Arch Biol Sci. 2021;73(3):425-432 https://doi.org/10.2298/ABS210615036C

All-trans retinoic acid prevents oxidative stress-mediated cellular senescence via upregulation of insulin-like growth factor binding protein-6 in normal human epidermal keratinocytes

Hyangtae Choi[1,2,3], Jin Young Lee[3], Nok Hyun Park[3], Jin Nam3, Won-seok Park[3], Chang Seok Lee[4,*] and Beom Joon Kim[1,2,#]

[1]*Department of Dermatology, Chung-Ang University College of Medicine*, Seoul, Republic of Korea
[2]*Department of Medicine, Graduate School, Chung-Ang University*, Seoul, Republic of Korea
[3]*Basic Research & Innovation Division, Amorepacific Corp. R&D Unit*, Yongin, Republic of Korea
[4]*Department of Beauty and Cosmetic Science, Eulji University*, Seongnam, Republic of Korea

Corresponding authors: *cslee2010@eulji.ac.kr; #beomjoon@cau.ac.kr

Received: June 15, 2021; **Revised**: September 6, 2021; **Accepted**: September 9, 2021; **Published online**: September 13, 2021

Abstract: All-trans retinoic acid (ATRA) influences cellular proliferation and differentiation but its mechanisms of action are not understood in keratinocytes. To investigate the potential mechanisms of action of ATRA in keratinocytes, microarray analysis of ATRA-treated normal human epidermal keratinocytes (NHEKs) was performed. Based on microarray data, we focused on insulin-like growth factor binding protein-6 (IGFBP-6), which is known to inhibit cellular senescence but has not been previously investigated in the context of ATRA-induced signaling in NHEKs. We verified that ATRA significantly increased IGFBP-6 gene and protein expression in NHEKs. Next, the effects of ATRA and IGFBP-6 on cell proliferation and senescence in H2O2-treated NHEKs were examined. IGFBP-6 was knocked-down using siRNA or overexpressed using pCMV-IGFBP-6. Cellular proliferation was observed using the bromodeoxyuridine (BrdU) incorporation assay. Cellular senescence was determined by monitoring SA-β-Gal staining and p21 expression. When IGFBP-6 was knocked down, cellular proliferation was inhibited, and the cellular senescence markers were increased. IGFBP-6 overexpression or ATRA treatment of H2O2-treated NHEKs rescued these effects. Taken together, our results suggest that ATRA prevents premature senescence-related skin damage at least in part by increasing IGFBP-6 expression, as shown herein in reactive oxygen species (ROS)-stimulated NHEKs.

Keywords: ATRA; cellular senescence; IGFBP-6; NHEK; ROS

INTRODUCTION

The human skin is a complex organ with a tangled structure basically consisting of two layers, the outer epidermis and the lower dermis, which are attached to each other through a basement membrane [1-3]. Keratinocytes are the most abundant cells in the epidermis. They protect the skin from external stimuli, such as ultraviolet (UV) irradiation and pathogens, but these external stimuli can induce keratinocyte senescence. All-trans retinoic acid (ATRA) is a natural derivative of vitamin A that influences various cellular processes, including cellular proliferation and differentiation [1,4,5]. It acts by inducing or maintaining the differentiation of keratinocytes. Studies have shown that ATRA can postpone the senescence of keratinocytes at least in part by inhibiting the expression of p16 and betaig-h3 and inducing telomerase activity [6]. ATRA can also exert proliferative effects on normal human epidermal keratinocytes (NHEKs) by upregulating P2Y purinoceptor 2 (P2Y2) [7]. Thus, ATRA has been used as a therapeutic agent for numerous skin disorders [1]. However, the action mechanisms of ATRA have not been well studied and need to be elucidated in greater detail.

Aged skin cells show altered secretion of growth factors, cytokines, chemokines and proteases, which

characterizes the senescence-associated secretory phenotype (SASP) [3,8]. The insulin-like growth factor (IGF) binding proteins (IGFBPs), which are known to be involved in SASP, bind to IGFs with a greater affinity than the IGF receptors. The IGFBPs thus play important roles in regulating IGF signaling, which is closely related to cellular proliferation [9,10]. About 98% of IGF-1 is constitutively bound to one of the six well-characterized IGFBP family members. IGFBP-6 is a recently discovered IGFBP that inhibits senescence and regulates cellular proliferation in fibroblasts [11,12]. Deletion of IGFBP-6 in human dermal fibroblasts decreases cell proliferation and induces apoptosis and senescence, whereas overexpression of IGFBP-6 prevents apoptosis and delayed senescence in fibroblasts [3]. Relatively few reports have addressed the function of IGFBP-6 in human epidermal keratinocytes [13,14].

