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Andrographolide의 Extracellular Signal-regulated Kinase Pathway (ERK)를 통한 상피 세포 줄기세포능 향상

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Andrographolide Promotes the Stemness of Epidermal Cells through the Extracellular Signal-regulated Kinase (ERK) Pathway

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Abstract – Andrographolide, the main compound of *Andrographis paniculata (A. paniculata),* shows various biological properties including anti-viral, anti-inflammatory, anti-diabetic, and hepatoprotective effects. Our previous study has shown that *A. paniculata* extract exerts antiaging effects by activation of stemness in epidermal stem cells (EpSCs). In this study, we investigated the effect of andrographolide as a main compound of *A. paniculata* on EpSCs and its mechnism of action using several *in vitro* assays. Andrographolide increased the proliferation of EpSCs and induced cell cycle progression. Additionally, andrographolide increased VEGF production and the expression of stem cell markers integrin β1 and p63. Furthermore, phosphorylation levels of extracellular signal-regulated kinase 1/2 (ERK1/2), S6 ribosomal protein (S6RP) and Akt were increased by andrographolide. Taken together, these results indicate that andrographolide-induced proliferation of EpSCs is mediated by the ERK1/2, Akt-dependent pathway with increased production of VEGF and upregulated stemness through integrin β1 and p63.

Keywords – Andrographolide, ERK1/2, Proliferation, Stemness, Vascular endothelial growth factor

Aged skin is characterized by decreased tissue thickness in the epidermis, marked atrophy, and reduced elasticity of the dermal connective tissue.[1] The epidermis, the most exposed part of the skin, needs to continuously renew itself due to constant desquamation which begins with the multiplication of proliferative cells. Epidermal stem cells (EpSCs) reside within the niche which is characterized with high expression of α6 integrin ($CD49f^+$) and integrin β1 ($CD29^+$). They can self-renew and develop into the distinct layers of the epidermis: the spinous layer, granular layer, and cornified layer. Thus, maintaining an epidermal stem cell population is of prime importance even during aging.[2,3] Several studies have shown that epidermal skin aging is associated with the reduced expression of stem cell markers such as p63 and integrin β1.[4,5] The expression of integrin β1 has a crucial role in the expansion of epidermal progenitor cells to maintain epidermal homeostasis. Reduction in integrin β1 levels with advancing age contributes to age-associated changes in the dermal thickness and skin vascularization. Therefore, aged human epidermal cells exhibit a reduced self-renewal ability. p63 is a p53 family member associated with epithelial development and maintenance. Expressed in high clonogenic cells, it can inhibit premature aging.[6,7]

MAPK/ERK and PI3K/Akt/mTOR are the major intracellular pathways controlling proliferation related to expression level of integrin β1 and p63.[8,9] Particularly, the crosstalk between the MAPK pathway and Akt is a well-defined signaling pathway through three protein kinase families: MAPK/ERK, SAPK/JNK, and p38.[10]

A. paniculata is a therapeutic herb used in several Asian countries as hepatoprotectant, antimicrobial, antioxidant, and

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anti-inflammatory agent. Our previous study showed that *A. paniculata* has an anti-skin aging effect through the upregulation of progenitor cell growth and the expression of epidermal stem cell marker integrin β1.[11] The characteristic components of *A. paniculata* are diterpenoids, including andrographolide (AGP) and its analogs, neoandrographolide, 14-deoxyandrographolide, and 14-deoxy-11-12-didehydro andrographolide. AGP is the most abundant diterpene in *A. paniculata* which has been analyzed by various techniques including TLC, HPLC and LC-MS methods[12,13,14]. AGP has various pharmaceutical effects, including antivirus, anti-inflammatory, and antimicrobial activities.[15] However, the involvement of AGP in EpSCs has not been reported yet. Therefore, the objective of this study was to examine the epidermal stemness effects of AGP on EpSCs and its possible mechanisms underlying such effects.

