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Original Research Article

The Stimulatory Effect of Porcine Placenta Extract on *in Vitro* Biological Activities of Keratinocytes and Fibroblasts

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ABSTRACT

Porcine placenta extract (PPE), a synergistic bioactive compound, enhances cellular functions such as proliferation, migration, and cellular signaling. Keratinocytes and fibroblasts play essential roles during wound healing, particularly in tissue regeneration. However, the direct impact of PPE on biological activities of keratinocytes and fibroblasts remains underexplored. This study aimed to elucidate the stimulatory impact of PPE on the proliferation, migration, and gene expression of matrix metalloproteinases (MMPs) and extracellular matrix (ECM)-related proteins in human keratinocytes and fibroblasts. Human keratinocyte (HaCaT) and fibroblast cell lines (Hs 895.Sk) were treated with specific concentrations of PPE (2.5, 5, 10, 12.5, 15, and 50 µg/mL). Cell proliferation and migration were assessed using MTT and scratch assays, respectively. Quantitative real-time PCR was performed to evaluate the expression of MMPs and ECM-related genes. Western blot was employed to investigate the activation of ERK1/2, AKT, JNK signaling pathways, and cyclin D1 expression. PPE significantly promoted the keratinocyte/fibroblast proliferation and migration in a concentration-dependent manner via activation of ERK1/2, AKT, and JNK signaling pathway. PPE also markedly upregulated the expression of MMP-2, MMP-10, and MMP-14 both in keratinocytes and fibroblasts, as well as α-SMA, fibronectin, collagen I, and collagen III in fibroblasts. Porcine placenta extract (PPE) enhances keratinocyte and fibroblast proliferation/migration by activating JNK, ERK1/2, and PI3K/AKT signaling pathways. PPE also upregulates MMP- and ECM-related gene expression including myofibroblast differentiation

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Keywords: Porcine Placenta Extract, Keratinocyte and fibroblast activities, Wound healing .

Introduction

Wound healing is an orchestrated process requiring various cells to restore the tissue damage. Among the pivotal healing-related cells, skin fibroblasts and keratinocytes respectively play key roles in granulation, tissue formation, and re-epithelialization, contributing to the remodeling phase of tissue repair.¹ The neighboring intact cells, including skin fibroblasts and keratinocytes, initially proliferate and migrate to the damage area, resulting in connective tissue and epithelial regeneration. Furthermore, the activated skin fibroblast differentiates to myofibroblast which synthesize the extracellular matrix-related proteins.

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The extracellular matrix (ECM), a complex and dynamic structural network, provides both structural supportive and critical signaling functions throughout the reparation.² In the healing process, myofibroblasts predominantly synthesize the collagen and fibronectin which attributes to essential ECM proteins.³ Matrix metalloproteinases (MMPs) are crucial modulators of ECM deposition by degrading excessive matrix components, thereby balancing ECM-related protein components.⁴ For decades, the scientific attention has been navigated toward the development of novel therapeutic modalities to enhance efficiency and efficacy of wound repair, with particular emphasis on bioactive compounds derived from natural sources. Among these, porcine placenta extract (PPE) has emerged as a particularly promising candidate due to its diverse regenerative properties, which are believed to be mediated through the regulation of key intracellular pathways.⁵

PPE contains a rich array of bioactive components, including growth factors and cytokines, which conjointly attribute its therapeutic potential. PPE facilitates cellular proliferation, attenuates inflammatory responses, and augments tissue regeneration. Particularly, key mediators such as basic fibroblast growth factor (bFGF) and transforming growth factor beta-1 (TGF-β1) that identified within PPE have been implicated to promote wound healing in rodent models.⁶ The intricate interplay between these bioactive molecules and cellular signaling cascades offers a compelling paradigm of PPE in modulating wound healing and optimizing clinical outcomes. At the molecular level, signal transduction pathways play critical roles in regulating

cellular responses during wound healing. PPE is able to modulate the wound microenvironment by engaging canonical pathways such as ERK1/2, AKT, and JNK, thereby enhancing cellular responses resulting in efficient regeneration.^{7, 8} A previous study has demonstrated the stimulatory effect of PPE on proliferation and migration in human osteoblasts via activation of these signaling pathways.⁹ Despite these promising results, the stimulatory effect of PPE on keratinocytes and fibroblasts remains limited, especially on ECM- and MMP-related gene expression.

The present study aimed to elucidate the stimulatory effects of PPE on keratinocyte and fibroblast proliferation and migration, with an emphasis on the expression of MMPs and ECM-associated gene. Furthermore, the signal transduction (ERK1/2, AKT, and JNK signaling pathway) under PPE stimulation was also evaluated using western blot analysis.