Reactive oxygen species (ROS), including H_2O_2, cause oxidative stress and DNA damage [15]. If DNA damage is not repaired, the cell will undergo apoptosis or premature senescence. Under pathological conditions, excessive ROS are especially harmful and induce cellular aging or cell death [16]. Recent studies showed that both long-term intracellular accumulation of H_2O_2 and exogenous exposure to subcytotoxic concentrations of H_2O_2 induce cells to undergo premature senescence [17,18].

In the present study, we used microarray analysis to identify genes that exhibit differential expression in ATRA-treated keratinocytes. Based on the obtained microarray data, we selected IGFBP-6 for further study. The relationship between ATRA and IGFBP-6 in keratinocytes is not clear, especially with respect to oxidative stress-mediated cellular senescence, so we decided to elucidate the expression and function of IGFBP-6 in ATRA-treated keratinocytes. We verified that ATRA significantly induces IGFBP-6 expression in NHEKs, and then examined the inhibitory effects of ATRA and IGFBP-6 on ROS-induced premature senescence in NHEKs. Our results offer the first insight into the relationship between ATRA and IGFBP-6 and their effects on oxidative stress-induced senescence of keratinocytes.

MATERIALS AND METHODS

Materials and cell culture

ATRA and H_2O_2 were purchased from Sigma Aldrich (St. Louis, MO, USA). NHEKs were purchased from Invitrogen (San Diego, CA, USA). Cultured NHEKs (early-passage cells) were used for all experiments. NHEKs were grown in keratinocyte basal medium (KBM)-gold supplemented with a bullet kit (KBM-Gold bullet kit; Lonza, Walkersville, MD, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Microarray analysis

Duplicate cultures of NHEKs were treated for 2 days with 1 μM of ATRA and RNA was isolated. The RNA sample integrity was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA (5 μg) was used for cDNA synthesis and labeling using a One-Cycle cDNA Synthesis Kit (Affymetrix). The total transcriptional profiles of ATRA treated- and untreated-NHEKs were compared using an Affymetrix Human Genome U133 plus 2.0 Array chip.

IGFBP-6 knock-down and gene overexpression systems

For IGFBP-6 knockdown, IGFBP-6-specific siRNA (ON-TARGETplus SMART pool siRNA; Dharmacon, CO, USA) was transfected into NHEKs using lipofectamine 2000 reagent (100 μM; Invitrogen, USA). For IGFBP-6 overexpression, pCMV-IGFBP-6 (Origene, Rockville, MD, USA) was transfected into NHEKs using the Effectene reagent (Qiagen, Valencia, CA, USA). In both cases, cells were harvested on day 3 post-transfection.

Cell proliferation assay

Cell proliferation was assessed using a BrdU kit (Roche Applied Science, IN, USA) according to the manufacturer's instructions. Briefly, cultured cells were incubated with BrdU labeling solution for 2 h at 37°C in a humidified incubator. The cells were washed twice with washing solution and fixed with fixative solution for 30 min, and the fixed cells were treated

with nuclease working solution. The cells were then incubated with anti-BrdU-POD for 30 min, the peroxidase substrate was added, and absorbance at 405 nm was measured using a microplate reader (Synergy 2; BioTek, Inc., VT, USA).

H_2O_2-induced premature senescence model and SA-β-Gal staining

For the induction of premature senescence, NHEKs were treated with 100 μM H_2O_2 for 2 h and cultured in fresh medium for 48 h. The senescent status was verified via *in situ* SA-β-Gal staining according to the manufacturer's instructions (Abcam, Cambridge, UK). Briefly, cultured NHEKs were fixed with fixative solution for 10 min at room temperature and the fixed cells were stained with staining solution containing 20 mg/mL X-gal for 24 h.

RNA isolation and real-time quantitative PCR

Total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's manual. First-strand cDNA was synthesized from 4 μg of total RNA using a Superscript™ III First-strand synthesis system (Invitrogen). Real-time qPCR was performed in an ABI 7500HT fast system using the TaqMan™ Universal PCR Master Mix (both from Applied Biosystems, Foster City, CA, USA). TaqMan probes for IGFBP-6 and ribosomal protein lateral stalk subunit P0 (RPLP0) were purchased from Applied Biosystems. Relative changes in the IGFBP-6 mRNA expression level were normalized with respect to the RPLP0 mRNA expression level in the same sample.