Materials and Methods

Chemicals and Antibodies – Andrographolide and dimethyl sulfoxide were purchased from Sigma-Aldrich (MO, USA). Surfact-Amps X-100 was purchased from Pierce (MA, USA). Integrin β1 antibody conjugated to FITC-fluorochrome (ab46920) and Anti-p63 antibody (ab110038) were bought from Abcam (Cambridge, UK). Phospho-Akt (Ser473) mouse monoclonal antibody (4051), Akt (pan) rabbit monoclonal antibody (4691), phospho-S6 ribosomal protein (Ser235/236) rabbit monoclonal antibody (4858), S6 ribosomal protein rabbit monoclonal antibody (2217), phospho-ERK1/2 mouse monoclonal antibody (9106), and ERK1/2 antibody (9102) were purchased from Cell Signaling Technology (MA, USA). Primary human antibodies, CD49f and CD29, were obtained from BD Bioscience (Oxford, UK).

Cell Culture – Epidermal stem cells were isolated from human foreskin using a method described by Lee *et al.*,[16] These cells were added to a Type 4 collagen-coated dish containing keratinocyte culture medium (EpiLife, Invitrogen, Carlsbad, CA, USA). After 4 h of incubation, the medium containing non-adherent cells was discarded and only rapidly adhering cells were cultured at 37°C in a 5% CO_2 incubator. These cells were sorted with the CD49f and CD29 antibodies.

Cell Viability Assay – Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the conversion of a substrate containing a tetrazolium ring into blue formazan by mitochondrial dehydrogenases. After incubation of the cells treated with AGP, the medium was changed to a serum-free keratinocyte medium (EpiLife) containing MTT (1 mg/ml). After the cells were incubated for 1 h and 30 min. at 37°C, formazan was solubilized with dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). The absorbance was measured at 570 nm using a spectrophotometer (Power Wave, Bio-Tek Inc, Winooski, VT, USA).

Cell Cycle Analysis – A flow cytometric analysis with propidium iodide (PI) was performed to measure cell cycle progression. Both adherent and floating cells were collected, washed with ice-cold PBS, fixed with 70% ice-cold ethanol for 1 h, repeatedly washed with PBS, and re-suspended with RNase A (0.5 mg/ml) in PBS. After 1 h of incubation, cellular DNA was stained with PI (50 μg/ml) for 20 min. protected from light at 4°C. The relative DNA concentration of the stained cells was measured using a flow cytometer (FACSCalibur™, Becton Dickinson, San Jose, CA, USA). All analyses were done following the manufacturer's recommended protocols.

Integrin β1 and p63 Flow Cytometry – To quantify the levels of integrin β1 and p63, EpSCs in serum-free medium were gathered with trypsin and analyzed following the flow cytometry protocol (eBioscience, San Diego, CA, USA). Aliquoted samples were separated into two parts (one for cell surface protein integrin β1 analysis and one for intracellular protein p63 analysis). The cell surface was stained with integrin β1 antibody conjugated to FITC-fluorochrome (Anti-integrin β1 antibody, Abcam, Cambridge, UK) for 30 min on ice. The protein level was detected using a flow cytometer. For detecting p63, cells were treated with 0.1% Surfact-Amps X-100 (Pierce, MA, USA) for membrane permeabilization and incubated on ice for 1 h in the dark. Supernatants were then collected, mixed with the primary antibody (Anti-p63 antibody, abcam, Cambridge, UK), and incubated at the same condition as described in the previous step. After washing with PBS and incubating with the secondary antibody conjugated to the FITC-fluorochrome (ab96871, Goat Anti-Mouse IgG H&L DyLight® 488, Abcam, Cambridge, UK), the samples were measured through by flow cytometry.

Cytokine Profiling – To quantify cytokines, the conditioned medium of the EpSCs in Serum-free DMEM was collected and analyzed using Platinum ELISA (eBioscience, San Diego, CA, USA). After adding the conditioned

medium, the samples were loaded into an antibody-coated microwell and incubated at RT for 2 h. After washing each well six times with washing buffer, biotin-conjugate was then added followed by shaking at RT for 1 h. Next, these samples were washed with PBS and reacted with Streptavidin-HRP Solution at RT for 1 h. After washing with PBS, TMB substrate solution was added and incubated for 30 min. at RT under dark condition. Finally, the reaction was stopped, and the cytokine level in the microwell was determined using a microplate reader.