Materials and Methods

Preparation of Crude Porcine Placenta Extract (PPE)

PPE was provided by the Faculty of Sciences, Mahidol University, Bangkok, Thailand. Briefly, porcine placentas were immediately obtained following full-term physiological deliveries as waste products of the agricultural industry. Then, it promptly rinsed with phosphate-buffered saline (PBS) solution. Mechanical homogenization in PBS was subsequently used, followed by sonication. The homogenate was centrifuged at 9,000g for 1 hour at 4°C. Subsequently, the supernatant was carefully collected and filtered through a sterile 0.2 µm filter. The protein concentration in crude PPE was quantified using the Bradford assay.¹⁰

Cell Culture

The human fibroblast cell line (Hs 895.Sk; American Type Culture Collection (ATCC)) and the human keratinocyte cell line (HaCaT; a generous gift from Assoc. Prof. Thaned Kangsamaksin, Mahidol University) were employed in all experiments. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in accordance with international standards for good cell culture practice. The doubling time of Hs 895.Sk and HaCaT is respectively 52.2 and 28 hours.

Assessment of Cell Proliferation via MTT Assay

Cell proliferation was assessed using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. Hs 895.Sk and HaCaT were seeded at a density of 5×10^3 cells per well in a 96-well plate and allowed to adhere overnight. Thereafter, cells were treated with specific concentrations of PPE (2.5, 5, 10, 12.5, 15, and 50 µg/mL) and incubated for 24, 48, or 72 hours. After the endpoint of specific incubation time, the conditioned medium was discarded and replaced with 0.5 mg/mL MTT in each well followed by incubation for 4 hours in humidified atmosphere. Formazan crystals were solubilized with dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader. Cell proliferation was calculated according to the following equation: ((Optical density of specific PPE treatment) / (Optical density of control)) $\times$ 100%.¹¹

Evaluation of Cell Migration via Scratch (Wound Healing) Assay

For scratch assays, Hs 895.Sk and HaCaT were cultured in 6-well plates until 90–100% confluency. Linear wounds were created by scraping the cell monolayer with a 200-µL sterile pipette tip. Then, the cell debris was removed by washing with PBS. Cells were then treated with PPE at specified concentrations (2.5, 5, 10, 12.5, 15, and 50 µg/mL) and incubated for 24 hours. The scratch wound was imaged at initial timepoint (0 hour) and 24 hours using an inverted microscope equipped with a 10 $\times$ objective. The wound area was quantified using ImageJ software. The percentage of wound reduction was calculated using the

following equation: (wound area at initial timepoint – wound area at 24 h) / wound area at initial timepoint $\times$ 100%.¹²

Analysis of Intracellular Protein Expression via Western Blot

To investigate intracellular signaling, Hs 895.Sk and HaCaT were treated with specific concentration of PPE (10 and 50 µg/mL) and incubated according to indicated timepoint. Cells were then lysed using RIPA lysis buffer supplemented with a protease inhibitor cocktail. Cell debris were then eliminated by centrifugation at 12,000g for 10 minutes at 4°C. After collecting supernatant, total protein concentrations were determined using the Bradford assay. The protein was separated using SDS-PAGE and transferred onto PVDF membranes. Then, membranes were blocked with 5% non-fat dry milk in TBST for 1 hour, washed, and incubated overnight at 4°C with specific primary antibodies for pAKT (cat. no. 9271), Akt (cat. no. 4691), pERK (cat. no. 4370), ERK (cat. no. 4695), pJNK (cat. no. 4668), JNK (cat. no. 9252), β -actin (cat. no. 4970), and cyclin D1 (cat. no. 2978) (all from Cell Signaling Technology, Danvers, MA, USA). After incubation overnight, membranes were washed and incubated with HRP-conjugated Goat anti-rabbit IgG (cat. no. 7074; Cell Signaling Technology) at room temperature for 1 hour. The protein band was detected using enhanced chemiluminescence (ECL), and band intensities were analyzed using Image Lab software (Bio-Rad, CA, USA).

To confirm the signal transduction from PPE stimulation, pretreatment with or without 15 µM inhibitors of PI3K (LY294002), ERK1/2 (PD98059), or JNK (SP600125) (Med Chem Express, NJ, USA) for 2 hours, followed by PPE treatment were also performed.

Quantification of Gene Expression via RNA Extraction and Real-Time PCR

Hs 895.Sk and HaCaT were treated with specific concentrations of PPE for 6 hours. Total RNA was isolated using the Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA) regarding to the manufacturer's protocol. Complementary DNA (cDNA) synthesis was performed using the Tetro cDNA Synthesis Kit (Bioline, UK), followed by quantitative real-time PCR analysis using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA). Primer sequences specific for ECM- and MMP-related genes are listed in Table 1. Gene expression was quantified using the 2^{- $\Delta\Delta$ CT} method, with normalization to 18S ribosomal RNA expression.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by appropriate post hoc tests. A p-value < 0.05 was considered as statistically significant difference. All statistical analyses were conducted using GraphPad Prism software (version 9.0; GraphPad Software, San Diego, CA, USA).