Western blotting

To prepare intracellular protein samples, cells were lysed in lysis buffer containing 150 mM NaCl, 1.0% IGEPAL® CA-630 (Sigma-Aldrich, St. Louis, MO, USA), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris (pH 8.0). Lysates were centrifuged, the soluble fractions were separated by SDS-polyacrylamide-gel electrophoresis (PAGE), and the resolved proteins were transferred to a nitrocellulose membrane. The blots were incubated with anti-p21, anti-IGFBP-6 (Abcam, Cambridge, MA, USA), or anti-β-actin (Santa Cruz Biotechnology, CA, USA), and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

The results are expressed as the mean±standard deviation (SD) of at least three independent measurements. Statistically significant differences were determined using the t-test and a two-tailed value of $P<0.05$ was considered statistically significant. (Three levels of statistical significance were considered ($P<0.05$, $P<0.01$ and $P<0.001$), as will be indicated).

RESULTS

ATRA induces IGFBP-6 expression in NHEKs

Microarray analysis of about 47000 genes in ATRA-treated versus -untreated NHEKs revealed that 163 and 75 genes were significantly (>2-fold) upregulated and downregulated by ATRA, respectively. Of the top 10 upregulated genes (Table 1), we selected IGFBP-6

Table 1. Top 10 upregulated differentially expressed genes.

UniGene ID	Gene Symbol	Gene name	Fold change* (2 days)
Hs.654568	*KRT 19*	*keratin 19*	5.7
Hs.654570	*KRT 15*	*keratin 15*	5.7
Hs.148641	*CTSH*	*cathepsin H*	4.7
Hs.438231	*TFP I2*	*tissue factor pathway inhibitor 2*	4.5
Hs.274313	***IGFBP 6***	***insulin-like growth factor binding protein 6***	**4.2**
Hs.371240	*AKAP 12*	*A kinase (PRKA) anchor protein 12*	4.1
Hs.226307	*APOBEC 3B*	*apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B*	3.6
Hs.297413	*MMP 9*	*matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)*	3.6
Hs.654422	*TUBA1A*	*tubulin, alpha 1a*	3.5
Hs.531561	*EMP 2*	*epithelial membrane protein 2*	3.4

*Based on microarray analysis of the fold increase in gene expression for NHEKs treated with ATRA for 2 days vs non-treated NHEKs (control).

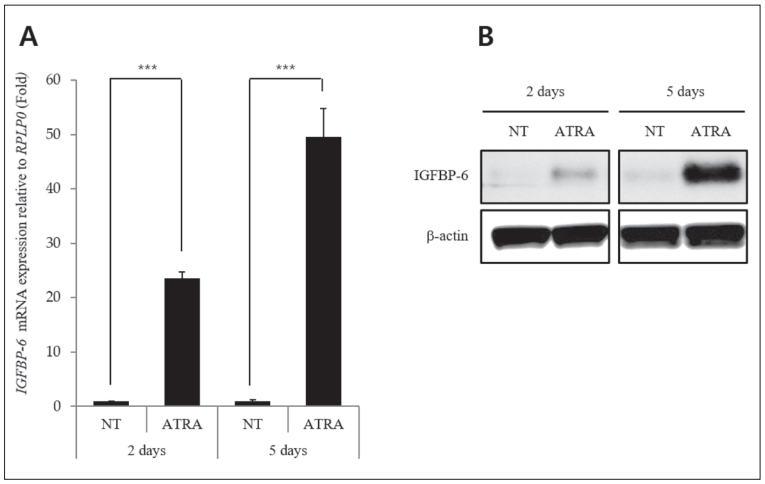

Fig. 1. ATRA induces IGFBP-6 expression in NHEKs. NHEKs were treated with 1 μM of ATRA, and (**A**) real-time qPCR and (**B**) Western blotting were performed on days 2 and 5. Data are expressed as the mean±SD of at least three independent measurements. Statistically significant differences were determined by the t-test. ***P<0.001 vs non-treated (NT) control group.

