Polydeoxyribonucleotide exerts opposing effects on ERK activity in human skin keratinocytes and fibroblasts

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Abstract. Polydeoxyribonucleotide (PDRN) is a mixture of deoxyribonucleotides. It serves as an anti-inflammatory and tissue-regenerating agent. The mitogen-activated protein kinase pathway modulates cell growth and collagen accumulation. It also regulates inflammation by suppressing the expression of proinflammatory cytokines. In the present study, it was attempted to elucidate the molecular mechanism of PDRN in skin healing by confirming the effects of PDRN treatment on skin keratinocytes and fibroblasts, and by assessing the levels of collagen and inflammatory cytokines regulated by the extracellular signal-regulated kinase (ERK) pathway. The potential effects of PDRN on skin regeneration were investigated. Fibroblast and keratinocyte proliferation and migration were analyzed using the water-soluble tetrazolium-8 and wound healing assays. The upregulation of collagen synthesis by PDRN-induced ERK activation was analyzed in fibroblasts with or without an ERK inhibitor. Inflammatory cytokine expression levels in keratinocytes were determined using reverse transcription-quantitative polymerase chain reaction. PDRN promoted the proliferation and migration of keratinocytes and fibroblasts. However, PDRN-induced ERK phosphorylation differed between keratinocytes and fibroblasts; PDRN increased ERK phosphorylation and collagen accumulation in fibroblasts, while it inhibited matrix metalloproteinase expression. By contrast, PDRN inhibited ERK phosphorylation in keratinocytes, and it decreased inflammatory cytokine expression levels. PDRN affects skin cell proliferation and migration, and collagen and inflammatory cytokine expression levels via ERK signaling. Overall, PDRN exerts a positive effect on skin regeneration, but the mechanism by which it promotes skin regeneration varies among different skin cell types.

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Abbreviations: cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HDFs, human dermal fibroblasts; HEKs, human epidermal keratinocytes; IL, interleukin; INOS, inducible nitric oxide synthase; IRB, Institutional Review Board; JNK, cjun Nterminal kinase; MAPK, mitogen-activated protein kinase; MCP1, monocyte chemotactic protein 1; MMPs, matrix metalloproteinases; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDRN, polydeoxyribonucelotide; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TNF, tumor necrosis factor; UV, ultraviolet; WST-8, water-soluble tetrazolium-8

Key words: extracellular signal-regulated kinase, fibroblasts, keratinocytes, polydeoxyribonucleotide, skin regeneration

Introduction

Skin damage due to ultraviolet (UV) radiation, known as photoaging, and due to over time, known as intrinsic aging, are considered distinct processes. Photoaged skin has various appearances, such as wrinkles, mottled pigmentation and telangiectasia. Intrinsic skin aging causes thinning of the epidermis and fine wrinkles. These processes occur naturally over time, and they depend on the accumulation of inflammatory mediators, such as free radicals (1). Intrinsic aging is also caused by extracellular matrix (ECM) destruction during dermal fibroblast aging. ECM-like collagen fibers and elastins are produced and maintained by dermal fibroblasts (2). Therefore, the impairment of fibroblast function affects the mechanical properties of skin connective tissue. In addition, inflammatory mediators, including cytokines, can induce skin aging (3). For example, UVB exposure induces the production of tumor necrosis factor (TNF)-α (4). TNF-α activates matrix metalloproteinases (MMPs), increasing inflammation and degradation of several types of collagen (5-7). Thus, the regulation of TNFα activity may be a novel target for treating aged skin.

The regulation of cell proliferation and differentiation in multicellular organisms involves complex processes primarily regulated by external growth factors provided by the microenvironment of proliferating cells. The mitogen-activated protein kinase (MAPK) pathway regulates the cell cycle by influencing cell death and survival processes (8). Among various

MAPKs, extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 are associated with skin aging. The ERK pathway mainly mediates cellular responses to growth factors, such as epidermal growth factor and phorbol ester. On the other hand, the JNK and p38 pathways mediate cellular responses to cytokines, such as TNF and interleukin (IL)-1, and physical stressors, such as UV radiation or osmotic shock (9,10). ERK activity is reduced, while JNK activity is increased in aged human skin compared with young skin *in vivo* (11). The MAPK pathway plays a role in modulating cell growth and procollagen synthesis (12), and it is also involved in MMP-1-regulated signaling pathways (13).

Polydeoxyribonucleotide (PDRN), a mixture of deoxyribonucleotides derived from salmon sperm, is commonly used for dermatological purposes, such as treating chronic wounds and diabetic foot ulcers (14,15). PDRN activates dermal fibroblasts and enhances the synthesis of vascular endothelial growth factor. These biological mechanisms of PDRN suggest that it could exert a major influence on ameliorating skin cell aging (16-19). Multiple studies have reported the beneficial effects of PDRN on skin health (14-19). However, the detailed molecular mechanisms remain poorly understood. Previously, keratinocytes or fibroblasts were studied alone or in co-culture in skin aging studies (14,16,18). In these studies, no notable differences in experimental PDRN effects were elucidated between the aforementioned types of skin cells. In the current study, it was confirmed that PDRN did not differentially affect the cell viability of fibroblasts and keratinocytes. However, the effective PDRN concentration and intracellular signaling transduction differed between epidermal keratinocytes and dermal fibroblasts. The aim was to elucidate the molecular mechanism by which PDRN promoted skin healing by confirming the effect of PDRN treatment on epidermal keratinocytes and dermal fibroblasts, and by assessing collagen and inflammatory cytokine levels, including those of TNF-α, IL-1β, IL-6, monocyte chemotactic protein (MCP)1, inducible nitric oxide synthase (iNOS), regulated by ERK signaling.