Results and Discussion

This study indicated porcine placenta extract stimulates the proliferation and migration of human fibroblasts and keratinocytes through ERK1/2, AKT, and JNK signaling cascades. PPE significantly enhanced proliferation—the increase of optical density compared to the control group—of both human fibroblasts and keratinocytes in a concentration-dependent manner, with the most obvious effect observed at 72 hours (Figure 1A). Notably, 10 µg/mL PPE markedly stimulated keratinocyte proliferation at 48 hours, whereas fibroblast proliferation was significantly increased at concentrations of 25 and 50 µg/mL. Cell migration was evaluated using the scratch assay. PPE significantly promoted the migration—the reduction of the scratched gap on cell monolayers—of both human fibroblasts and keratinocytes at 24 hours compared with vehicle-treated controls (Figure 1B). Specifically, keratinocyte migration was significantly enhanced at 5 and 10 µg/mL, whereas fibroblast migration was markedly increased at 25

Table 1: Primer sequences for quantitative real-time PCR

Gene name	Primer Sequences
Metalloproteinase-1 (<i>MMP-1</i>)	F: 5'-GATGTGGAGTGCCTGATGTG-3' R: 5'-CTGCTTGACCTCAGAGACC-3'
Metalloproteinase-2 (<i>MMP-2</i>)	F: 5'-GCAGTGAATCTACAGGGACGC-3' R: 5'-ATCCTGATCCAACCAATCACC-3'
Metalloproteinase-9 (<i>MMP-9</i>)	F: 5'-CCTTCCTTATCGCCGACAAG-3' R: 5'-TGAACAGCAGCATCTTCCCC-3'
Metalloproteinase-10 (<i>MMP-10</i>)	F: 5'-CATTCCTTGTGCTGTGTGTC-3' R: 5'-TGTCTAGCTTCCCTGTCACC-3'
Metalloproteinase-14 (<i>MMP-14</i>)	F: 5'-ATAAACCCAAAAACCCACC-3' R: 5'-AAACACCCAATGCTGTCTC-3'
Type 1 Collagen (<i>Col1</i>)	F: 5'-GTGCTAAAGTGCCAATGGT-3' R: 5'-ACCAGGTTACCGCTGTTAC-3'
Type 3 Collagen (<i>Col3</i>)	F: 5'-CCAGGAGCTAACGGTCTCAG-3' R: 5'-CAGGGTTTCCATCTCTTCCA-3'
Fibronectin	F: 5'-AATGGCCAGATGATGAGCTG-3' R: 5'-TGGCACCGAGATATTCCTCC-3'
Alpha-smooth muscle actin (<i>α-Sma</i>)	F: 5'-ATCACCAACTGGGACGACAT-3' R: 5'-CATACATGGCTGGGACATTG-3'
18S ribosome	F: 5'-CCATCCAATCGGTATGTAGCG-3' R: 5'-GTAACCCGTTGAACCCATT-3'