Western Blot Analysis – Cells were seeded into a 6-well cell culture plate at density of 1×10^5 cells/well. After 24 h of incubation, the medium was replaced with serum-free medium. After incubation for 4 h, the cells were treated with AGP. The cells were then harvested and lysed with lysis buffer (PRO-PREP, iNtRON Biotechnology, Seongnam, Korea). Cell lysates were centrifuged at 16,200 × g for 10 min. to collect the supernatants. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using a transfer kit (iBlot, iBlot gel transfer stacks, Life technologies, MA, USA). The membranes were then blocked with 5% skim milk in 1 × PBS containing 0.1% Tween 20 (PBST) at RT for 1 h and incubated with phospho-Akt (Ser473) antibody (1:1000), Akt (pan) antibody (1:1000), phospho-S6 ribosomal protein (Ser235/236) antibody (1:1000), S6 ribosomal protein antibody (1:1000), phospho-ERK1/2 antibody (1:2000) and ERK1/2 antibody (1:1000) (Cell Signaling Technology, MA, USA) at 4 °C overnight. After washing with PBST, the membranes were incubated with mouse or rabbit IgG secondary antibody (1:1000, Cell Signaling Technology, MA, USA) at RT for 1 h. After washing with PBST, the membranes were developed with chemiluminescent substrate (GloBrite ECL Reagent kit, R&D Systems, Detroit, MI, USA). Detected proteins were normalized by the individual non-phosphate form.

Statistical Analysis – All experiments were conducted at least three times. Data are expressed as the mean ± standard deviation (SD). Data were analyzed by student's t-test. A *P* value of less than 0.05 was considered statistically significant.

Results

AGP Induces the Proliferation of EpSCs – To determine the proliferation capacity of AGP at 1, 10, and 30 μg/ml, we performed the MTT assay. EGF at 10 ng/ml was used as a positive control. Compared to the control, the AGP treatment at 1, 10, 30 μg/ml increased the cell proliferation rate to 142%, 174%, and 165% of the control (100%), respectively (Fig. 1A). We then examined the cytotoxic effect of AGP and its effect on cell cycle by PI staining. The results showed that the S and G_2/M phases were increased after treatment with AGP at a concentration of 1 or 10 μg/ml without showing any cytotoxicity (Fig. 1B). These results indicate that AGP has proliferation effects on EpSCs by inducing the cell cycle progression.

AGP Increases Cytokine Secretion from EpSCs – Cell proliferation is a complex process regulated by many kinds of molecules. To determine what kinds of molecules were involved in AGP-induced cell proliferation, we measured fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), interleukin (IL-6), transforming growth factor beta 1 (TGF-β1), and vascular endothelial growth factor A (VEGF-A) from EpSCs. As shown in figure 2, VEGF-A was found to be dramatically upregulated by the AGP treatment. After treatment with AGP at 1, 10, and 30 μg/ml, VEGF-A was secreted at 250, 273, and 437 pg/ml, respectively, which was significantly more than that of the control. These results suggest that the AGP induced proliferation of EpSCs is related to VEGF-A secretion.

AGP Regulates Integrin β1 and p63 Expression Levels in EpSCs – Integrin β1 and p63 are considered stem cell markers in keratinocytes. The relationship between the stem cell and proliferation capacity has been well defined in many previous studies. Therefore, we investigated whether AGP affects the stemness characteristics of the EpSCs by flow cytometry. Integrin β1 and p63 expression levels in cells treated with AGP were increased compared to those in the control cells. The integrin β1 expression levels in the samples treated with 1 and 10 μg/ml AGP showed a significant increase of up to 46.5% and 56.3%, respectively (Fig. 3A). In the sample treated with 10 μg/ml AGP, the p63 expression level was increased up to 47.7% compared to that in the control (Fig. 3B). Thus, AGP upregulates skin stem cell markers such as integrin β1 and p63 expression.