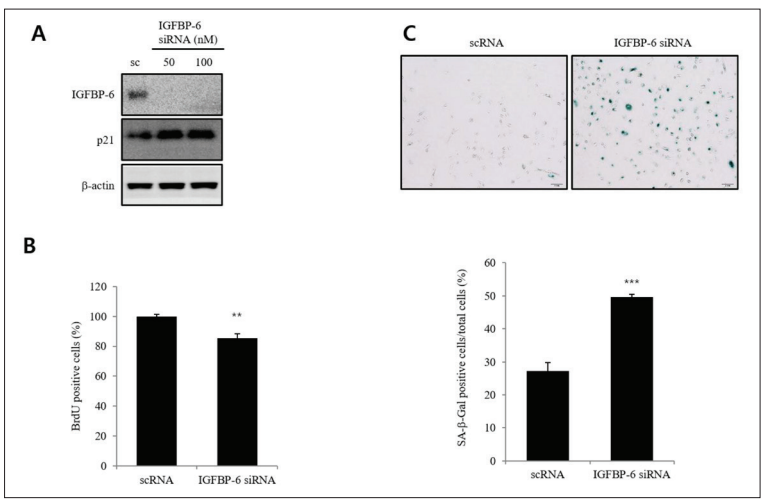

Fig. 2. IGFBP-6 silencing decreases cell proliferation and increases senescence in NHEKs. IGFBP-6 was knocked down using a specific small interfering RNA (IGFBP-6 siRNA). **A** – Protein levels of IGFBP-6 and p21 examined by Western blotting. **B** – Cell proliferation by BrdU incorporation in NHEKs transfected with scRNA or IGFBP-6 siRNA. The bar graphs represent the mean of the percent change compared to the scRNA-transfected control. **C** – Changes in the number of senescent cells monitored by SA-β-Gal staining. SA-β-Gal positive cells are blue. Data are expressed as the mean±SD of at least three independent measurements. Statistically significant differences were determined using the t-test. **P<0.01, ***P<0.001 vs the scRNA-transfected control group.

for further study based on a previous report that IGFBP-6 is associated with aging in fibroblasts [3]. We first performed real-time qPCR and Western blotting to confirm that IGFBP-6 expression was increased in ATRA-treated NHEKs at both the mRNA and protein levels. Based on the results of our microarray analysis, ATRA significantly enhanced IGFBP-6 expression in NHEKs at both the mRNA (Fig. 1A) and protein (Fig. 1B) levels. IGFBP-6 expression was powerfully increased after ATRA treatment for 5 days.

IGFBP-6 knock-down decreases cell proliferation and increases senescence in NHEKs

To begin assessing the significance of IGFBP-6 for cellular proliferation and senescence in NHEKs, we transfected NHEKs with scrambled (sc) RNA or IGFBP-6 siRNA (50 nM or 100 nM siRNA), incubated the cells for 3 days, and conducted Western blot analysis of IGFBP-6 expression. We confirmed that both 50 and 100 nM of IGFBP-6 siRNA successfully inhibited IGFBP-6 protein expression in NHEKs, whereas scRNA treatment did not affect IGFBP-6 protein expression (Fig. 2A). This indicated that IGFBP-6 expression was effectively knocked down by IGFBP-6 siRNA transfection in NHEKs. Interestingly, we also noted that IGFBP-6 siRNA treatment of NHEKs significantly upregulated the expression of p21 protein, which is an inhibitor of cyclin-dependent kinases and a marker of cellular senescence. We next used a BrdU incorporation assay to assess how IGFBP-6 knock-down affected cell proliferation in NHEKs. As shown in Fig. 2B, cellular proliferation was reduced in IGFBP-6-depleted NHEKs, indicating that IGFBP-6 positively influenced the proliferation of NHEKs. We then used SA-β-Gal staining to elucidate whether the knock-down of IGFBP-6 induces cellular senescence in NHEKs. As shown in Fig. 2C, the number of SA-β-Gal-positive (senescent) NHEKs increased under IGFBP-6 depletion, suggesting that IGFBP-6 is indeed involved in regulating cellular senescence in NHEKs. These data