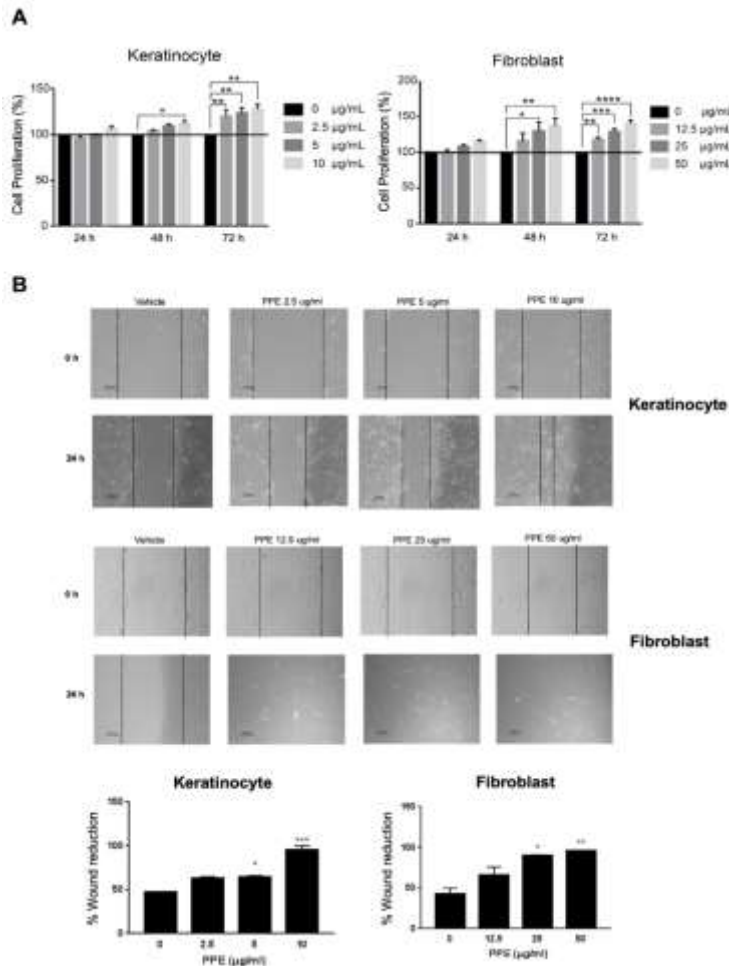


Figure 1: Porcine placenta extract (PPE) promotes proliferation and migration of human keratinocytes and fibroblasts. (A) Quantitative analysis of cell proliferation in human keratinocytes and fibroblasts following treatment with indicated concentrations of PPE (0, 2.5, 5, and 10 µg/mL for keratinocytes; 0, 12.5, 25, and 50 µg/mL for fibroblasts), assessed using the MTT assay at 24, 48, and 72 hours post-treatment (n = 9). (B) Quantification of wound closure in keratinocytes and fibroblasts using the scratch wound healing assay (n = 3) following PPE treatment at the specified concentrations. All data represent the mean ± SEM from three independent experiments. Statistical significance was determined using appropriate tests, and differences are considered significant where indicated. *P < 0.05 compared to 0 µg/mL PPE; **P < 0.01 compared to 0 µg/mL PPE; ***P < 0.001 compared to 0 µg/mL PPE.

and 50 µg/mL. In addition to evaluating the biological activities of human fibroblasts and keratinocytes, the activation of canonical intracellular signaling pathways was also evaluated. Western blot analysis showed PPE induced the phosphorylation of ERK1/2, AKT, and JNK compared to control (without PPE treatment). Additionally, PPE upregulated the expression of the cell cycle regulator cyclin D1 (Figure 2A). To confirm pathway involvement, specific pharmacological inhibitors (LY294002 (PI3K inhibitor), PD98059 (ERK1/2 inhibitor), and SP600125 (JNK inhibitor)) were employed prior to PPE treatment. The results indicated the stimulation of PPE depended on ERK1/2, AKT, and JNK signaling pathways (Figure 2B). Additionally, biological assays demonstrated the PPE-induced human keratinocyte proliferation was activated through ERK1/2, AKT, and JNK signaling pathways, whereas the PPE-induced

human fibroblast proliferation was predominantly mediated via the JNK signaling pathway (Figure 3A). Regarding cell migration, PPE-induced human keratinocyte migration involved ERK1/2, AKT, and JNK signaling pathways. Interestingly, PPE-induced human fibroblast migration involved only AKT and JNK signaling pathways (Figure 3B). Furthermore, the regulatory impact of PPE on extracellular matrix (ECM) and matrix metalloproteinase (MMP) gene expression was also investigated by treating cells with specific concentrations of PPE (0, 2.5, 5, and 10 µg/mL for keratinocytes; 0, 12.5, 25, and 50 µg/mL for fibroblasts). The mRNA expression levels were quantified using real-time PCR. PPE (10 µg/mL) significantly upregulated the expression of MMP-1, MMP-2, MMP-9, MMP-10, and MMP-14 in human keratinocytes.

