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Identification of novel markers for skin senescence associated with 1st ageing peaks of life span

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Introduction

Aging underlies declining skin function and is the predominant risk factor for numerous skin problems [1]. Thus, a deeper understanding of aging is likely to provide insight into the mechanisms of skin diseases and to facilitate the development of novel anti-aging skincare. Recent studies have revealed three significant changes in age-related molecules in the blood over a human lifetime. The results support our group's hypothesis that non-linear age-related changes in the skin will also occur. The first peak of aging proteins is around the fourth decade of life, and the downregulation of proteins related to structures such as the extracellular matrix is mainly observed at this time, which is expected to be a period that is closely associated with skin aging [2]. The aim of this study was to screen for skin aging markers corresponding to the first aging peak and to develop an ingredient that modulates them.

Materials & Methods

Keratinocytes (HaCaT) cells and human primary fibroblast cells (HDFn, C-12300, PromoCell) were used.. HDFn cells were serially passaged from passage 6 through passage 41. Bulbine frutescens extract (BFE), Ganoderma Lucidum (Mushroom) Stem Extract (GLSE), and Crocin (Sigma-Aldrich) were dissolved in PBS.

UVA/ UVB irradiation: HDFn cells were then exposed to UVA/ UVB light (Bio-Sun system, Vilber Lourmat, Inc.) at a total dose of 50 mJ/cm2 (UVA) and 150 mJ/cm2 (UVB).

RT-PCR and Quantitative Real-Time polymerase chain reaction (qRT PCR): RNA extraction and RT-PCR was performed from the cells and the samples were purified following the manufacturer's protocol. RT-PCR product was used for electrophoresis on an agarose gel. Gel pictures were captured by UV transillumination using a GelDoc apparatus (Bio-Print, Vilber-Lourmat, Inc.). Using a SYRB Green Realtime PCR Master Mix (Power SYBRTM Green PCR Master Mix, 4367659, Applied BiosystemsTM) and QuantStudioTM 3 (QuanStudioTM 3 Real-Time PCR instrument, A28132, Thermo Fisher Scientific, Inc.) real-time PCR machine, the gene expression levels were standardized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*).

Gene Knockdown (siRNA Transfection): Human *Fstl3* siRNA and *Mmp12* siRNA (Bioneer Co.) was incubated with Lipofectamine RNAi max in serum-free DMEM for 5 min following the manufacturer's protocol. All cells were supplemented with the siRNA by directly adding it to the cell culture media followed incubation for 24 h.

Measurement of pro-collagen type I generation: The concentration of pro-collagen type I in the cell culture medium was measured using a commercially available ELISA kit (Procollagen type I C-peptide EIA Kit, MK101,TaKaRa Bio, Inc) according to the manufacturer's instructions. Each sample was analyzed in three replicates.

Results & Discussion

1. Age-related gene expression in skin cells

The *Wfdc2, Fstl3, Ptn, Mmp12, Gdf15* and *Arfip2* genes were expressed in the HDFn and HaCaT cells. The *Svep1, Ccdc80, Smoc1, Ptgds, Scarf2* and Msmp genes were expressed only in the HDFn cells. The *Sost* gene was expressed only in the HaCaT cells. The *Chrd1, Rspo4, Nppb, Ephb6, Scg3, Serpine2, Omd, Chad, Col11A2* and *Ret* genes were not detected in both cell types.

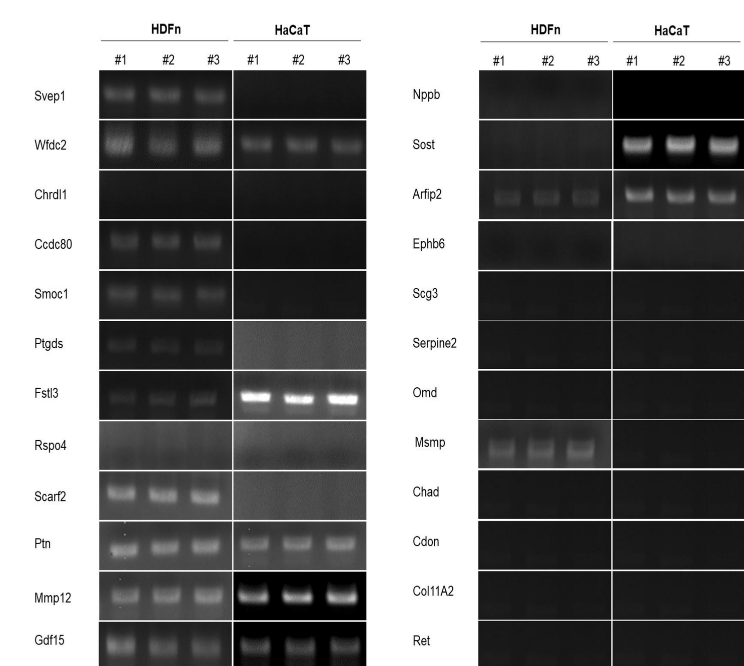


Fig 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the age-related gene expression observed in the HDFn and HaCaT cells.