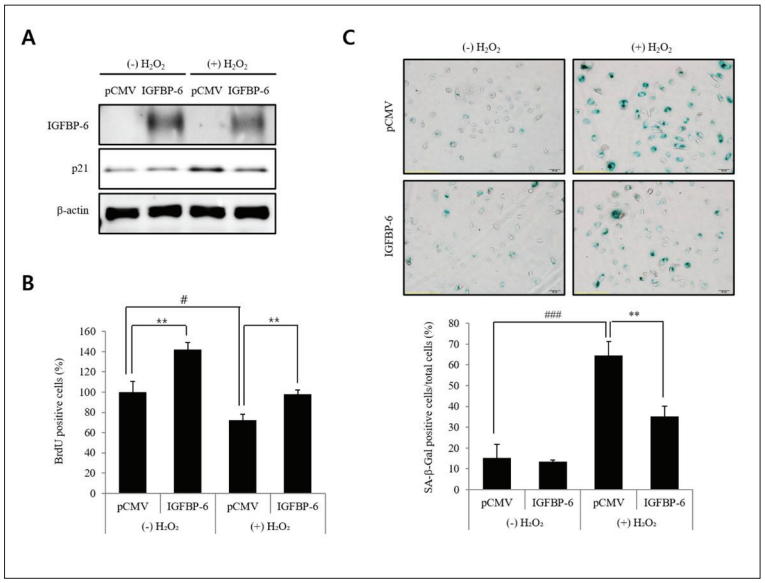

Fig. 3. IGFBP-6 overexpression increases cell proliferation and decreases senescence in H_2O_2-treated NHEKs. IGFBP-6 was overexpressed using pCMV-IGFBP-6. **A** – Protein expression levels of IGFBP-6 and p21 examined by Western blotting. **B** – Cell proliferation of IGFBP-6-overexpressing NHEKs was assessed by BrdU incorporation. The bar graphs represent the mean of percent change compared to the vector control (pCMV). **C** – Changes in the numbers of senescent cells monitored by SA-β-Gal staining. SA-β-Gal positive cells are blue. Data are expressed as the mean ± SD of at least three independent measurements. Statistically significant differences were determined using the t-test. **P<0.01 vs pCMV transfected-control group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 *vs* H_2O_2 non-treated control group.

suggest that IGFBP-6 may promote cellular proliferation and prevent cellular senescence in NHEKs.

IGFBP-6 overexpression increases cell proliferation and decreases senescence in H_2O_2 -treated NHEKs

Based on our observation that IGFBP-6 depletion promoted cellular senescence in NHEKs, we next investigated whether elevated IGFBP-6 expression could reduce cellular senescence induced by an external oxidative stress, such as that triggered by H_2O_2 exposure. To elucidate whether IGFBP-6 prevents H_2O_2-induced oxidative damage, we transfected NHEKs with an IGFBP-6 overexpression vector or empty control vector (pCMV) and exposed the transfected cells to 100 μM H_2O_2 (identified as a subcytotoxic dose in preliminary experiments; data not shown) for 3 days. As shown in Fig. 3A, NHEKs transfected with the pCMV-IGFBP-6 overexpression vector showed a high level of expression of IGFBP-6 in the presence or absence of H_2O_2. Exposure to H_2O_2 was found to increase p21 in pCMV-transfected cells, but this upregulation of p21 was clearly reduced in IGFBP-6-overexpressing NHEKs. The BrdU incorporation assays revealed that although H_2O_2 treatment inhibited cell proliferation in both groups, IGFBP-6-overexpressing NHEKs exhibited higher proliferation than control pCMV-transfected NHEKs in both the presence and absence of H_2O_2 (Fig. 3B). H_2O_2 treatment dramatically induced cellular senescence, but the H_2O_2-induced increase of SA-β-Gal-positive cells was lower in IGFBP-6-overexpressing cells (Fig. 3C). Taken together, the data indicate that IGFBP-6 contributes to defending NHEKs against oxidative stress.

ATRA increases cell proliferation and decreases senescence in H_2O_2-exposed NHEKs

Finally, we examined whether ATRA could also prevent cellular damage in H_2O_2-stimulated NHEKs. One μM of ATRA was applied to NHEKs in the presence or absence of H_2O_2 for 3 days, and BrdU incorporation and the SA-β-Gal staining assays were performed. As shown in Fig. 4A, H_2O_2 exposure reduced the cell numbers of NHEKs, but ATRA treatment recovered cell viability up to the control level in H_2O_2-treated NHEKs. The cellular senescence induced by H_2O_2 exposure of NHEKs was also significantly inhibited by ATRA (Fig. 4B). In addition, IGFBP-6 expression was increased by ATRA treatment of NHEKs regardless of the presence or absence of H_2O_2 (Fig. 4C).

DISCUSSION

Aged human skin is characterized by structural and functional alterations of the epidermis and dermis, including decreased epidermal thickness, flattening of the dermal-epidermal junction and decreased keratinocyte proliferation [19,20]. The epidermis is exposed to UV light, which causes oxidative stress and DNA damage that in turn predisposes the skin