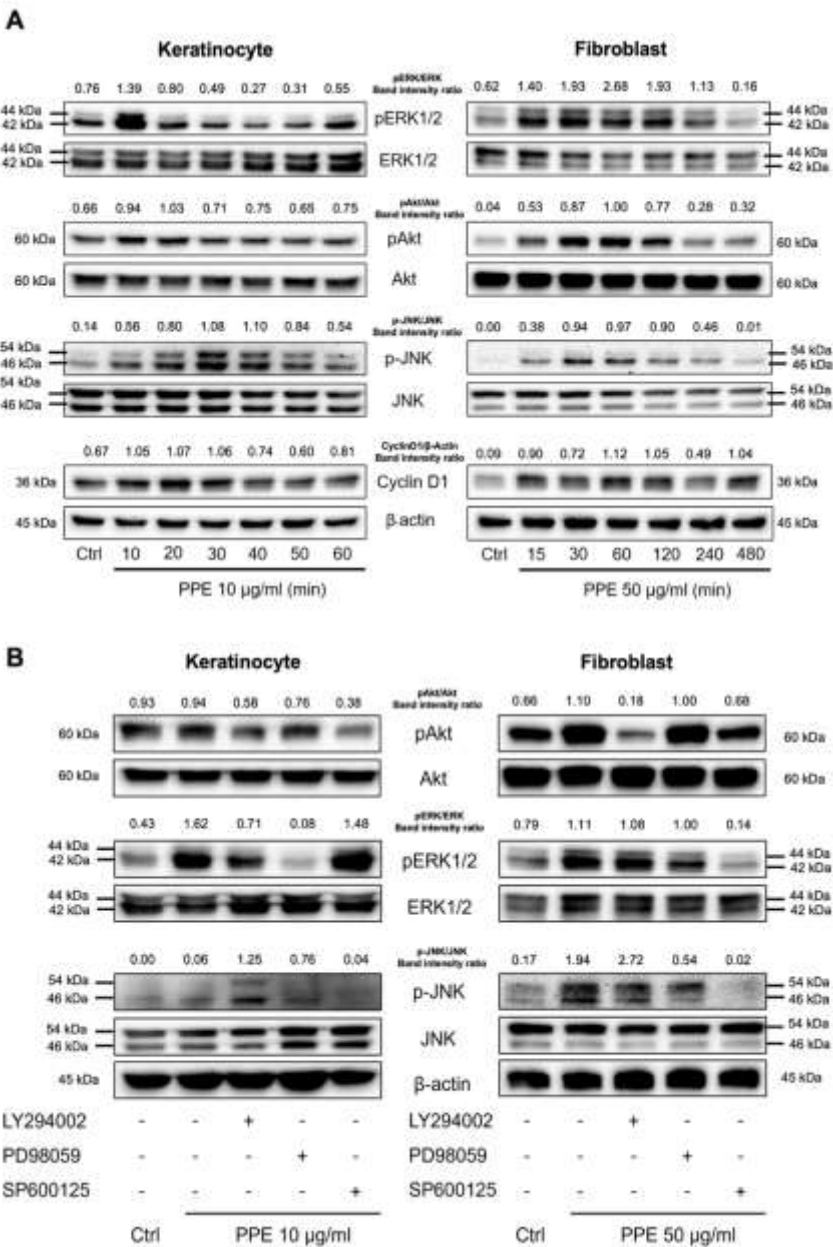


Figure 2: PPE induces phosphorylation of ERK1/2, AKT, and JNK signaling pathways in human keratinocytes and fibroblasts. **(A)** Keratinocytes and fibroblasts were treated with 10 µg/mL and 50 µg/mL of PPE, respectively, under serum-starved conditions and incubated for the indicated time points. Phosphorylation of ERK1/2, AKT, and JNK was assessed via immunoblotting. **(B)** To investigate pathway specificity, cells were pretreated for 2 hours with LY294002 (PI3K inhibitor), PD98059 (ERK1/2 inhibitor), or SP600125 (JNK inhibitor), followed by PPE treatment. Protein lysates were collected at 20 and 30 minutes for keratinocytes and fibroblasts, respectively. Full-length immunoblots are provided in Additional File 1 (Full-length blots).