AGP Activates EpSCs Proliferation by Regulating Phosphorylated ERK1/2, S6 Ribosomal Protein, and Akt – MAPK/ERK and PI3K/Akt/mTOR are major intracellular pathways controlling proliferation related to cancer therapy. To define the signaling pathway inducing prolifera-

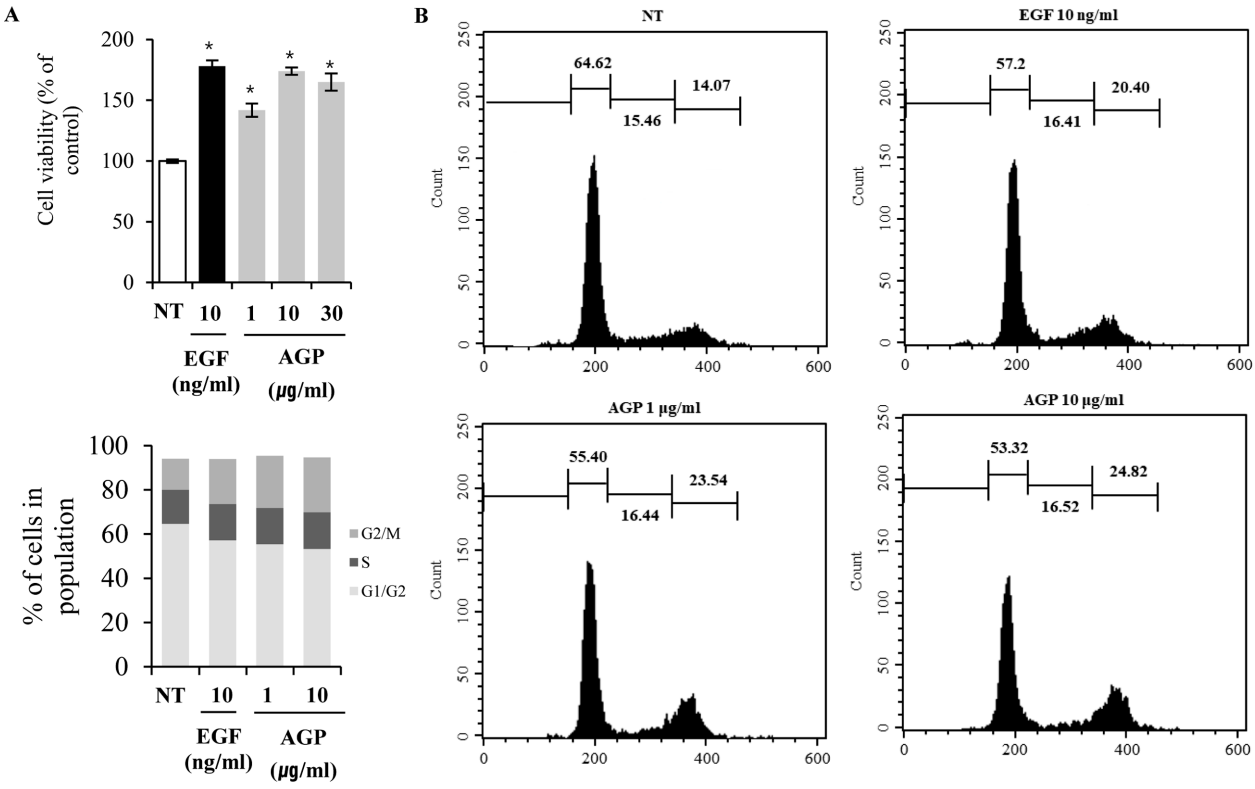

Fig. 1. AGP induced proliferation of EpSCs. (A) EpSCs were cultured with AGP in serum free media for 72 h. and the cell growth rate was detected using MTT. (B) PI staining assays under the same conditions for 48 h. EGF was used as positive controls in proliferation and cell cycle assays, respectively, n=3. The data are presented as the mean ± SD, **P* < 0.05 *vs*. AGP-untreated control

