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# Laminin peptide YIGSR induces collagen synthesis in Hs27 human dermal fibroblasts

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ABSTRACT

The dermal ECM is synthesized from fibroblasts and is primarily compromised of fibrillar collagen and elastic fibers, which support the mechanical strength and resiliency of skin, respectively. Laminin, a major glycoprotein located in the basement membrane, promotes cell adhesion, cell growth, differentiation, and migration. The laminin tyrosine-isoleucine-glycine-serine-arginine (YIGSR) peptide, corresponding to the 929–933 sequence of the β1 chain, is known to be a functional motif with effects on the inhibition of tumor metastasis, the regulation of sensory axonal response and the inhibition of angiogenesis through high affinity to the 67 kDa laminin receptor.

In this study, we identified a novel function of the YIGSR peptide to enhance collagen synthesis in human dermal fibroblasts. To elucidate this novel function regarding collagen synthesis, we treated human dermal fibroblasts with YIGSR peptide in both a time- and dose-dependent manner. According to subsequent experiments, we found that the YIGSR peptide strongly enhanced collagen type 1 synthesis without changing cell proliferation or cellular MMP-1 level. This YIGSR peptide-mediated collagen type 1 synthesis was modulated by FAK inhibitor and MEK inhibitor. This study clearly reveals that YIGSR peptide plays a novel function on the collagen type 1 synthesis of dermal fibroblasts and also suggests that YIGSR is a strong candidate peptide for the treatment of skin aging and wrinkles.



## 1. Introduction

The dermal ECM is synthesized from fibroblasts and is primarily compromised of fibrillar collagen and elastic fibers, which support the mechanical strength and resiliency of skin, respectively [1]. Collagen is the most abundant structural protein in connective tissue [2]. There are numerous types of collagen in normal skin, of which type I collagen is the most abundant (~90% of dry weight) [3]. Collagen is synthesized in fibroblasts in the dermis. Collagen synthesis can be regulated both transcriptionally and post-transcriptionally, and several signaling pathways are known to regulate collagen synthesis. After its mRNA is transcribed and translated by stimuli like TGF-beta and other cytokines [4,5], procollagen, a precursor of collagen, is synthesized through hydroxylation of proline and lysine by prolyl hydroxylase and lysyl hydroxylase, respectively, and finally converted to collagen by several cofactors such as ascorbic acid, oxygen and ketoglutarate. [6,7]. Collagen is negatively regulated by matrix metalloproteinases (MMPs)-induced proteolysis. [8]. Collagen synthesis is important for maintaining tissue integrity, and the loss of type I collagen in the dermal layer of skin is the major cause of wrinkle formation in skin aging. Although a number of polyphenolic compounds are suggested to prevent ultraviolet-induced wrinkles, very few of them are able to directly increase type I collagen synthesis [9].

Laminin, a major glycoprotein located in the basement membrane, promotes cell adhesion, cell growth, differentiation, and migration [10]. These effects are known to be mediated by the 67 kDa laminin receptor (67LR). Increases in the expression of 67LR have been reported in common cancers, such as breast, cervical, colon, gastric, hepatocellular, lung, ovary, pancreatic, prostate and thyroid carcinomas [11–20]. A positive correlation with aggressiveness or metastatic potential has been found in many cases [21]. In addition, it has also been reported that modulation of the synthesis and secretion of laminin is associated with some cancers in which laminin chains may be either up-regulated or

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absent compared with normal tissues [22]. The laminin tyrosine-isoleucine-glycine-serine-arginine (YIGSR) peptide, corresponding to the 929–933 sequence of the β1 chain, is known to be a functional motif with effects on the inhibition of tumor metastasis, the regulation of sensory axonal response and the inhibition of angiogenesis through high affinity to 67LR [23]. Therefore, the YIGSR peptide is used for studying laminin functions, which are mainly related to ECM protein-induced cell adhesion by surface coating or competition with soluble peptides. Although many studies have been conducted so far, involvement of the laminin pathway in collagen regulation has not yet been reported.