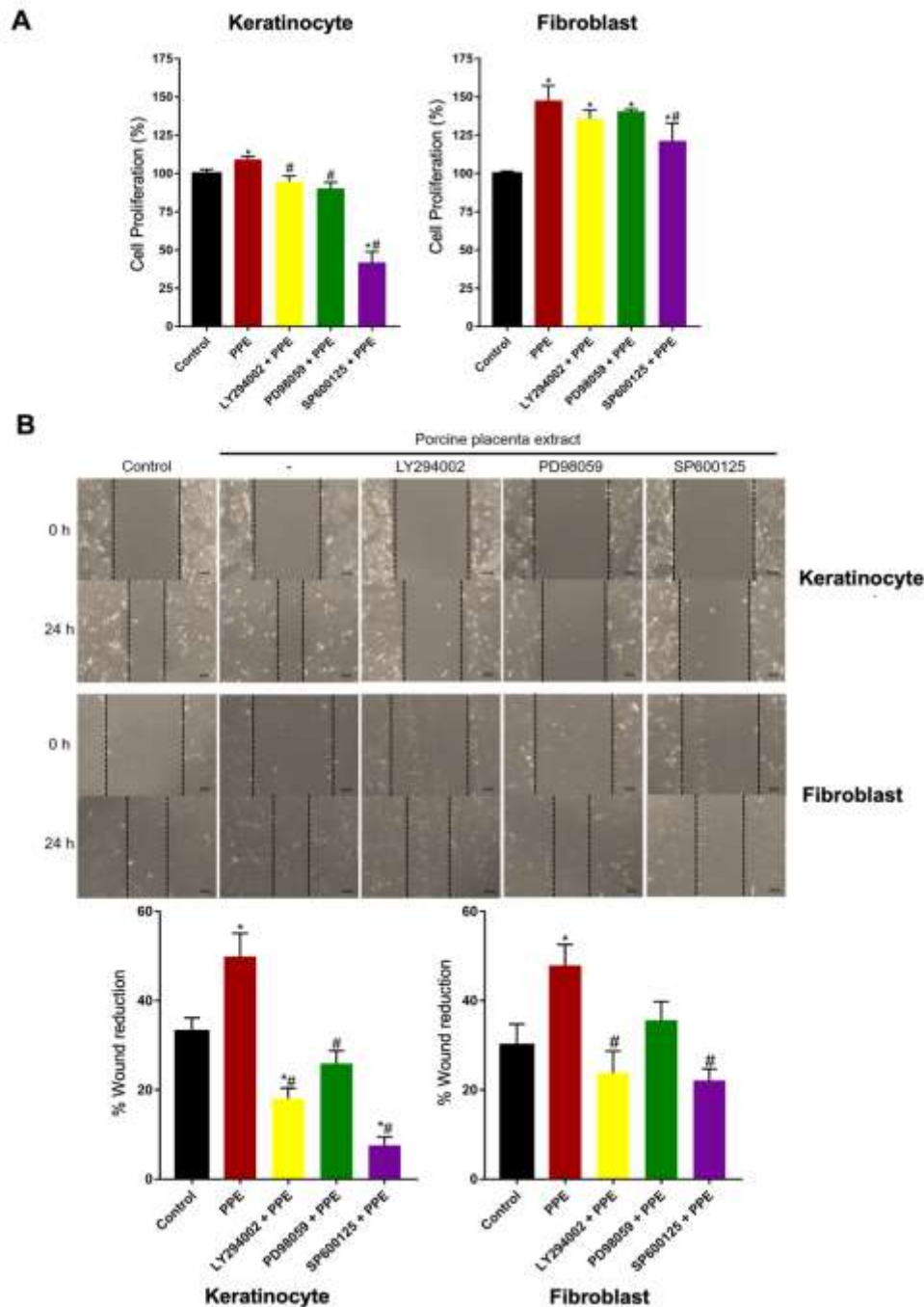


Figure 3: PPE promotes the proliferation and migration of keratinocytes and fibroblasts via activation of the PI3K/AKT, ERK1/2, and JNK signaling pathways. Keratinocytes and fibroblasts were treated with PPE in the presence or absence of specific pathway inhibitors: LY294002 (PI3K inhibitor), PD98059 (ERK1/2 inhibitor), and SP600125 (JNK inhibitor). **(A)** Cell proliferation was assessed using the MTT assay at 48-hour post-treatment. **(B)** Cell migration was evaluated by wound healing assay, and the percentage of wound closure was quantified. Data are presented as mean $\pm$ SEM from three independent experiments ($n = 3$). * $P < 0.05$ compared to control; # $P < 0.05$ compared to PPE alone.

However, PPE (50 $\mu\text{g/mL}$) significantly upregulated the expression of MMP-2, MMP-10, and MMP-14 in human fibroblasts (Figure 4A). Moreover, PPE markedly upregulated the expression of collagen III, collagen I, fibronectin, and alpha-smooth muscle actin (α -SMA) in fibroblasts (Figure 4B). These findings suggest PPE enhanced MMP gene expression and stimulated fibroblast differentiation, resulting in the balancing between ECM deposition/degradation.

Porcine placenta extract (PPE), a bioactive compound derived from agricultural waste products, contains plenty of growth factors and

signaling molecules. Proteomic analysis of PPE, followed by sequence alignment against the *Sus scrofa* proteome database and subsequent analysis via DAVID bioinformatics tools, revealed the presence of a variety of signal-transducing, phosphorylated, and disulfide-bonded proteins.^{10, 13} The essential growth factors (epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF)) alongside chemokines and antioxidants, have been identified as major functional constituents. Previous studies have

highlighted PPE is able to enhance cellular proliferation and migration, reduce oxidative stress, and attenuate inflammation.^{9, 13-15} In this study, PPE significantly promoted the proliferation and migration of both keratinocytes and fibroblasts in a concentration-dependent manner (Figure 1). Moreover, PPE induced the expression of MMP-2, MMP-9,

MMP-10, and MMP-14 in both cells (Figure 4A). It suggests a potential role of PPE in modulating ECM degradation to prevent hypertrophic scar or keloid formation. Concurrently, PPE markedly upregulated the gene expression of myofibroblast marker (α -SMA) and ECM-related genes (fibronectin, type I collagen, and type III collagen) (Figure 4B).