Materials and methods

Cell culture. The present study was approved by the Institutional Review Board (IRB) of Hallym University Sacred Heart Hospital (IRB no. HALLYM 2019-07-029; Republic of Korea). Human epidermal keratinocytes (HEKs) were isolated from foreskins of patients through circumcisions (20). Epidermal layers were separated from dermis via enzymatic digestion. Tissues were incubated with 25 U/ml dispase in Hank's Balanced Salt Solution for 20 h at 4˚C. The following day, the epidermis was separated from whole tissue, and keratinocytes were dissociated from the epidermal layer by digesting the layer with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) solution for 15 min. Cells were cultured in 0.07 mM Ca^{2+} 154CF medium (Thermo Fisher Scientific, Inc.) containing human keratinocyte growth supplement (Thermo Fisher Scientific, Inc). All cell types were incubated in a humidified 5% CO_2 incubator at 37˚C. Primary cells at passages 2-8 were used, with enzymatic dissociation using trypsin-EDTA for every passage.

Human dermal fibroblasts (HDFs) were prepared as previously described (21,22). Briefly, human dermal tissue samples were sectioned to a thickness of 4 mm. Within the first week, keratinocytes migrated out of the biopsy tissue. Fibroblasts appeared 7-10 days after the first outer growth of keratinocytes. Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.), high glucose media supplemented with 20% fetal bovine serum (FBS; Cytiva) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Thermo Fisher Scientific, Inc.) favored the growth of fibroblasts over keratinocytes. Homogenous fibroblast cultures were obtained via keratinocyte dilution after two passages. HDFs were maintained in DMEM supplemented with 10% FBS.

Cell proliferation and cytotoxicity assay. HDFs ($1x10^3$ cells/well) and HEKs ($5x10^3$ cells/well) were seeded in 96-well culture plates (SPL Life Sciences). After incubation for 24 h, cells were serum-starved for 16 h and treated with PDRN (Placentex®; Mastelli srl Officina Bio-Farmaceutica) at different concentrations (0, 1, 5, 10, 50 and 100 μg/ml) for an additional 24, 48, and 72 h. Cell viability was measured by using the water-soluble tetrazolium-8 (WST-8) Cell Proliferation kit (Quanti-MAX™; Biomax Co. Ltd.) in order to investigate cytotoxicity and cell proliferation. Cytotoxicity was measured within 24 h, and proliferation rate was measured at 24, 48 and 72 h post-treatment. Briefly, medium-containing plates were incubated with WST-8 reagent at 37˚C for 30 min, and absorbance at a wavelength of 450 nm was measured using a microplate reader.

Cell migration assay. HDFs ($2x10^5$ cells/well) and HEKs ($4x10^5$ cells/well) were plated in six-well culture plates (SPL Life Sciences). When the cells reached 80-90% confluence, serum was removed for 16 h. Cells were scratched in the middle with a 200-μl pipette tip. The starting point was marked with a marker pen at the bottom of the plate. Medium was replaced with either serum-depleted medium containing PDRN (1, 5, 10, 50 and 100 μg/ml) or serum-depleted medium without PDRN as a control, and the cells were incubated for 0, 12 and 24 h. Digital images of the scratches were acquired using a phase-contrast light microscope (TS100; Nikon Corporation). Images were randomly divided into three parts, the number of migrated cells was counted and the experiment was repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA) of collagen type I and III. HDFs ($2x10^5$ cells/well) and HEKs ($4x10^5$ cells/well) were seeded in six-well culture plates in complete growth medium. After starving cells for 16 h, PDRN was added for 24 h. The culture supernatants were then collected for ELISA to determine the concentrations of specific proteins. Supernatants were assayed using commercially available Human Pro-Collagen I α 1 (cat. no. ab210966; Abcam) and collagen type III (cat. no. MBS761779; MyBioSource, Inc.) ELISA kits according to the manufacturer's instructions. Measurements were performed at least three times. The mean values of samples were used to calculate concentrations on the basis of a standard curve obtained with standard proteins provided from the kits.

Western blotting. To investigate the molecular mechanism involved in PDRN induction, HDFs ($2x10^5$ cells/well) and HEKs ($4x10^5$ cells/well) were seeded in six-well culture