In this study, we identified a novel function of YIGSR peptide to enhance collagen synthesis on human dermal fibroblasts. To elucidate this novel function regarding collagen synthesis, we treated dermal fibroblasts with YIGSR peptide in both a time- and dose-dependent manner. According to subsequent experiments, we found that YIGSR peptide strongly enhanced collagen type 1 synthesis without changing cell proliferation. YIGSR peptide-mediated collagen type 1 synthesis was clearly modulated by FAK inhibitor and MEK inhibitor. This study clearly reveals that YIGSR peptide plays a novel role in the collagen type 1 synthesis of dermal fibroblasts and also suggests YIGSR peptide as a new wrinkle care material.

## 2. Materials and methods

### 2.1. Cell culture and YIGSR treatment

Hs27, human dermal fibroblasts, were obtained from American Type Culture Collection (ATCC). Hs27 cells were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) maintained in a 5% $CO_2$ humidified atmosphere at 37 °C. Hs27 cells were used between passage numbers 5 and 20. For the time and dose-dependent treatment, Hs27 cells were serum starved for 24 h and treated by YIGSR peptide in the specific doses and times. The synthetic YIGSR peptide was purchased from Anygen (Korea).

### 2.2. MTT assay

Hs27 cells were seeded on a 96-well plate ($1 \times 10^4$ cells/well) and cultured for 24 h. After serum starvation for 24 h, YIGSR peptide was treated for the given conditions. After suction removal of the media, cells were treated with 0.5 mg/ml of MTT (Sigma–Aldrich, St. Louis, MO) dissolved in serum-free media for 3 h in a 37 °C $CO_2$ incubator. After suction removal of the solution, 100 μl of DMSO were added to each well, and the plate was vortexed for 10 min. Absorbance of the solution was measured at 540 nm.

### 2.3. Inhibitor study

PF573228 and PD98059 were used for experiments on the inhibition of FAK and MEK, respectively. After Hs27 cells were seeded on 24-well plates, the cells were serum starved for 24 h. Cells were treated with 1 μM of each inhibitor for 24 h, along with 1 μM of YIGSR peptide. The inhibitors were purchased from Tocris Bioscience (Bristol, United Kingdom).

### 2.4. Quantitative RT-PCR

Total RNA was extracted using TRIzol agent (Invitrogen Corp.). cDNA was then reverse-transcribed from 1 μg of total RNA using oligo (dT) primers and murine leukemia virus reverse transcriptase. PCR amplification mixtures (20 μl) contained 10 μl of 2× SYBR Green I Premix ExTaq (TAKARA Co., Ltd.), 2 μl of 1 μM forward and 1 μM reverse primer mix, and 8 μl of a diluted cDNA template. Real-time quantitative PCR was performed using the Bio-Rad CFX96 Real-time PCR detection system using the following amplification conditions: 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 15 s, annealing at 60 °C for 15 s, and 72 °C for 30 s. After amplification, a melting curve analysis was performed according to the manufacturer's instructions (Bio-Rad). Primer sequences for type I collagen were as follows: 5′-GAACGCGTGTCATCCCTTGT-3′ (forward) and 5′-GAACGAGGTAGTCTTTCAGCAACA-3′ (reverse).

### 2.5. Immunoblotting

The following antibodies were used for immunoblotting: antibodies against type I collagen (Rockland Immunochemicals Inc., PA), phospho-FAK ($Tyr^{397}$, Cell signaling), phospho-pyk2 ($Tyr^{402}$, Cell signaling), and phospho-ERK ($Thr^{202}/Tyr^{204}$, Abcam). Immunoblotting was performed as follows: proteins were denatured by boiling in Lammeli sample buffer for 5 min at 95 °C, separated by SDS–PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS, pH 7.6), then incubated with primary antibodies. After washing membranes several times with TTBS, blots were incubated with HRP-conjugated secondary antibody, washed with TTBS, and detected by enhanced chemiluminescence (ECL system; GE Healthcare). Densitometric analysis was performed using the Image J program (Ver 1.38, http://rsb-web.nih.gov/ij/index.html).

### 2.6. Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses between two groups were performed by unpaired 2-tailed Student's *t*-test. *P*-values < 0.05 were considered significant.