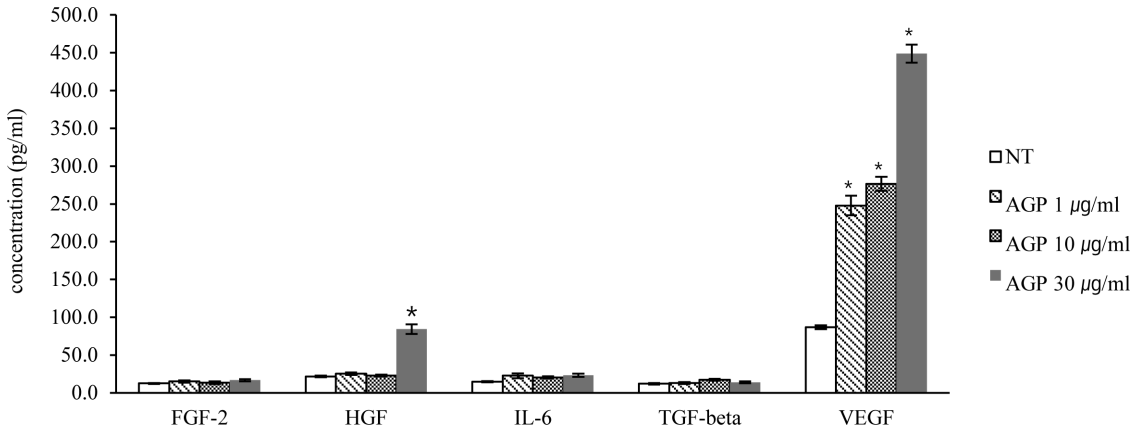

Fig. 2. AGP stimulated cytokine secretion of EpSCs. EpSCs were cultured in serum free condition with AGP for 72 h. The conditioned media were analyzed by Platinum ELISA coated with FGF-2, HGF, IL-6, TGF-beta and VEGF antibodies, and their expression levels were detected by cytometer, n=3. The data are presented as the mean ± SD, **P* < 0.005 vs. AGP-untreated control

tion, we did an immunoblot analysis of the phosphorylation levels of proteins related to these pathways. Our results revealed that ERK1/2, S6 ribosomal protein, and Akt (Ser473) were meaningfully phosphorylated depending on the concentration of AGP (Fig. 4). These results indicate that AGP can increase EpSCs proliferation through the cell growth pathway related to MAPK/ERK and Akt signaling molecules.