plates in growth medium for 24 h. Cells were serum-starved for 16 h before supplementation with PDRN for 0, 5, 15, 30, 60 and 120 min. Confirming changes in MAPK phosphorylation kinetics under stimulation within 2 h has been widely used as a method to monitor MAPK activation *in vitro* (23,24). Protein expression levels of phosphorylated ERK1/2, JNK and p38 were determined by immunoblotting. Cells were washed using ice-cold phosphate-buffered saline. After washing, the cells were lysed in RIPA buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris pH 7.5, 2 mM EDTA, protease inhibitor cocktail solution and phosphatase inhibitor cocktail solution, producing a 1X final concentration (GenDEPOT LLC) on ice for 30 min. After centrifugation at 20,000 x g at 4˚C for 30 min, a BCA protein assay (cat. no. 23227; Pierce; Thermo Fisher Scientific, Inc.) was used for protein quantitation and the resulting supernatant was boiled at 95˚C for 5 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples (25 μg/lane) were subjected to 10% SDS-PAGE and electrotransferred to immobilon polyvinylidene fluoride membranes (Merck KGaA). Non-specific binding was blocked with skimmed milk (5%) for 1 h at room temperature. Membranes were then stored at 4˚C overnight with specific primary antibodies (1:1,000 dilution) against phospho-ERK (sc-7383), ERK 1/2 (sc-514302), phospho-p38 (sc-166182), p38α/β MAPK (sc-7972), phospho-JNK (sc-6254), JNK (sc-7345) and α-tubulin (sc-5286) purchased from Santa Cruz Biotechnology, Inc. The membranes were washed using Tris-buffered saline with 0.05% Tween 20 and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (1:3,000 dilution; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.). Protein bands were visualized using an enhanced chemiluminescence detection system (Amersham imager 600; GE Healthcare). Phosphorylation was semi-quantified with Image J software (National Institutes of Health) by comparing the density of the phosphorylated form and total protein.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated with TRIzol® reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. complementary DNA (cDNA) was synthesized using Maxime RT PreMix kit (Intron Biotechnology, Inc.) according to the manufacturer's instructions. qPCR was performed using SYBR Green dye using the KAPA SYBR® FAST qPCR Master Mix (2X) kit (Roche Diagnostics GmbH). A comparative Cq method ($2^{-\Delta\Delta Cq}$) (25) was used to quantify relative gene expression, and β-actin was used as an endogenous reference control for all transcripts. A LightCycler 96 Real-Time PCR System (Roche Diagnostics GmbH) was used, with thermal cycling conditions consisting of 95˚C for 3 min, followed by 45 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 1 sec. Primers were designed according to published cDNA or genomic sequences (26,27). The sequences of the primers were as follows: *β-actin* (forward, GAGACCTTCAACACCCCAGC; reverse, ATGTCACGCACGATTTCCCT; NG_007992), *TNF-α* (forward, CCCAGGGACCTCTCTCTAATC; reverse, ATGGGCTACAGGCTTGTCACT; NG_007462), interleukin (*IL*)*-1β* (forward, GATGAAGTGCTCCTTCCAGG; reverse, GCATCTTCCTCAGCTTGTCC; NG_008851), *IL-6* (forward, CAATCTGGATTCAATGAGGAGAC; reverse, TTTTTCTGCAGGAACTGGATCAG; NG_011640), monocyte chemotactic protein 1 (*MCP1*; forward, TTCCCCTAGCTTTCCCCAGA; reverse, TCCCAGGGGTAGAACTGTGG; NG_012123) and inducible nitric oxide synthase (*iNOS*; forward, AAAGTTTGACCAGAGGACCC; reverse, TCCTTTGTTACCGCTTCCAC; NG_011470).

Statistical analysis. All experiments were performed at least three times, and data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using various methods. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test using Microsoft Excel (Microsoft Corporation) and GraphPad Prism 9 software (Dotmatics). For the analysis of the proliferation assay, two-way ANOVA was performed followed by Dunnett's and Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PDRN increases proliferation and migration of human skin cells. The proliferation of HDFs and HEKs was investigated *in vitro* to examine the effect of PDRN on the bioactivity of these cells. The cytotoxicity effects of PDRN were demonstrated in HDFs and HEKs. HDFs survived in medium supplemented with 100 μg/ml of PDRN; however, cytotoxicity towards HEKs was observed at a PDRN concentration of >500 μg/ml (Fig. 1A). Next, the optimal concentration of PDRN was determined for maximum intensity of cell response with minimum cytotoxicity. The proliferation and migration of HDFs and HEKs was assessed at 24, 48 and 72 h after PDRN treatment at dose of 0, 1, 5, 10, 50 and 100 μg/ml. HDF and HEK proliferation increased with PDRN treatment, but HEK proliferation decreased upon addition of 100 μg/ml of PDRN at 48 and 72 h (Fig. 1B). These results show that PDRN increases skin cell proliferation, but a high concentration of PDRN can inhibit HEK proliferation. HDF migration increased with increasing PDRN concentration, and there was no difference in migration upon treatment with >10 μg/ml PDRN (Fig. 2A and C). HEK migration was highest upon treatment with 1 μg/ml PDRN but suppressed at ≥50 μg/ml PDRN (Fig. 2B and D). Based on these results, it was confirmed that PDRN increased the proliferation and migration of skin cells. The optimal PDRN concentration for the treatment of HDFs and HEKs was determined to be 10 and 1 μg/ml, respectively.

Intracellular signaling cascades in PDRN-treated human skin cells. Transcription-regulating protein kinases, including MAPK family members ERK and JNK, have previously been shown to be phosphorylated after PDRN treatment in fibroblasts or melanocytes (28,29). The activation of the MAPK family members was thus investigated using western blotting. HDFs and HEKs were serum-starved for 16 h, followed by treatment with a serum-free medium supplemented with 10 μg/ml of PDRN for HDFs, and 1 μg/ml of PDRN for HEKs. The cells were harvested after 0, 5, 15, 30, 60 and 120 min. As shown in Fig. 3A and C, treatment with PDRN significantly increased ERK phosphorylation without altering total ERK levels in HDFs. Phosphorylation