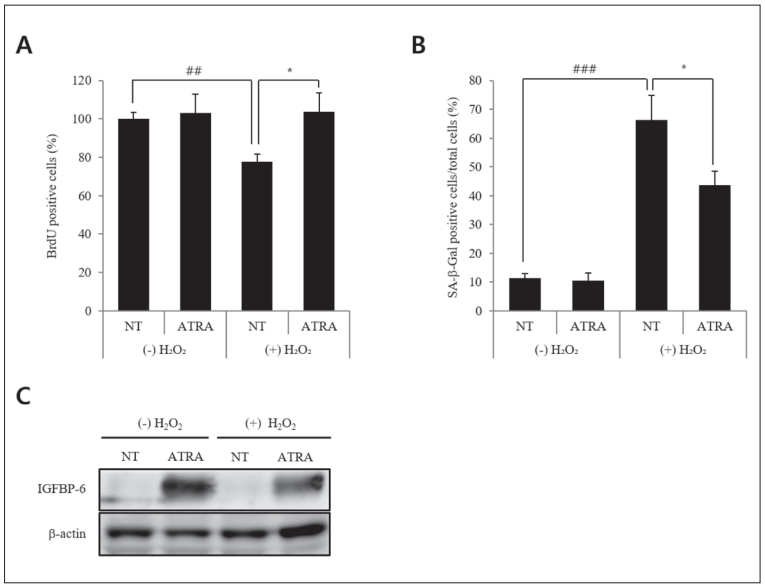

Fig. 4. ATRA increases cell proliferation and decreases senescence in H_2O_2-treated NHEKs. **A** – Cell proliferation of ATRA-treated NHEKs assessed via BrdU incorporation. **B** – Changes in percentages of senescent cells detected by SA-β-Gal staining. **C** – Protein expression levels of IGFBP-6 examined by Western blotting. Data are expressed as the mean±SD of at least three independent measurements. Statistically significant differences were determined using the t-test. *P<0.05 vs non-treated (NT) control group; ##P<0.01, ###P<0.001 vs H_2O_2 non-treated control group.

to damage and different diseases (e.g. cancers), and is responsible for the characteristic aged appearance [21]. Premature senescence can be caused by oxidative and other stresses that cause DNA damage, chromatin perturbation, oncogene expression [22].

ATRA is a representative anti-aging agent that is used in cosmetics, although its mechanisms of action have not been well elucidated. Herein, we have defined for the first time the gene alterations that are related to the actions of ATRA in human keratinocytes. We first performed microarray analysis in an effort to identify genes related to the effect of ATRA on NHEKs. This analysis suggested that ATRA significantly increased IGFBP-6 gene expression in NHEKs; we confirmed this increase at the protein and mRNA levels. ATRA was previously reported to regulate the synthesis of IGF and IGFBP in cancer cells [23-25], suggesting that it could be a potent regulator of IGFBP expression. Among the IGFBPs, IGFBP-6 is known to prevent aging by regulating p21 in fibroblasts [3]. However, no previous study had examined the possible relationship between ATRA and IGFBP-6 in NHEKs. Based on our initial observations and previous reports, we posited the novel hypothesis that ATRA might prevent oxidative stress-induced cell damage via an IGFBP-6-mediated pathway.

Subcytotoxic concentrations of exogenous H_2O_2 stimulate intracellular ROS generation [26], and prolonged exposure to H_2O_2 leads to premature cellular senescence, which is characterized by the inhibition of proliferation and increased levels of SA-β-Gal [27-29]. In the present study, we demonstrated that both ATRA and IGFBP-6 recovered cell viability and prevented keratinocyte senescence in H_2O_2-treated NHEKs. These findings suggest that ATRA and IGFBP-6 are effective in preventing the cellular senescence induced by oxidative stress.

The current study is limited in that we did not identify a direct correlation between ATRA and IGFBP-6 in NHEKs. To show that ATRA-induced IGFBP-6 directly regulates cell proliferation and senescence in H_2O_2-treated NHEKs, we performed further experiments using IGFBP-6 siRNA in ATRA-treated, H_2O_2-exposed NHEKs. IGFBP-6 siRNA transfection neutralized the increase in cell numbers and reduced cellular senescence associated with ATRA treatment of H_2O_2-exposed NHEKs. These data supported our hypothesis that ATRA controls cell viability and senescence via an IGFBP-6-dependent pathway in H_2O_2-treated NHEKs. Cell viability differed between scRNA- and IGFBP-6 siRNA-transfected, H_2O_2-treated NHEKs, but not between the corresponding non-treated NHEKs. We speculate that the experimental conditions for both siRNA transfection and H_2O_2 stimulation could have complex effects on cell survival.