## 3. Results

### 3.1. YIGSR induced collagen synthesis at both the protein and mRNA level with no change in cell survival

To investigate the effects of YIGSR on dermal fibroblasts, we initially measured type I collagen level after Hs27 human dermal fibroblasts were treated with YIGSR. The fibroblasts were incubated in YIGSR for 24 h, and the level of type I collagen was measured by immunoblotting. As shown in Fig. 1A, YIGSR can significantly enhance the production of type I collagen in a dose-dependent manner. A micromolar concentration of YIGSR ($10^3$ nM) is the maximum concentration for enhancing collagen production, which was more than 6 times that of the not-treated condition in the densitometric analysis. Collagen levels, which were traced through time courses, increased continuously until the level in the 24-h treatment reached around 5 times greater than in the not-treated condition (Fig. 1B). The effects of YIGSR on the mRNA expression of type I collagen were also observed in a similar pattern (Fig. 1C), indicating the maximum level of gene expression to be a micromolar concentration, meaning that YIGSR can regulate type I collagen at the transcriptional level.

To verify the effect of YIGSR on cellular viability, we performed an MTT assay. There were no significant changes in the dose-dependent treatment (Fig. 2A) or in the time-dependent treatment with a fixed dose of 1 μM (Fig. 2B). These results clearly indicate that YIGSR increases type I collagen level by transcriptional up-regulation without any change in cellular viability.

### 3.2. YIGSR did not change MMP-1 level

MMP-1 is a well-known protease that degrades collagen proteins. To investigate whether YIGSR affects the cellular level of