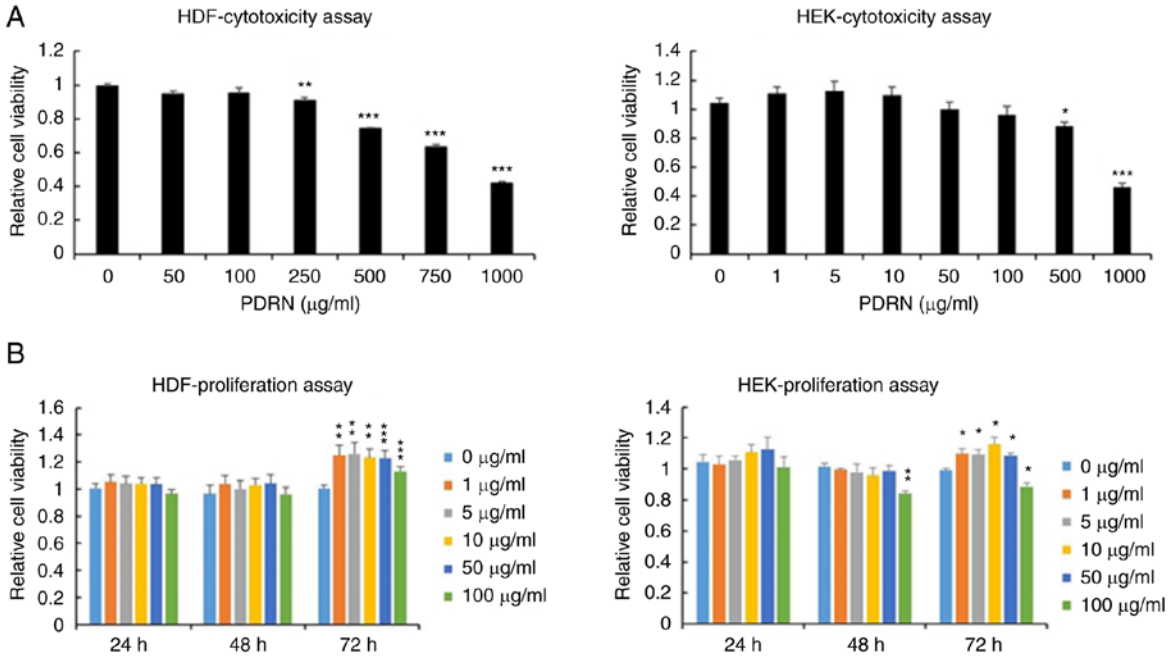

Figure 1. Cytotoxicity and proliferation induced by PDRN treatment. (A) PDRN cytotoxicity on HDFs and HEKs was determined using a WST-8 assay 24 h after addition of PDRN to culture medium. (B) HDF and HEK proliferation rates were determined using the WST-8 assay at 24, 48 and 72 h after addition of PDRN to the culture medium. The results were normalized and expressed as fold change ± SEM compared with the control without PDRN. *P<0.05, **P<0.01 and ***P<0.005 vs. 0 μg/ml. PDRN, polydeoxyribonucleotide; HDF, human dermal fibroblasts; HEK, human epidermal keratinocytes; WST-8, water-soluble tetrazolium-8.

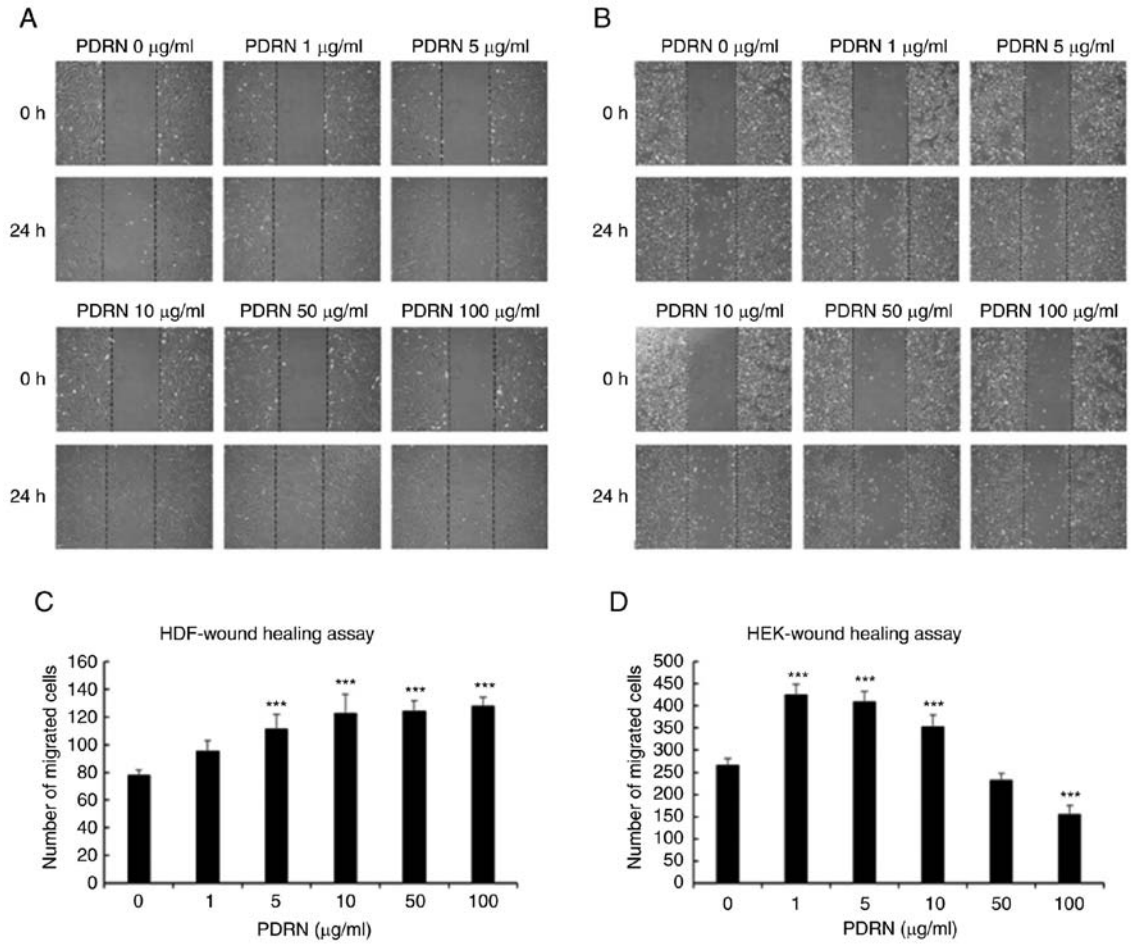

Figure 2. Increased cell migration by PDRN-treated human skin cells. Migration of (A) HDFs and (B) HEKs 24 h after PDRN treatment was observed under a phase-contrast microscope. Original magnification, x40. The number of migrated (C) PDRN-treated HDFs and (D) HEKs at 24 h were counted. ***P<0.005 vs. 0 μg/ml. PDRN, polydeoxyribonucleotide; HDF, human dermal fibroblasts; HEK, human epidermal keratinocytes.