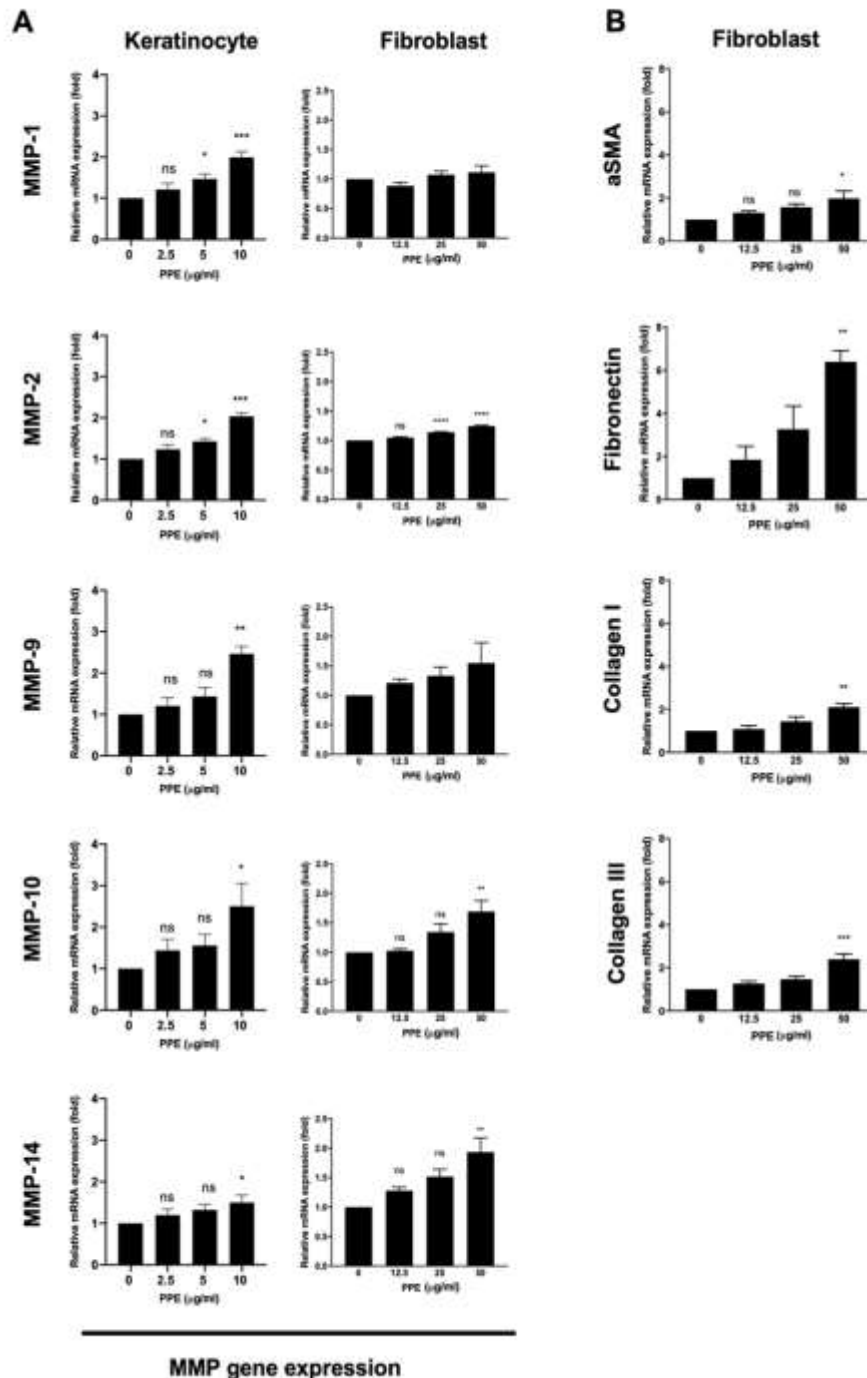


Figure 4: PPE upregulates the expression of MMP- and ECM-related genes in human keratinocytes and fibroblasts. **(A)** Relative mRNA expression levels of matrix metalloproteinase (MMP) genes (MMP-1, MMP-2, MMP-9, MMP-10, and MMP-14), and **(B)** extracellular matrix (ECM)-associated genes including fibronectin, collagen type I, collagen type III, and α -smooth muscle actin (α -SMA) in fibroblasts following treatment with the indicated concentrations of PPE for 6 hours. Gene expression was quantified by real-time PCR and normalized to the untreated control group (fold change). All values represent the mean $\pm$ SEM from three independent experiments (n = 9). *P < 0.05 compared to 0 μ g/mL PPE; **P < 0.01 compared to 0 μ g/mL PPE; ***P < 0.001 compared to 0 μ g/mL PPE.