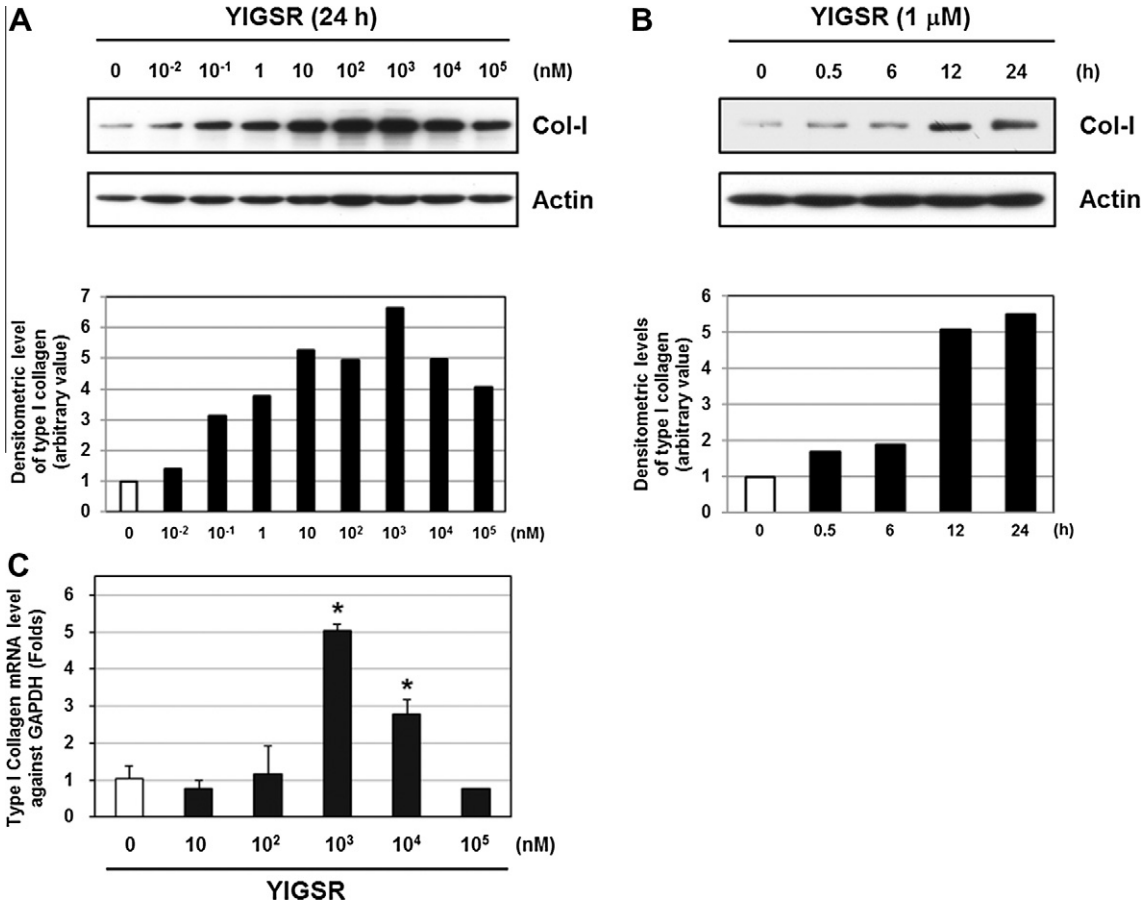


**Fig. 1.** YIGSR induced collagen synthesis in Hs27 dermal fibroblasts. (A) Immunoblotting of type I collagen after dose-dependent YIGSR treatment. After treatments with 0–$10^5$ nM YIGSR for 24 h, cell lysates were electrophoresed and blotted. (B) Immunoblotting of type I collagen after time-dependent YIGSR treatment. After treating with 1 μM YIGSR for 0–24 h, cell lysates were electrophoresed and blotted. Col-I indicates type I collagen. Densitometric analysis was normalized by actin, and results are presented below each immunoblotting result. (C) Q-PCR analysis of type I collagen after dose-dependent YIGSR treatment for 24 h. The *Y*-axis indicates relative fold-change against the value of "0 nM" (*$P < 0.05$, Student's *t* test).

MMP-1, we measured changes in the MMP-1 level following time-dependent YIGSR treatment (Fig. 2C). There was no significant change in MMP-1 level according to a densitometric analysis, indicating that YIGSR had no effect on MMP-1 level. In conclusion, the increase in type I collagen caused by YIGSR was not the result of down-regulating MMP-1, but rather the result of up-regulating the signaling pathway that enhances the gene expression of type I collagen.

### 3.3. YIGSR induced the phosphorylation of FAK, pyk2 and ERK

To investigate a signaling pathway that could regulate type I collagen levels, we focused on the down-signaling pathway of the laminin receptor. YIGSR is known to bind to the laminin receptor and evoke subsequent signaling in cells; for example, the phosphorylation of FAK and Pyk2. FAK is a tyrosine kinase involved in cellular adhesion and the spreading process [24]. FAK is recruited as a participant in focal adhesion dynamics between cells and has a role in motility and cell survival [25]. Pyk2 plays an important role in cell spreading and migration through mediating signals for the G-protein-coupled receptors and MAP kinase pathway [26]. We monitored these events in the fibroblasts following time-dependent YIGSR treatment.

Through subsequent immunoblotting, we found that the tyrosine phosphorylation ($Tyr^{397}$) of FAK is significantly increased in response to YIGSR treatment (Fig. 3). The phosphorylation level was increased as early as 0.1 h (6 min) and for upto 24 h (Fig. 3A). In the densitometric analysis, the YIGSR-induced phosphorylation level was increased about 2-fold compared to the non-treated condition, and this degree of phosphorylation was sustained for 24 h (Fig. 3B). The tyrosine phosphorylation of Pyk2 ($Tyr^{402}$) was also increased but only from 6 h onward, not as early of a time point as for FAK (Fig. 3A). The level in the 12 h treatment was around 1.5-fold higher than in the non-treated condition according to the densitometric analysis (Fig. 3B). In the case of MAPK/ERK, the phosphorylation level was rapidly increased in early time points to around 2.5-fold and was sustained at this level until 24 h (Fig. 3). In conclusion, YIGSR clearly induced the phosphorylation of laminin receptor downsignaling molecules in Hs27 human dermal fibroblasts including FAK, Pyk2 and ERK.

### 3.4. Inhibitors of FAK and MEK prevent YIGSR-induced collagen-1 synthesis

To investigate whether YIGSR-induced type I collagen expression was mediated by FAK and MAPK, an inhibitor study was performed using inhibitors for FAK and MEK. As a first step, we treated with a selective FAK inhibitor named PF573228 for 24 h. The increase in type I collagen caused by YIGSR treatment was mostly inhibited by PF573228 treatment (Fig. 4A), clearly indicating that YIGSR-induced type I collagen expression is mediated by FAK activation.