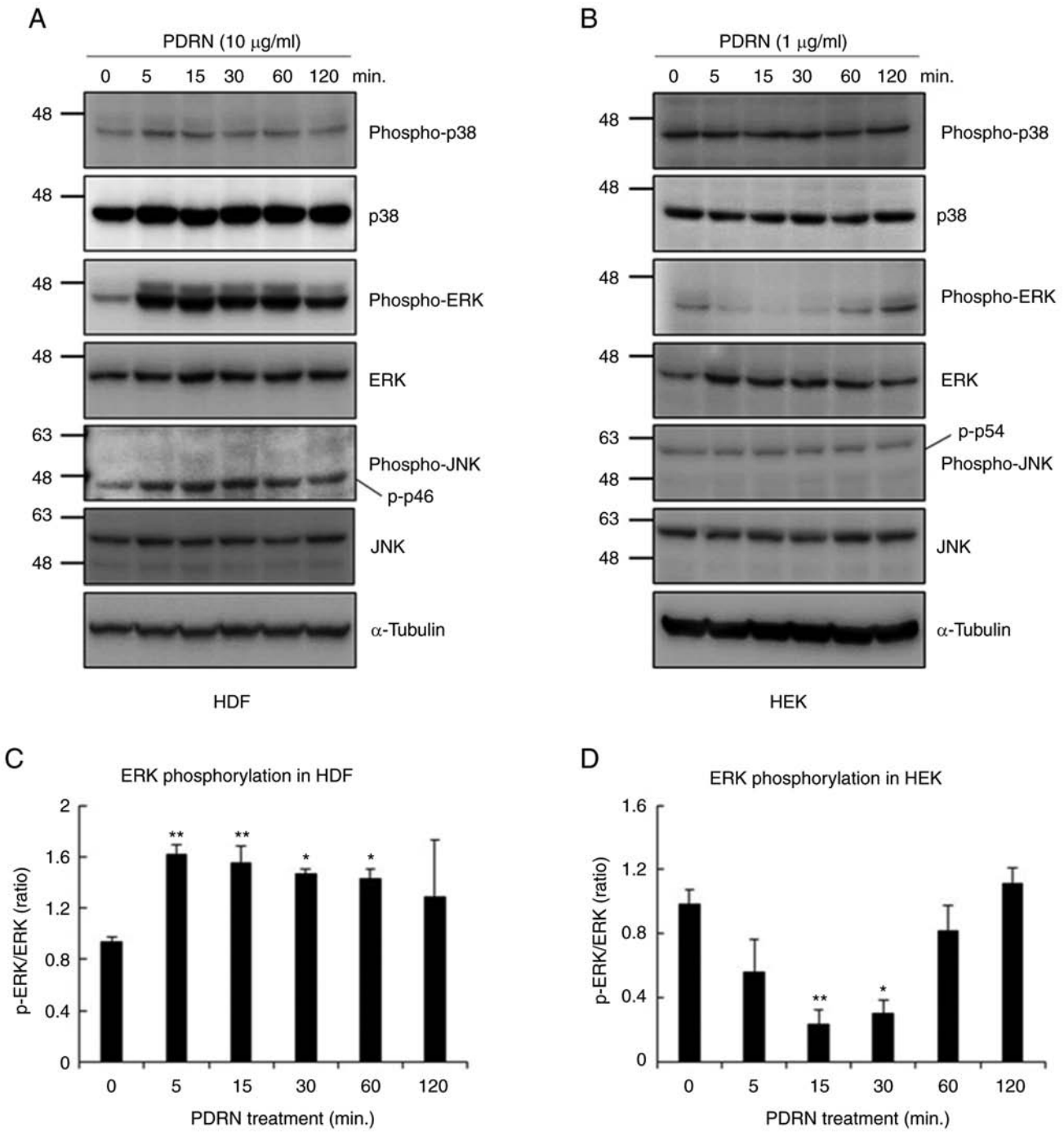

Figure 3. Effects of PDRN on mitogen-activated protein kinase pathway activation in human skin cells. Cells were cultured without serum for 16 h, followed by addition of PDRN to culture medium for 0, 5, 15, 30, 50 and 120 min. Cell lysates were analyzed by western blotting using targeted protein antibodies in (A) HDFs and (B) HEKs. Phosphorylated ERK was quantified with Image J software by comparing the density of phospho-ERK and ERK in (C) HDFs and (D) HEKs. Similar results were obtained in three different experiments. *P<0.05 and **P<0.01 vs. 0 min. PDRN, polydeoxyribonucleotide; HDF, human dermal fibroblasts; HEK, human epidermal keratinocytes; ERK, extracellular signal-regulated kinase; JNK, c jun N terminal kinase.

of the p46 JNK isoform was also increased by PDRN treatment, but the level of the phosphorylated-p54 JNK isoform did not change and there was no statistically significant differences (Fig. S1A). The increase of p38 phosphorylation was not statistically significant. However, ERK phosphorylation decreased significantly after PDRN treatment in HEKs, before they adapted to the treatment (Fig. 3B and D). Phosphorylated JNK and p38 levels did not change during PDRN treatment in HEKs (Fig. S1B). Based on these results, it was concluded that PDRN induces opposite effects on MAPK activation depending on the skin cell type, and it is necessary to investigate the underlying intracellular signal transduction mechanisms.