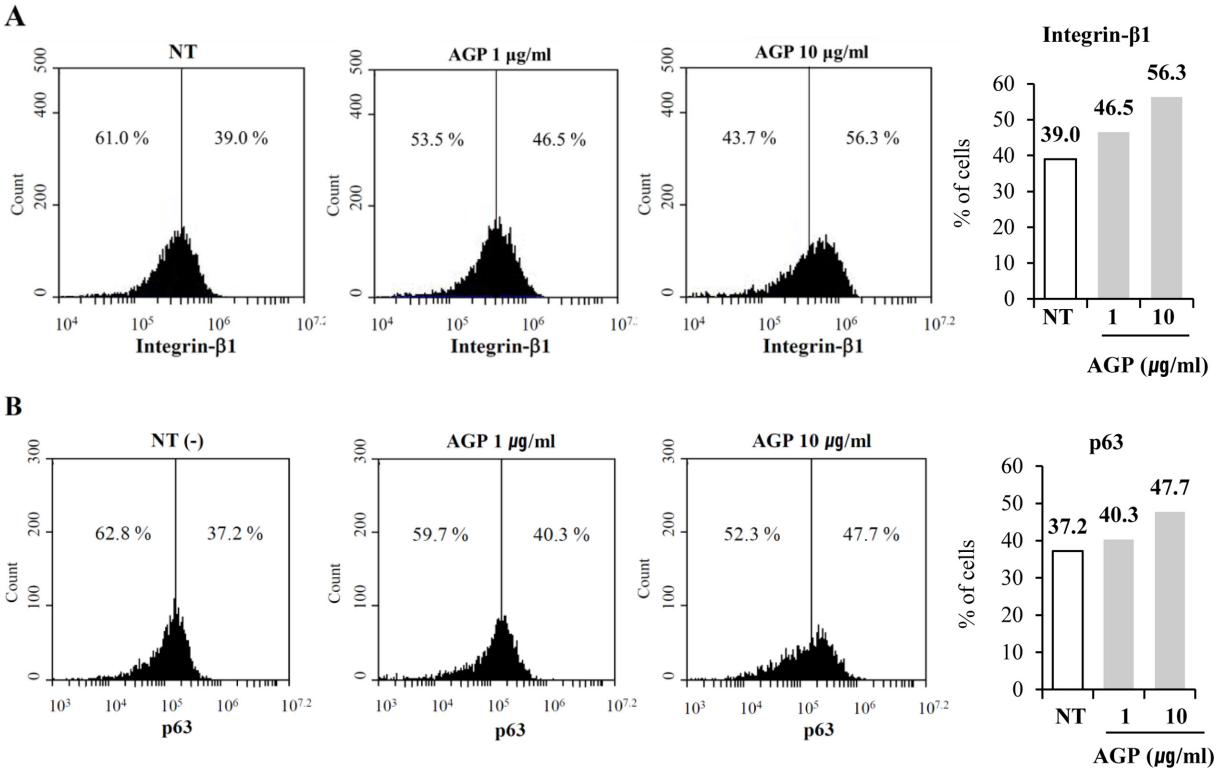

Fig. 3. AGP had a positive effect on integrin β1 and p63 expression in EpSCs. EpSCs were cultured in serum free condition with AGP for 48 h. The EpSCs were collected and divided into two groups of cells. One group was stained with the integrin β1 antibody (A) on the membrane and the other group was permeabilized and detected with the p63 antibody (B), and then, the expression was detected using flow cytometry, n=3

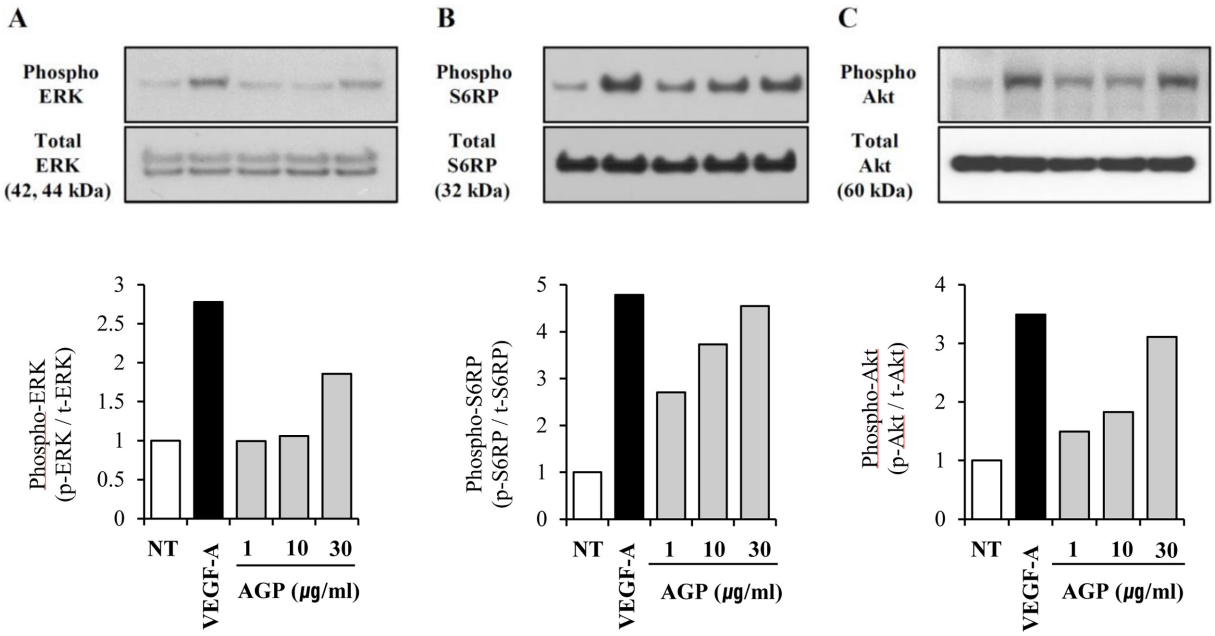

Fig. 4. AGP increased proliferation associated proteins of EpSCs. EpSCs were cultured in a serum free condition with AGP. After cell lysis, the proteins were analyzed with western blot using phospho-S6, phospho-ERK1/2, phospho-Akt(Ser473) and normalized to their total forms of proteins, respectively, n=3

Discussion

Aging is associated with decreased regenerative properties of many tissues, including bone, skin, muscle, brain, and so on. In skin epithelial tissues, homeostasis relies on the self-renewing capacity of stem cells within the basal layer. p63, a p53 family member, is expressed in basal layer. It is an indispensable factor for epithelial morphogenesis and stemness.[17] As transmembrane receptors, integrins are responsible for the attachment of the basal layer of the epidermis to the basement membrane, its underlying substratum.[18] To confirm the involvement of p63 and integrin b1 in AGP-related stemness signaling, the effects of AGP on the levels of cell proliferation and stemness were investigated. AGP treatment significantly increased proliferation by regulating cell cycle. It also upregulated the expression levels of these stemness genes in EpSCs. Our data suggest that the effect of AGP on the proliferation of epithelial stem cells is associated with the subsequent induction of stemness.

