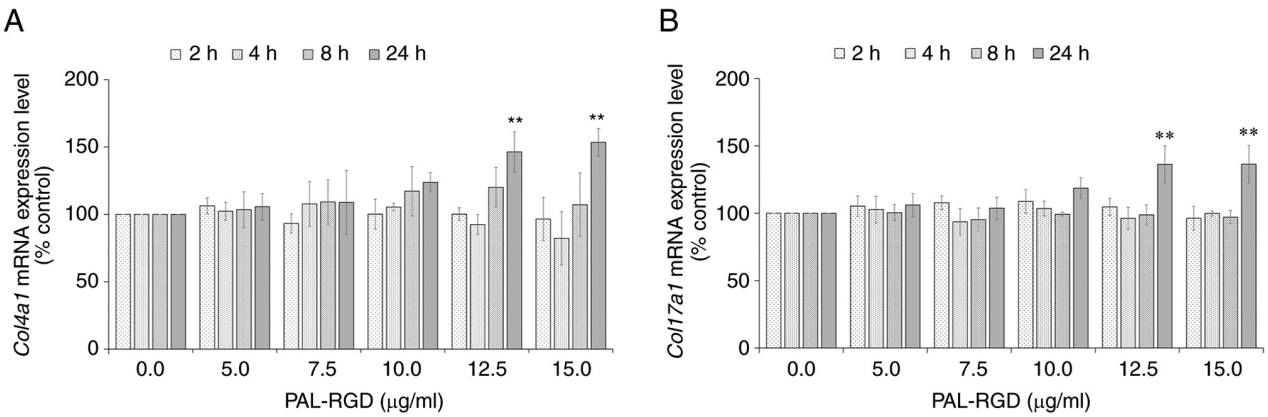

Figure 4. Effect of PAL-RGD on expression of *COL4A1* and *COL17A1* in HaCaT keratinocytes. mRNA expression levels of (A) *COL4A1* and (B) *COL17A1* following PAL-RGD treatment. Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. 0. PAL-RGD, palmitoyl-Arg-Gly-Asp; COL4A1, collagen type IV α 1 chain; COL17A1, collagen type XVII α 1 chain.

at 4 h for all concentrations of PAL-RGD compared with the control. *LAMB3* mRNA expression was increased in a dose-dependent manner at 2 h prior to dropping to the basal level at 8 h. The 2 h increase of *LAMB3* mRNA expression was significant from 7.5 to 15 μg/ml of PAL-RGD used compared with the control. Furthermore, mRNA expression of *LAMC2* significantly increased 2 h after PAL-RGD treatment. At 2 h increase of *LAMC3* mRNA expression was significantly for all concentrations of PAL-RGD used compared with the control. The maximum increases in *LAMA3*, *LAMB3* and *LAMC2* in response to 15 μg/ml PAL-RGD treatment were significant by 201% (4 h, P<0.0001), 146% (2 h, P=0.0003) and 460% (4 h, P<0.0001) respectively, compared with the control.

Effect of PAL-RGD on COL4 α 1 chain (COL4A1) and COL17A1 mRNA expression in HaCaT cells. The mRNA expression of *COL4A1* and *COL17A1* was increased by PAL-RGD treatment in HaCaT cells (Fig. 4). Although unchanged following exposure to PAL-RGD for 2 and 8 h, mRNA expression levels increased in a dose-dependent

manner at 24 h. Treatment with 12.5 μg/ml PAL-RGD significantly induced *COL4A1* and *COL17A1* mRNA levels by 146 and 136%, respectively. The mRNA expression of *COL4A1* and *COL17A1* in HaCaT cells treated with 15 μg/ml PAL-RGD significantly increased by 153 and 136%, respectively, compared with the control.

Discussion

In the present study, PAL-RGD treatment increased both mRNA and protein expression levels of DEJ components, such as laminin-332, collagen IV and collagen XVII, in HaCaT keratinocytes. The mRNA expression levels of laminin-332 components increased before 8 h and returned to normal at 24 h; however, protein expression levels in media were significantly increased at 48 h. Laminin-332 is a secreted protein that matures via specific proteolytic processing, which may contribute to the time lag between mRNA expression in cells and protein accumulation in media (19) Previous studies have reported that transforming growth factor-β (TGF-β) upregulates expression of *LAMA3*, *LAMB3*, *LAMC2*, *COL4A1* and *COL17A1* (20-22). PAL-RGD may induce TGF-β signaling; further studies are required to investigate the signaling pathway underlying the PAL-RGD-mediated upregulation of laminin-332, collagen IV and collagen XVII.

Laminins are glycoproteins composed of three chains (α, β and γ). At least 15 different isoforms exist with a combination of five distinct α, three β and three γ subunits (23,24). The functional domains of LAMs serve distinct roles, such as assembly into trimeric molecules, binding to other ECM molecules and interaction with cell surface receptors. Laminins are involved in various cellular functions, such as cell adhesion, migration, proliferation and differentiation (25). In particular, laminin-332 activates the signaling through integrin α3β1 and α6β4 (26). The binding of laminin-332 and integrin α3β1 activates the mitogen-activated protein kinase signaling pathway, which regulates keratinocyte proliferation (27). Moreover, the interaction between laminin-332 and integrin α3β1 regulates Src kinase signaling through focal adhesion kinase, promoting polarized lamellipodium extension, which regulates keratinocyte migration (28). The interaction between laminin-332 and integrin α6β4 mediates assembly of the hemidesmosome cytoskeleton and recruitment of adaptor proteins Shc/growth factor receptor-bound protein 2 (29). Therefore, PAL-RGD may promote proliferation and migration of keratinocytes by increasing laminin-332 expression.

Collagen IV is a non-fibrillar collagen that constitutes ~50% of the BM, forming a structural scaffold for interactions between BM components, such as laminin networks, perlecan and proteoglycans, during BM assembly (30,31). Other extracellular molecules, including growth factors, laminins, proteoglycans and nidogens, are attached to the scaffolds, where they serve various biological roles, such as cell adhesion, migration, tissue regeneration, wound healing, immobilization of growth factors and enzymes and molecular sieving (32,33). Given these features of collagen and related molecules, PAL-RGD may strengthen BM scaffolds by promoting synthesis of collagen IV.

Collagen XVII is a hemidesmosomal transmembrane protein involved in epidermal-dermal attachment (34). Congenital collagen XVII deficiency results in junctional epidermolysis bullosa and acquired autoimmunity to collagen XVII leads to bullous pemphigoid (35). Furthermore, collagen XVII binds to laminin-332 in hemidesmosomes and collagen IV in the ECM, linking cytoplasmic structural components with ECM (36). Based on these functions of collagen XVII, PAL-RGD may enhance BM assembly by inducing collagen XVII synthesis.

In summary, the present study suggested that PAL-RGD may serve a role in strengthening the DEJ by promoting expression of laminin-332, collagen IV and collagen XVII in keratinocytes. Together with previous studies demonstrating that PAL-RGD decreases facial wrinkles by increasing collagen I and decreasing MMP-1, this compound could improve skin aging by increasing production of components of the DEJ and BM. A limitation of the present study was lack of data on the biological effect of PAL-RGD on the function of the DEJ. Further studies are needed to determine the biological effect of PAL-RGD on the function of the DEJ.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JHL conceived and designed the study, performed experiments, collected the data and wrote the manuscript. JSB designed the study, performed experiments and revised the manuscript. JHL and JSB confirm the authenticity of all the raw data. SKL conceived the study and revised the manuscript. DHL interpreted the data and revised the manuscript. All authors discussed the results. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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