PDRN enhances collagen accumulation by activating the ERK pathways in HDFs. To further understand the mechanism underlying PDRN-induced ERK activation, the selective ERK inhibitor PD98059 was used in HDFs. A total of 1 μM PD98059

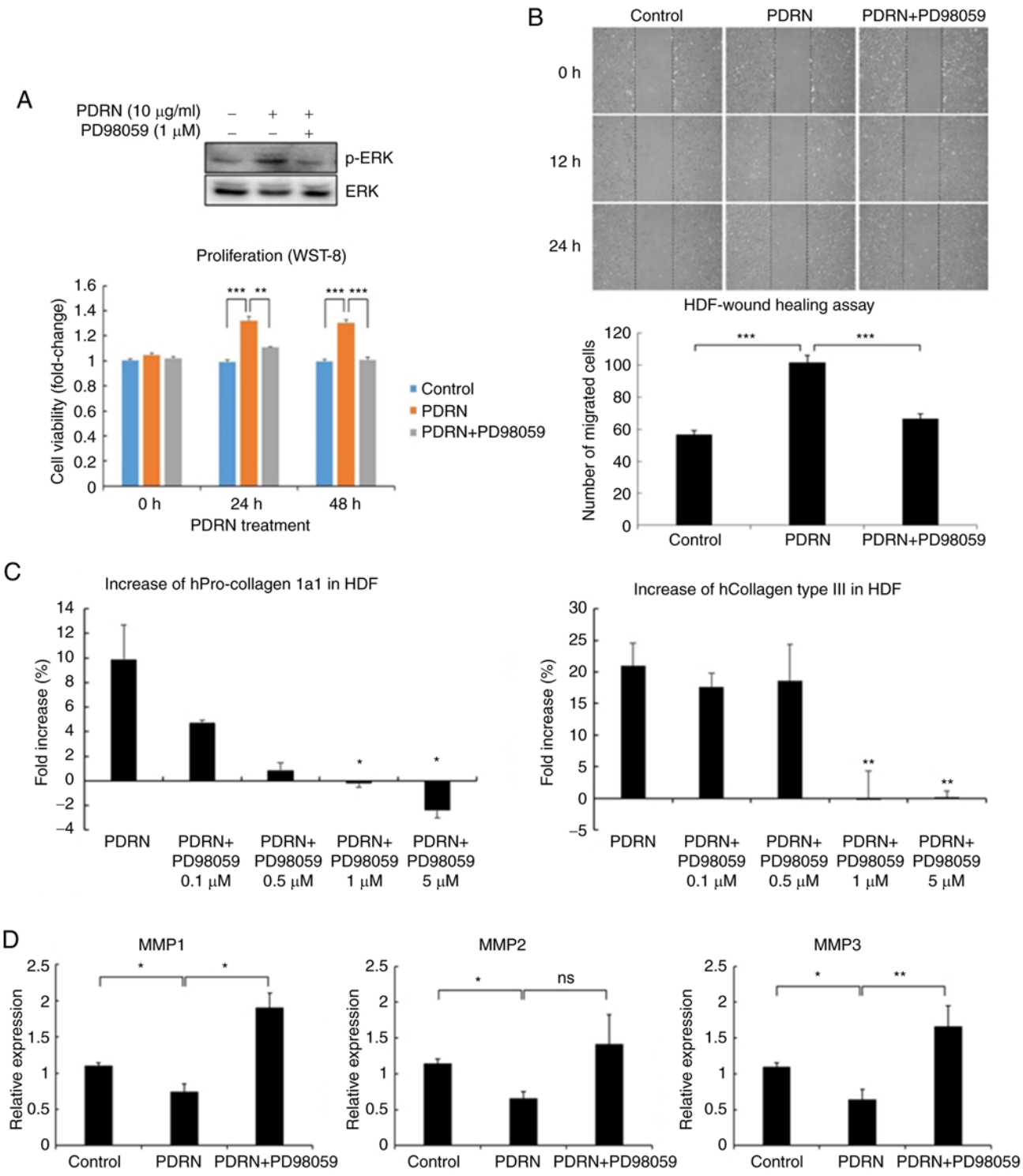

Figure 4. Effect of ERK inhibition on PDRN-induced collagen accumulation around HDFs. To explore the underlying mechanism of PDRN-induced ERK activation, the proliferation and migration of HDFs stimulated with PDRN in the presence of PD98059 were measured using (A) WST-8 and (B) wound healing assays. Original magnification, x40. Cells were cultured without serum for 16 h, followed by addition of PDRN and PD98059 to the culture medium for 24 h. (C) hPro-Collagen I α 1 and hCollagen type III expression levels were determined using ELISA in HDFs. (D) MMP-1, 2 and 3 expression levels were examined by reverse transcription-quantitative PCR. Each treatment was performed in triplicate at least, and the data are presented as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.005 vs. PDRN or indicated control. PDRN, polydeoxyribonucleotide; HDF, human dermal fibroblasts; ERK, extracellular signal-regulated kinase; WST-8, water-soluble tetrazolium-8; p-, phosphorylated; h, human; MMP, matrix metalloproteinases.

was added to HDFs, and cells were incubated for 30 min before PDRN treatment. Fig. 4A shows that PD98059 significantly suppressed PDRN-induced ERK phosphorylation, and the WST-8 assay showed that the significant increase in cell proliferation induced by PDRN treatment was reversed by treatment with PD98059. Moreover, PDRN-mediated HDF migration was inhibited by PD98059 treatment (Fig. 4B). Next, collagen protein expression was investigated by ELISA after 24 h of PDRN treatment of HDFs (Fig. 4C). Collagen type I and III protein levels were increased after PDRN treatment compared