To examine the involvement of the MAPK/ERK pathway, Hs27 cells were co-treated with YIGSR and one of the potent and selec-

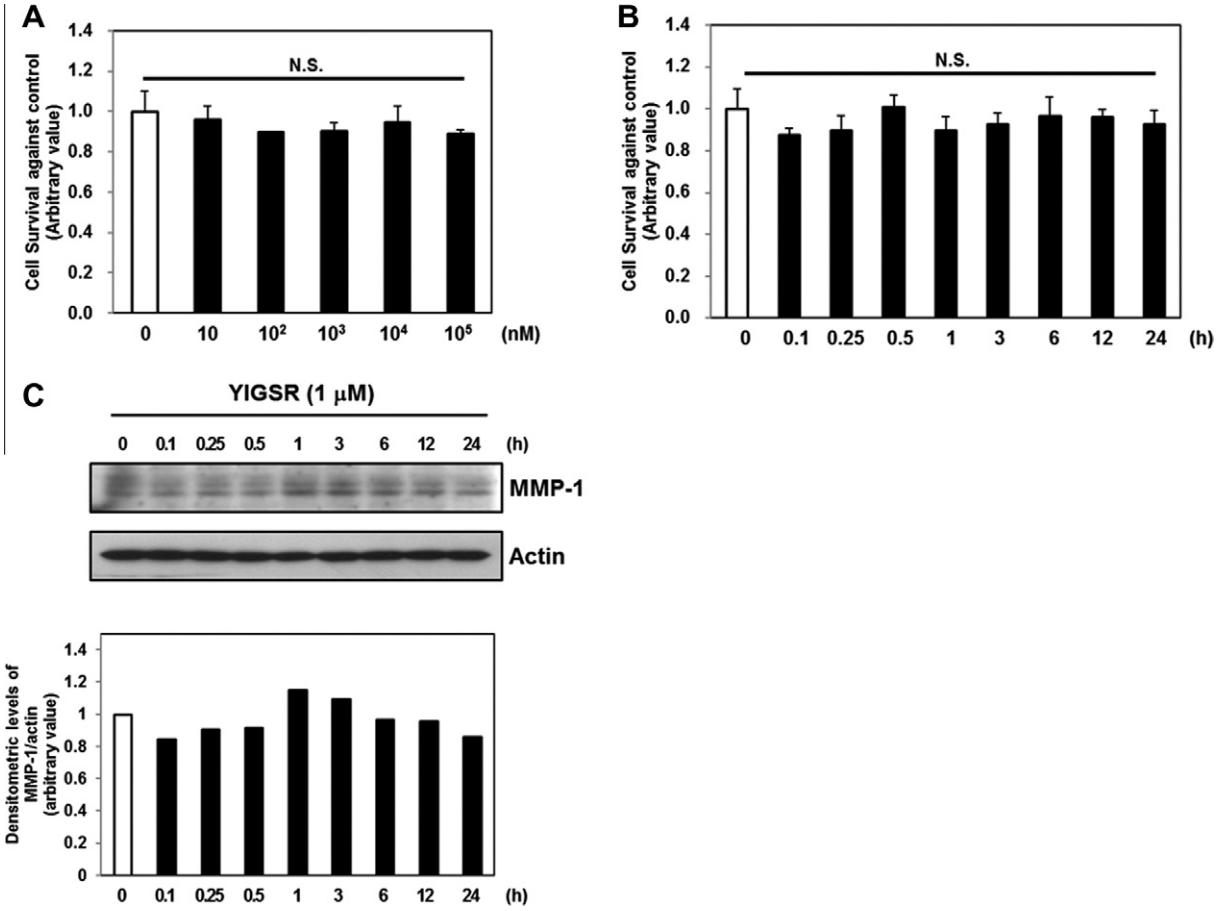


**Fig. 2.** Cell survival assay and cellular MMP-1 protein levels after YIGSR treatment. After treatment with dose- and time-dependent YIGSR, an MTT assay was performed. (A) The cell survival level of Hs27 after dose-dependent YIGSR treatment for 24 h. (B) The cell survival level of Hs27 after time-dependent treatment of 1 μM of YIGSR. The *Y*-axis indicates relative fold-change against a value of "0" for each experiment. N.S. indicates not significant. (C) Immunoblotting of MMP-1 after time-dependent treatment. After treating with 1 μM of YIGSR for 0–24 h, cell lysates were electrophoresed and blotted. Densitometric analysis was normalized by actin, and results are presented below each immunoblotting result.

tive MAPK/ERK inhibitors, PD98059. PD98059 treatment obviously inhibited YIGSR-induced type I collagen expression (Fig. 4B). Therefore, the MAPK/ERK pathway is involved in YIGSR-induced type I collagen expression.