No previous report has examined the role and action mechanism of IGFBP-6 in human epidermal keratinocytes. In contrast, retinoic acid is well known to function as an anti-aging agent by acting on various pathways in keratinocytes. However, to the best of our knowledge it has not been demonstrated that retinoic acid mediates IGFBP-6 gene expression in

NHEKs. Our microarray data show that ATRA markedly increases IGFBP-6 expression, which led us to propose that the anti-aging efficacy of ATRA could be related to changes in IGFBP-6 expression. We then examined whether ATRA and IGFBP-6 can affect cellular senescence and oxidative stress, which are major phenomena in aging. Our results revealed that cellular proliferation was inhibited, and the levels of cellular senescence markers (SA-β-Gal and p21) were increased after knock-down of IGFBP-6. Notably, the premature senescence phenotype induced by oxidative stress was counteracted by IGFBP-6 overexpression. These findings show for the first time that IGFBP-6 regulates premature senescence in NHEKs. In addition, we confirmed that ATRA powerfully induces IGFBP-6 expression in NHEKs, as assessed using microarray analysis, real-time qPCR and Western blotting. Taken together, our findings indicate that ATRA may prevent the oxidative stress-mediated cellular senescence of NHEKs at least partly via upregulation of IGFBP-6 in NHEKs, although further studies are needed to discover the relationship between ATRA and IGFBP-6.

In conclusion, the results of examination of alterations of gene expression in ATRA-treated NHEKs presented herein allowed us to conclude that ATRA induces IGFBP-6 expression in NHEKs, and that ATRA or IGFBP-6 can alleviate cellular damage (e.g., subcytotoxicity and senescence) in oxidatively stressed NHEKs. These findings suggest that IGFBP-6 proteins regulate the proliferation and senescence of human keratinocytes and that ATRA may act as an anti-aging agent at least partly via an IGFBP-6-dependent pathway. The current study provides important new insight into the mechanism of action through which ATRA provides anti-aging effects on the skin.

Funding: The study was supported by grants from the Amorepacific R&D Center.

Author contributions: H Choi, CS Lee and BJ Kim designed the study. H Choi performed most of the experiments. JY Lee, NH Park and WS Park supported the experiments. CS Lee and BJ Kim analyzed the data and contributed to the writing of the manuscript.

Conflict of interest disclosure: The authors have nothing to disclose.

REFERENCES

1. Mukherjee S, Date A, Patravale V, Korting H, Roeder A, Weindl G. Retinoids in the treatment of skin aging: an overview of clinical efficacy and safety. Clin Interv Aging. 2006;1(4):327-48. https://doi.org/10.2147/ciia.2006.1.4.327
2. Rinnerthaler M, Bischof J, Streubel MK, Trost A, Richter K. Oxidative stress in aging human skin. Biomolecules. 2015;5(2):545-89. https://doi.org/10.3390/biom5020545
3. Micutkova L, Diener T, Li C, Wrzesinska A, Mueck C, Heutter E, Weinberger B, Loebenstein B, Roepstorff P, Zeng R, Duerr P. Insulin-like growth factor binding protein-6 delays replicative senescence of human fibroblasts. Mech Ageing Dev. 2011;132(10):468-79. https://doi.org/10.1016/j.mad.2011.07.005
4. Schroeder M, Zouboulis CC. All-trans-retinoic acid and 13-cis-retinoic acid: pharmacokinetics and biological activity in different cell culture models of human keratinocytes. Horm Metab Res. 2007;39(2):136-40. https://doi.org/10.1055/s-2007-961813
5. Jean J, Soucy J, Pouliot R. Effects of retinoic acid on keratinocyte proliferation and differentiation in a psoriatic skin model. Tissue Eng Part A. 2011;17(13-14):1859-68. https://doi.org/10.1089/ten.tea.2010.0463
6. Min BM, Oh JE, Choi CM. Retinoic acid delays keratinocyte senescence by suppression of betaig-h3 and p16 expression and induction of telomerase activity. Int J Mol Med. 2004;13(1):25-31. https://doi.org/10.3892/ijmm.13.1.25
7. Fujishita K, Koizumi S, Inoue K. Upregulation of P2Y2 receptors by retinoids in normal human epidermal keratinocytes. Purinergic Signal. 2006;2(3):491-8. https://doi.org/10.1007/s11302-005-7331-5
8. Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci USA. 2001;98(21):12072-7. https://doi.org/10.1073/pnas.211053698
9. Bach LA. IGFBP-6 five years on; not so 'forgotten'? Growth Horm IGF Res. 2005;15(3):185-92. https://doi.org/10.1016/j.ghir.2005.04.001
10. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev. 2002;23(6):824-54. https://doi.org/10.1210/er.2001-0033
11. Xie L, Tsaprailis G, Chen QM. Proteomic identification of insulin-like growth factor-binding protein-6 induced by sublethal H2O2 stress from human diploid fibroblasts. Mol Cell Proteomics. 2005;4(9):1273-83. https://doi.org/10.1074/mcp.M500032-MCP200
12. Beilharz EJ, Russo VC, Butler G, Baker NL, Connor B, Sirimanne ES, Dragunow M, Werther GA, Gluckman PD, Williams CE, Scheepens A. Co-ordinated and cellular specific induction of the components of the IGF/IGFBP axis in the rat brain following hypoxic-ischemic injury. Brain Res Mol Brain Res. 1998;59(2):119-34. https://doi.org/10.1016/S0169-328X(98)00122-3
13. Kato M, Ishizaki A, Hellman U, Wernstedt C, Kyogoku M, Miyazono K, Heldin CH, Funa K. A human keratinocyte cell line produces two autocrine growth inhibitors, transforming