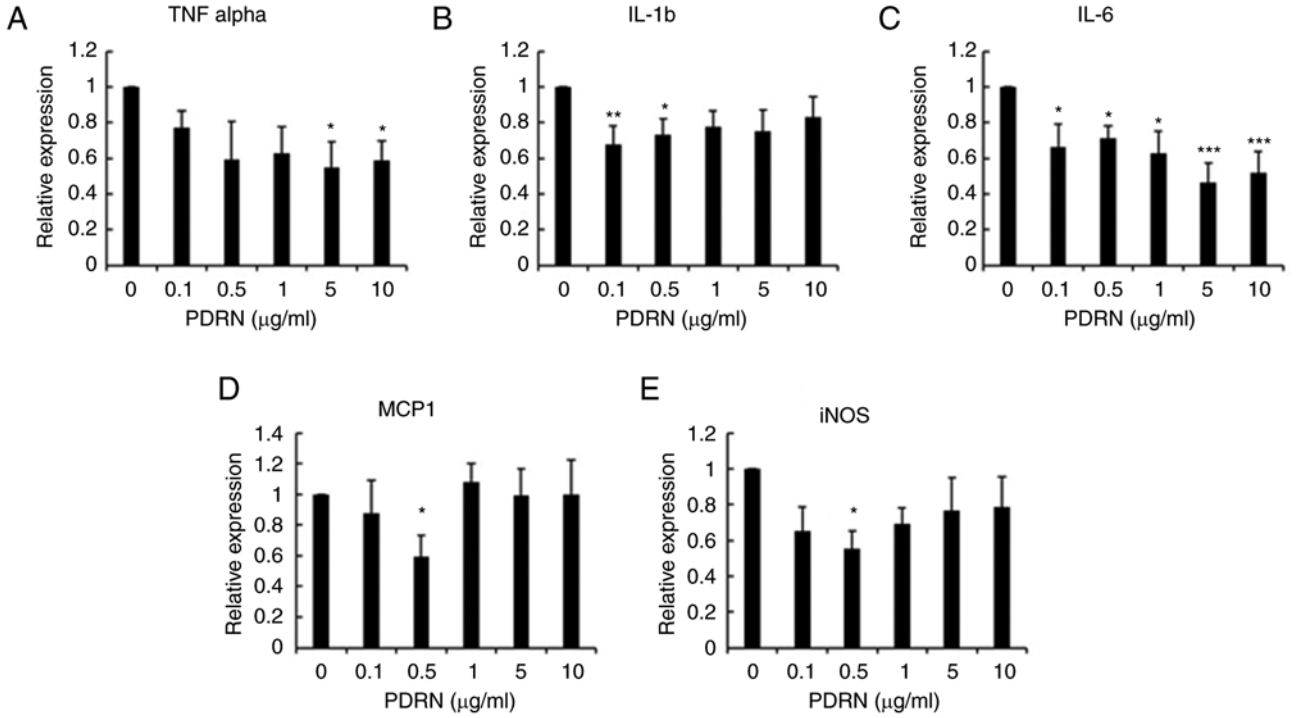

Figure 5. Decreased expression levels of pro-inflammatory cytokines in PDRN-treated HEKs. HEKs were cultured without serum for 16 h, followed by PDRN addition to the culture medium for 24 h. Inflammatory cytokine expression levels were detected by reverse transcription-quantitative PCR. Each treatment was performed in at least 5 times, and data presented as means ± SEM. *P<0.05, **P<0.01 and ***P<0.005 vs. 0 μg/ml. PDRN, polydeoxyribonucleotide; TNF, tumor necrosis factor; IL, interleucin; MCP1, monocyte chemotactic protein 1; iNOS, inducible nitric oxide synthase; HEKs, human epidermal keratinocytes.

with those in the untreated control, but PD98059 decreased collagen secretion in a dose-dependent manner. The regulation of MMP expression by PDRN treatment was also examined via RT-qPCR (Fig. 4D). PDRN decreased the expression of MMP1, 2 and 3 in HDFs, whereas PD98059 rescued MMP expression in these cells. These results suggest that PDRN treatment triggers collagen accumulation in HDFs via ERK phosphorylation.

PDRN suppresses the pro-inflammatory effect by inhibiting the ERK pathway in HEKs. As ERK phosphorylation was reduced in HEKs, collagen levels were also assessed, but these could not be measured due to low expression levels (data not shown). It was speculated that ERK inhibition by PDRN would manifest different effects in keratinocytes compared with fibroblasts. ERK activation is related to inflammatory responses, and their regulation is a crucial factor in skin repair. To examine the effects of PDRN on ERK signaling-mediated inflammatory responses, the expression levels of proinflammatory cytokines in HEKs were determined after 24 h PDRN treatment (Fig. 5). The expression levels of TNF-α and IL-6 decreased with PDRN treatment in a dose-dependent manner, while those of IL-1β and iNOS were inhibited within the non-toxic concentration range for HEKs of ≤1 μg/ml. MCP1 expression levels were suppressed when 0.5 μg/ml of PDRN was used, but expression levels were not suppressed when cells were treated with >0.5 μg/ml. It is noteworthy that expression levels of inflammatory cytokines in HDFs could not be determined due to either low expression or no significant change was observed even after treatment with PDRN (data not shown).

Discussion

PDRN is a mixture of deoxyribonucleotides with a molecular weight range of 50-1,500 kDa and derived from human placenta or salmon sperm (30). According to previous experiments, the most relevant mechanism of action of PDRN is the engagement of adenosine A2A receptors. PDRN binds to the adenosine A2A receptor, and it induces the synthesis of vascular endothelial growth factor, which improves angiogenesis and consequently promotes wound healing (30,31). PDRN inhibits apoptosis and exerts anti-inflammatory effects by downregulating inflammatory cytokines, including iNOS, IL-1β, IL-6 and TNF-α (32). Also, PDRN stimulates nucleic acid synthesis through a salvage pathway to recover bases and nucleosides generated from the breakdown of DNA and RNA (33). Therefore, PDRN reactivates normal cell proliferation by generating nucleotides and nucleosides that contribute to DNA formation. Although it was confirmed that PDRN exerts opposite effects on ERK activity is HDFs and HEKs in the present study, PDRN-treated HDFs and HEKs showed increased proliferation with reduced mRNA expression levels of cell cycle arrest proteins p21, p27, p57 and p53 (Fig. S2). These results suggest that the PDRN-induced increase in the proliferation of ERK-inhibited keratinocytes may be due to the PDRN-induced salvage pathway.