VEGF is a signaling protein that promotes the growth of new blood vessels in embryo development as well as wound healing called angiogenesis and vasculogenesis. VEGF is a mitogen for endothelial cells. It stimulates proliferation through VEGFR-induced activation of the RAS/RAF/ERK and PI3K-Akt pathways.[19,20] Recently, it was reported that AGP can inhibit angiogenesis in tumors by inhibiting the VEGF signaling pathway.[21] However, it is currently unknown in what manner AGP acts in normal cell growth conditions. Our study results show that AGP possesses proliferative properties in EpSCs by upregulating VEGF expression through AKT and ERK activation. These results suggest that the regulation of VEGF signaling by AGP has an important role in regulating cell growth between normal and cancer cells.

AGP is a labdane diterpenoid. It is a well-described pharmaceutical component of *Andrographis paniculata*. AGP is known to inhibit oxidative stress with anti-inflammatory and antimicrobial effects.[22,23] A recent study showed that andrographolide sodium bisulfate (ASB), the sodium form of sulfonated AGP, is effective in inhibiting UV-induced skin photoaging.[24] Although AGP and its derivatives have attracted attention because they can improve skin conditions, their relationship with the stemness of epidermal stem cells has not been reported yet. In this study, we propose that the AGP-derived stem cell potency by increased proliferation of EpSCs might be an extracellular factor that improves self-renewal.

Conclusions

Andrographolide, a major component of *Andrographis Paniculata* enhances the proliferation and cell cycle progression of EpSCs. The proliferative properties of andrographolide are regulated through the expression of integrin β1, p63 and VEGF, which is mediated by the activation of ERK1/2, Akt and S6RP signaling. These findings suggest that andrographolide may be used to prevent skin aging by enhancing the self-renewal potency of EpSCs.

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References

1. Lavker, R. M., Zheng, P. S. and Dong, G. (1987) Aged skin: a study by light, transmission electron, and scanning electron microscopy. *J. Invest. Dermatol*. **88**: 44s-51s.
2. El-Domyati, M., Attia, S., Saleh, F., Brown, D., Birk, D. E., Gasparro, F., Ahmad, H. and Uitto, J. (2002) Intrinsic aging vs. photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin. *Exp. Dermatol*. **11**: 398-405.
3. Farage, M. A., Miller, K. W., Elsner, P. and Maibach, H. I. (2008) Intrinsic and extrinsic factors in skin ageing: a review. *Int. J. Cosmet. Sci*. **30**: 87-95.
4. Naylor, E. C., Watson, R. E. and Sherratt, M. J. (2011) Molecular aspects of skin ageing. *Maturitas* **69**: 249-256.
5. Varani, J., Dame, M. K., Rittie, L., Fligiel, S. E., Kang, S., Fisher, G. J. and Voorhees, J. J. (2006) Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. *Am. J. Pathol*. **168**: 1861-1868.
6. Candi, E., Amelio, I., Agostini, M. and Melino, G. (2015) MicroRNAs and p63 in epithelial stemness. *Cell Death Differ* **22**: 12-21.
7. Su, X., Paris, M., Gi, Y. J., Tsai, K. Y., Cho, M. S., Lin, Y. L., Biernaskie, J. A., Sinha, S., Prives, C., Pevny, L. H., Miller, F. D. and Flores, E. R. (2009). TAp63 prevents premature aging by promoting adult stem cell maintenance. *Cell Stem Cell* **5**: 64-75.
8. Sears, R. C. and Nevins, J.R. (2002) Signaling networks that link cell proliferation and cell fate. *J. Biol. Chem*. **277**: 11617-11620.