To elucidate the signal transduction under PPE stimulation, canonical pathways were investigated. PPE activated key intracellular signaling pathways involved in cell proliferation and migration. PPE induced phosphorylation of ERK1/2 and AKT, as well as upregulation of the cell cycle regulator cyclin D1, in both keratinocytes and fibroblasts (Figure 2A). The inhibition of these pathways using LY294002 (PI3K inhibitor) and PD98059 (ERK1/2 inhibitor) attenuated PPE-induced proliferation and migration (Figures 2B and 3). Additionally, PPE activated the c-Jun N-terminal kinase (JNK) pathway, a regulator of cellular differentiation and transcription factor activity. The inhibition of JNK using SP600125 (JNK inhibitor) mitigated the proliferative and

migratory effects of PPE in both keratinocytes and fibroblasts (Figures 2 and 3). These findings are consistent with previous reports that JNK gene knockout in keratinocytes and fibroblasts impairs cell proliferation and migration, whereas JNK activation in fibroblasts enhances collagen synthesis.^{16, 17} Furthermore, JNK signal stimulatory effects of PPE ing functions as a downstream cascade of TGF- β and PDGF to regulate MMP expression, ECM production, and growth factor secretion, in dermal fibroblasts.¹⁸ Taken together, the activation of JNK pathway by PPE may represent a dominant mechanism which PPE facilitates wound healing, including modulation of MMP expression and ECM remodeling. (Figure 5)

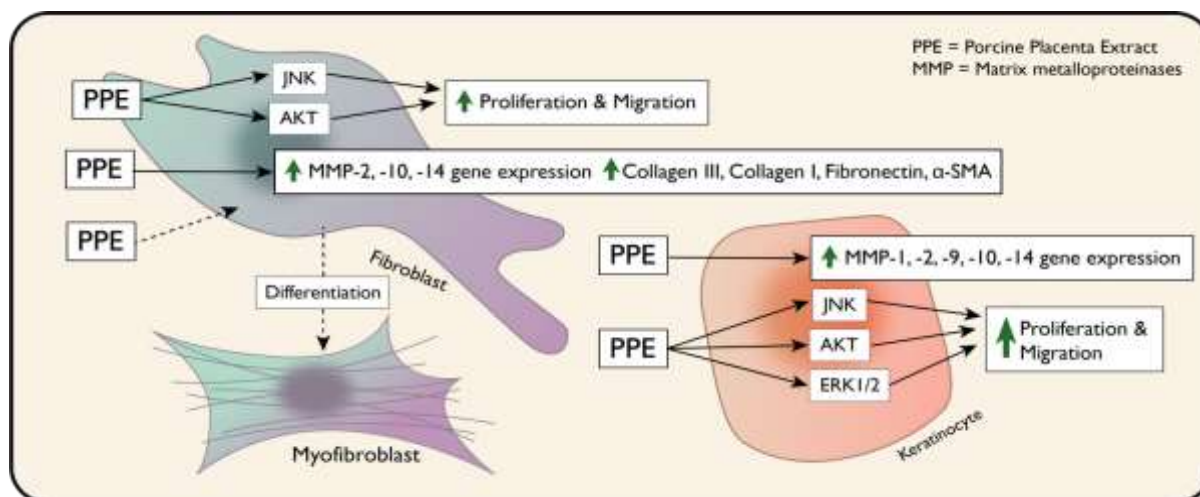


Figure 5: Schematic representation of the stimulatory effects of PPE on biological activities of keratinocytes and fibroblasts. PPE enhances keratinocyte and fibroblast proliferation and migration through activation of the JNK, ERK1/2, and PI3K/AKT signaling pathways. PPE upregulates MMP gene expression in both cell types and promotes ECM-related gene expression (fibronectin, collagen I, collagen III) in fibroblasts. PPE also upregulates α -SMA gene expression in fibroblasts, in which may indicate myofibroblast differentiation.

Even though our study presented the evidence of PPE on tissue regeneration, there are some limitations. Firstly, the immortalized cell lines were entirely utilized and may not fully recapitulate the behavior of primary human dermal cells. However, a previous study has reported there is no difference in terms of morphology and functional responses between primary keratinocytes and HaCaT cells, as well as between primary fibroblasts and Hs895. Sk cells.¹⁹ Secondly, since the complex components in PPE (multiple growth factors, cytokines, and antioxidants) have been implied as a synergistic bioactive compound, the fractionation or purification of PPE into a single substance may compromise its synergistic bioactivity. Lastly, an *in vivo* experiment or clinical trial should be further conducted to affirm the stimulatory effect on wound healing. However, this is the first study to comprehensively evaluate the effect of PPE on MMP and ECM gene expression, in conjunction with its proliferative, migratory, and signal transduction in both keratinocytes and fibroblasts.

In conclusion, PPE represents a promising bioactive agent for wound healing, exerting multifaceted effects on keratinocyte and fibroblast. PPE promotes cell proliferation, migration, MMP gene expression, and myofibroblast differentiation through the activation of ERK1/2, AKT, and JNK signaling pathways. These findings suggest the potential clinical utility of PPE, particularly for patients with impaired wound healing or as a candidate agent to mitigate scar or keloid formation.

Conclusion

Porcine placenta extract (PPE) enhances keratinocyte and fibroblast proliferation/migration by activating JNK, ERK1/2, and PI3K/AKT signaling pathways. PPE also upregulates MMP- and ECM-related gene expression including myofibroblast differentiation.

Conflict of interest

The author's declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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