Most studies on the effects of PDRN on skin regeneration have been conducted using fibroblasts (17,19,29,33). However, under physiological conditions, the normal wound healing process is characterized by complex and integrated processes that require the interactions of inflammatory cells, fibroblasts, keratinocytes and endothelial cells, and the involvement of

growth factors and enzymes. Normal wound healing proceeds through four phases: Hemostasis, inflammation, proliferation, maturation and remodeling (34,35). In the present study, it was confirmed that PDRN not only improved cell proliferation and migration, but it also promoted accumulation of collagen. The most notable histological changes in intrinsic and photo-aged skin occur within the dermis (36,37), and changes in collagen have been suggested to be the cause of wrinkling observed in photoaged and naturally aged skin (38,39). UV irradiation enhances MMP synthesis in human skin *in vivo*, and MMP-mediated collagen destruction constitutes a notable proportion of connective tissue damage that occurs during photoaging. Collagen destruction in intrinsic skin aging may be due to elevated MMP expression and an accompanying reduction in collagen synthesis, similar to photoaging (39,40). Increased MMP-mediated degradation of aged and photoaged skin results in decrease in collagen, causing wrinkles in aged skin. Fibroblasts were treated with PD98059, an ERK inhibitor, and a decrease in PDRN-mediated collagen accumulation was observed along with an increase in MMP expression levels. These results suggest that PDRN activates collagen synthesis via ERK phosphorylation.

Inflammation during skin aging reduces collagen activity and increases MMP levels (41,42). Inflammation also disrupts the rate of cell proliferation in all skin layers, leading to thinning of the epidermis, flattening of the dermo-epidermal junction and increased irregular pigment production, eventually resulting in an increased incidence of skin cancer (43). UVR can activate signaling pathways (44,45), and it promotes the downstream signaling pathway intermediates, such as ERK1/2 and p38 (46-48). During skin aging, cytokines produced by keratinocytes and dermal fibroblasts potentiate inflammation by binding to their receptors on adjacent cells. The increased production of MCP1 induces the expression of reactive oxygen species, MMPs and other inflammatory mediators that can damage the dermal matrix (49). Finally, inflammatory cytokines can upregulate the synthesis of their receptors or nuclear factors, such as NF-κB, iNOS and JNK, further augmenting the inflammatory response (50,51) and aggravating the damaging effects on the skin. In the current study, it was confirmed that proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, MCP-1 and iNOS related to skin aging, were reduced after PDRN treatment of HEKs. By contrast, ERK phosphorylation was found to be reduced by PDRN in keratinocytes, and the effective concentration of PDRN was also lower than that required by fibroblasts.

The expression changes of IL-6 and TNF-α in keratinocytes and fibroblasts were additionally investigated using ELISA kits. IL-6 was decreased in both cell types, and TNF-α was decreased only in fibroblasts (data not shown). The reason TNF-α expression was decreased in keratinocytes is likely due to the absence of inflammation-inducing factors. Therefore, it can be expected that TNF-α will be reduced by PDRN treatment in future experiments when keratinocytes are inflamed (52,53). In the results of the present study, mRNA expression levels of inflammatory cytokines in fibroblasts could not be determined due to either low levels or there was no significant change even after treatment with PDRN. Transcriptional regulation of inflammatory cytokines does not appear to be affected by PDRN in fibroblasts, but the translation and secretion of cytokines seems to have been affected by PDRN through signaling pathways other than ERK signaling transmission.

PDRN is commonly used for dermatological purposes. In the present study, the effects of PDRN on fibroblasts and keratinocytes were investigated *in vitro* and the possible underlying molecular mechanisms were examined by investigating the bioactivities of skin cells. PDRN affects cell proliferation and migration, the expression levels of fibrotic proteins such as collagen type I and III, and inflammatory cytokines via the ERK pathway. PDRN has a positive effect on skin regeneration, but the mechanism that regulates it differs depending on cell type. Therefore, it may exert different effects on skin regeneration in different cell types. Increased ERK phosphorylation in PDRN-treated HDFs induced the synthesis of collagen type I and III proteins. However, the suppression of ERK phosphorylation in PDRN-treated HEKs resulted in decrease of pro-inflammatory cytokine levels. The findings of the current study indicate that PDRN has potential for skin rejuvenation via dermal remodeling and epidermal anti-inflammation effects, and that signal transductions can exert opposing effects in different cell types, which needs to be taken into consideration in drug effect tests in skin research.

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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

EJP and SMS designed the project. SMS and EJB performed the experiments. SMS, EJB, KJK, KHK and EJP evaluated data. SMS and EJB wrote this article. SMS, EJB, KJK, KHK and EJP edited the manuscript. EJP supervised the project. SMS and EJP confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This experiment was approved by the IRB of Hallym University Sacred Hospital (Anyang, Republic of Korea) (approval no. HALLYM 2019-07-029). All procedures in the present study were conducted in accordance with the IRB of Hallym University Sacred Hospital approved protocols. Written informed consent was obtained for patient participation at the initial time that the samples were taken.

Patient consent for publication

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Competing interests

The authors declare that they have no competing interests.

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