9. Steelman, L. S., Chappell, W. H., Abrams, S. L., Kempf, R. C., Long, J., Laidler, P., Mijatovic, S., Maksimovic-Ivanic, D., Stivala, F., Mazzarino, M. C., Donia, M., Fagone, P., Malaponte, G., Nicoletti, F., Libra, M., Milella, M.., Tafuri, A., Bonati, A., Bäsecke, J., Cocco, L., Evangelisti, C., Martelli, A. M., Montalto, G., Cervello, M. and McCubrey, J. A. (2011) Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging (Albany NY)* **3**: 192-222.
10. Zhang, W. and Liu, H. T. (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research* **12**: 9-18.
11. You, J., Roh, K. B., Li, Z., Liu, G., Tang, J., Shin, S., Park, D. and Jung, E. (2015) The antiaging properties of *Andrographis paniculata* by activation epidermal cell stemness. *Molecules* **20**: 17557-69.
12. Lim, J. C., Chan, T. K., Ng, D. S., Sagineedu, S. R., Stanslas, J., Wong, W. (2012) Andrographolide and its analogues: versatile bioactive molecules for combating inflammation and cancer. *Clin. Exp. Pharmacol. Physiol.* **39**: 300-10.
13. Karioti, A., Timoteo, P., Bergonzi, M. C., Bilia, A. R. (2017) A validated method for the quality control of *Andrographis paniculata* preparations. *Planta Med.* **83**: 1207-1213
14. Lin, H. C., Li, C.C., Yang, Y. C., Chiu, T. H., Liu, K.L., Li, C. K., Chen, H. W. (2019) *Andrographis paniculata* diterpenoids and ethanolic extract inhibit TNFα-induced ICAM-1 expression in EA.hy926 cells. *Phytomedicine* **52**: 157-167.
15. Shen, Y. C., Chen, C. F. and Chiou, W. F. (2002) Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect. *Br. J. Pharmacol*. **135**: 399-406.
16. Lee, J., Jung, E., Kim, Y. S., Park, D., Toyama, K., Date, A. and Lee, J. (2013) Phloridzin isolated from *Acanthopanax senticosus* promotes proliferation of α6 integrin (CD 49f) and β1 integrin (CD29) enriched for a primary keratinocyte population through the ERK-mediated mTOR pathway. *Arch. Dermatol. Res*. **305**: 747-54.
17. Melino, G., Memmi, E. M., Pelicci, P.G. and Bernassola, F. (2015) Maintaining epithelial stemness with p63. *Sci. Signal.* **8**: re9.
18. Alonso, L. and Fuchs, E. (2003) Stem cells of the skin epithelium. *Proc. Natl. Acad. Sci. USA.* 100 Suppl 1:11830-5.
19. Meadows, K. N., Bryant, P. and Pumiglia, K. (2001) Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. *J. Biol. Chem*. **276**: 49289-98.
20. Rodrigues, M., Griffith, L. G. and Wells, A. (2010) Growth factor regulation of proliferation and survival of multipotential stromal cells. *Stem Cell Res. Ther*. **1**: 32.
21. Shen, K., Ji, L., Lu, B., Xu, C., Gong, C., Morahan, G. and Wang, Z. (2014) Andrographolide inhibits tumor angiogenesis via blocking VEGFA/VEGFR2-MAPKs signaling cascade. *Chem. Biol. Interact*. **218**: 99-106.
22. Chiou, W. F., Lin, J. J. and Chen, C. F. (1998) Andrographolide suppresses the expression of inducible nitric oxide synthase in macrophage and restores the vasoconstriction in rat aorta treated with lipopolysaccharide. *Br. J. Pharmacol*. **125**: 327-334.
23. Shen, Y. C., Chen, C. F. and Chiou, W. F. (2000) Suppression of rat neutrophil reactive oxygen species production and adhesion by the diterpenoid lactone andrographolide. *Planta Med*. **66**: 314-317.
24. Zhan, J. Y., Wang, X. F., Liu, Y. H., Zhang, Z. B., Wang, L. Chen, J. N., Huang, S., Zeng, H. F. and Lai, X. P. (2016) Andrographolide sodium bisulfate prevents UV-induced skin photoaging through inhibiting oxidative stress and inflammation. *Mediators Inflamm*. **2016**: 3271451.

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