## 4. Discussion

In this study, we elucidated a novel function of the YIGSR peptide to enhance collagen synthesis in human dermal fibroblasts. YIGSR increased the cellular level of type I collagen, and this effect was mediated by increasing the transcription level of collagen without significant changes in cell viability or cellular MMP-1 level. YIGSR evoked various cellular signaling events such as phosphorylation in focal adhesion signaling and MAPK signaling, which is known to be critical for collagen synthesis.

Type I collagen is the major protein comprising the extracellular matrix, which provides structural support to surrounding tissues. It has been reported that progressive decline of type I collagen synthesis in dermal fibroblasts attributes to the formation of wrinkles and aging skin [9,27]. Cellular type I collagen level is regulated by diverse pathways. Gene expression of type I collagen is highly regulated in both a transcriptional and post-transcriptional manner. A number of well-characterized transcription factors such as CBF, NF-1, C/EBP, SMADS, AP1, Sp1 and nuclear receptors including peroxisome proliferator activated receptors (PPARs) have been shown to interact with the regulatory elements of collagen genes [9,28,29]. ERK is a well-known mediator of ECM expression involving type I collagen gene expression [30]. ERK phosphorylation mediates smad3 phosphorylation and induces type I collagen gene expression [30]. According to our inhibitor study, it is clear that the ERK pathway mediates YIGSR-induced type I collagen expression (Fig. 4B).

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase [31]. FAK regulates a wide range of signaling cascades through association with unique adaptor molecules at its SH2 or SH3 docking site, including ERK, phosphoinositide 3-kinase (PI3K) and the Rho family of small GTPases [30]. It has also been reported that FAK-PI3K-Akt phosphorylation induces type I and III collagen expression [32]. It had previously been shown that, when FAK related non-kinase (FRNK) plasmids are transfected into fibronectin (FN)-stimulated HSC using liposome transfection, the over-expression of FRNK significantly decreases the collagen synthesis of HSC *in vitro* [33]. In hepatic stellate cells, FAK knockdown results in decreases in type I collagen levels [33]. Since tyrosine phosphorylation ($Tyr^{397}$) of FAK is reported to be adhesion-dependent and is influenced by the laminin pathway [30], the YIGSR pathway seems to be similar to the cell adhesion pathway, which mediates the phosphorylation of FAK by $Tyr^{397}$. ERK is known to be a downstream effector of FAK [34]. It has been reported that FAK mediates adhesion-dependent ERK activity [35]. We also confirmed the phosphorylation of FAK and ERK by YIGSR treatment,