growth factor-beta and insulin-like growth factor binding protein-6, in a calcium- and cell density-dependent manner. J Biol Chem. 1995;270(21):12373-9. https://doi.org/10.1074/jbc.270.21.12373
14. Marinaro JA, Hendrich EC, Leeding KS, Bach LA. HaCaT human keratinocytes express IGF-II, IGFBP-6, and an acid-activated protease with activity against IGFBP-6. Am J Physiol. 1999;276(3):E536-42. https://doi.org/10.1152/ajpendo.1999.276.3.E536
15. Ksiazek K. A comprehensive review on mesenchymal stem cell growth and senescence. Rejuvenation Res. 2009;12(2):105-16. https://doi.org/10.1089/rej.2009.0830
16. Wei H, Li Z, Hu S, Chen X, Cong X. Apoptosis of mesenchymal stem cells induced by hydrogen peroxide concerns both endoplasmic reticulum stress and mitochondrial death pathway through regulation of caspases, p38 and JNK. J Cell Biochem. 2010;111(4):967-78. https://doi.org/10.1002/jcb.22785
17. Ho PJ, Yen ML, Tang BC, Chen CT, Yen B. H2O2 accumulation mediates differentiation capacity alteration, but not proliferative decline, in senescent human fetal mesenchymal stem cells. Antioxid Redox Signal. 2013;18(15):1895-905. https://doi.org/10.1089/ars.2012.4692
18. Burova E, Borodkina A, Shatrova A, Nikolsky N. Sublethal oxidative stress induces the premature senescence of human mesenchymal stem cells derived from endometrium. Oxid Med Cell Longev. 2013;2013:474931. https://doi.org/10.1155/2013/474931
19. Thuringer JM, Katzberg AA. The effect of age on mitosis in the human epidermis. J Invest Dermatol. 1959;33:35-9. https://doi.org/10.1038/jid.1959.119
20. West MD. The cellular and molecular biology of skin aging. Arch Dermatol. 1994;130(1):87-95. https://doi.org/10.1001/archderm.1994.01690010091014
21. Pelle E, Huang X, Mammone T, Marenus K, Maes D, Frenkel K. Ultraviolet-B-induced oxidative DNA base damage in primary normal human epidermal keratinocytes and inhibition by a hydroxyl radical scavenger. J Invest Dermatol. 2003;121(1):177-83. https://doi.org/10.1046/j.1523-1747.2003.12330.x
22. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007;8(9):729-40. https://doi.org/10.1038/nrm2233
23. Gabbitas B, Canalis E. Retinoic acid regulates the expression of insulin-like growth factors I and II in osteoblasts. J Cell Physiol. 1997;172(2):253-64. https://doi.org/10.1002/(SICI)1097-4652(199708)172:2<253::AID-JCP12>3.0.CO;2-A
24. Martin JL, Coverley JA, Pattison ST, Baxter R. Insulin-like growth factor-binding protein-3 production by MCF-7 breast cancer cells: stimulation by retinoic acid and cyclic adenosine monophosphate and differential effects of estradiol. Endocrinology 1995;136(3):1219-26. https://doi.org/10.1210/endo.136.3.7532580
25. Babajko S, Binoux M. Modulation by retinoic acid of insulin-like growth factor (IGF) and IGF binding protein expression in human SK-N-SH neuroblastoma cells. Eur J Endocrinol. 1996;134(4):474-80. https://doi.org/10.1530/eje.0.1340474
26. Forman HJ. Use and abuse of exogenous H2O2 in studies of signal transduction. Free Radic Biol Med. 2007;42(7):926-32. https://doi.org/10.1016/j.freeradbiomed.2007.01.011
27. Lu T, Finkel T. Free radicals and senescence. Exp Cell Res. 2008;314(9):1918-22. https://doi.org/10.1016/j.yexcr.2008.01.011
28. Passos JF, Von Zglinicki T. Oxygen free radicals in cell senescence: are they signal transducers? Free Radic Res. 2006;40(12):1277-83. https://doi.org/10.1080/10715760600917151
29. Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Exp Gerontol. 2000;35(8): 927-45. https://doi.org/10.1016/S0531-5565(00)00180-7