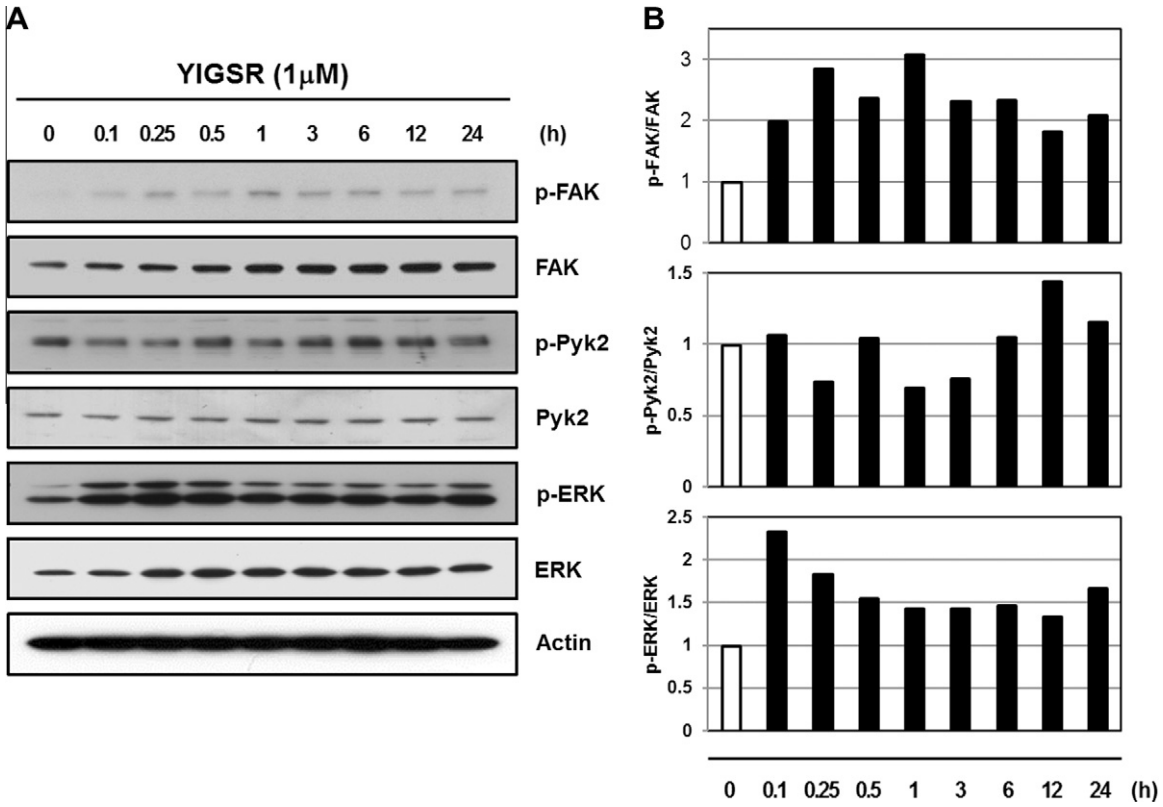


**Fig. 3.** YIGSR induced FAK, Pyk2 and ERK phosphorylation in Hs27 dermal fibroblasts. (A) Immunoblotting of downsignaling proteins of the laminin receptor after time-dependent treatment. After treating with 1 μM of YIGSR for 0–24 h, cell lysates were electrophoresed and blotted. (B) Densitometric analysis of immunoblotting. Normalization was performed by each total protein. The *Y*-axis indicates relative fold-change against a value of "0" for each experiment.

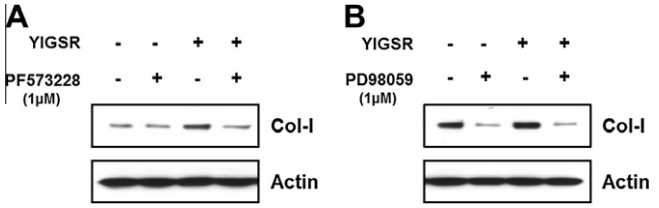


**Fig. 4.** MAPK signaling mediated the YIGSR effect on collagen synthesis. (A) Immunoblotting of type I collagen after co-treatment with YIGSR and FAK inhibitor (PF573228). After treating with 1 μM of YIGSR and PF573228 for 24 h, cell lysates were electrophoresed and blotted. (B) Immunoblotting of type I collagen after co-treatment of YIGSR and MEK inhibitor (PD98059). After treating with 1 μM of YIGSR and PD98059 for 24 h, cell lysates were electrophoresed and blotted.

indicating mediation of the FAK-ERK pathway in YIGSR-induced type I collagen expression.

Here, we found a novel function of YIGSR on collagen synthesis in human dermal fibroblasts. The YIGSR effect was mediated by increasing the transcription level of type I collagen without significantly changing cell viability or cellular MMP-1 level. Type I collagen in the dermis provides mechanical support for the structure of skin, and lower production of type I collagen is one of the major causes of wrinkle formation [9]. There are several compounds including anti-oxidants, such as vitamins C and E, co-enzyme Q10 and retinoids, that are used for treating UV-induced skin aging and wrinkles [9,36]. Although plant-derived natural compounds are widely used for the purpose of anti-wrinkle effects in the cosmetic market, most of these compounds do not possess the function of increasing collagen synthesis but rather are used for their anti-oxidant effects [9,27,37]. This study suggests that YIGSR is a strong candidate peptide for the treatment of skin aging and wrinkles.

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