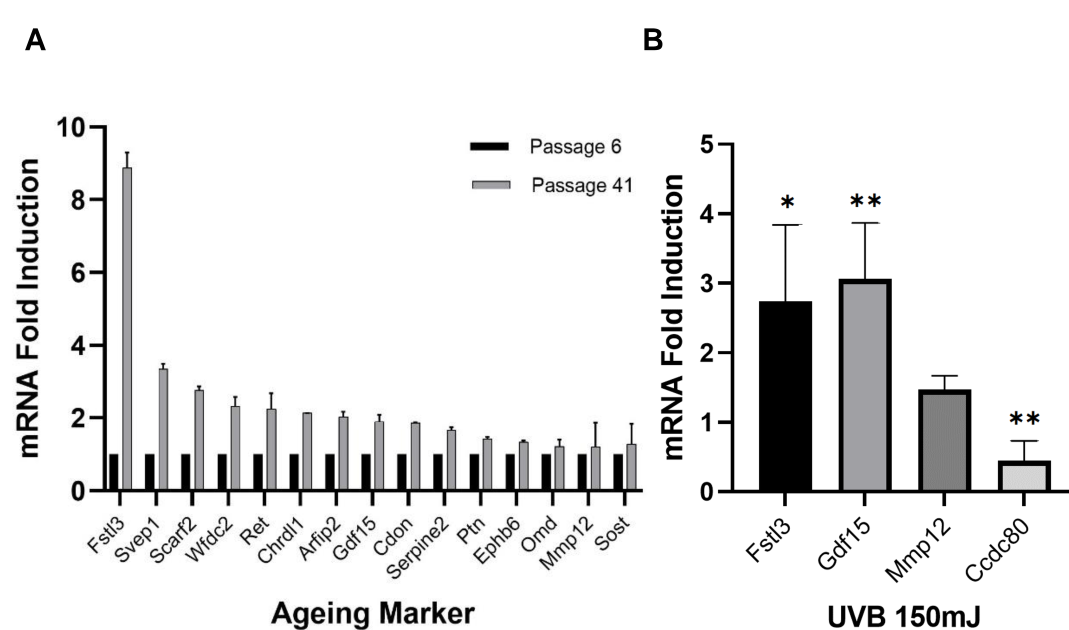


Fig2. Changes in age-related gene expression in HDFn cells (A) passage 6 vs. passage 41 (B) gene expression in UVB-irradiated cells (all changes in expression are relative to the control without any treatment). Data are shown as the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control.

2. Age-related gene expression changes in the HDFn cells

In senescent cells (passage 41), the expression of the *Fstl3* gene was significantly increased in the senescent cells compared to the young cells (passage 6). The *Svep1, Scarf2, Srfip2, Gdf15, Ptn,* and *Mmp12* genes were increased in senescent cells, too. In contrast, gene expression of Ccdc80, Msmp, Ptgds, Smoc1 was attenuated in the senescent cells (Fig2.A). It was evaluated whether the same gene expression change pattern appeared in the UVB-applied model as in the senescent cells. The results indicate that the expression of the *Fstl3, Gdf15* and *Mmp12* genes was increased, and the expression of the *Ccdc80* gene was decreased in the UVB irradiated cells (Fig2.B). Thus, the UVB-applied model is suitable for observing age-related gene expression.

Results & Discussion

3. Effects of the skincare ingredients on age-related gene expression

The three components used were Bulbine frutescens extract (BFE), Ganoderma Lucidum (Mushroom) Stem Extract (GLSE), and Crocin. *Col1A1* and *Eln* gene expression levels and collagen synthesis decreased when the *Fstl3* gene expression was decreased. However, even when the *Fstl3* gene expression was increased by UVB irradiation, *Col1A1* gene expression and collagen synthesis were decreased. Even when UVB-induced Fstl3 gene expression was suppressed using siRNA, the *Col1A1* and *Eln* mRNA levels and the production of collagen proteins were also reduced (Fig.4A, B, C)

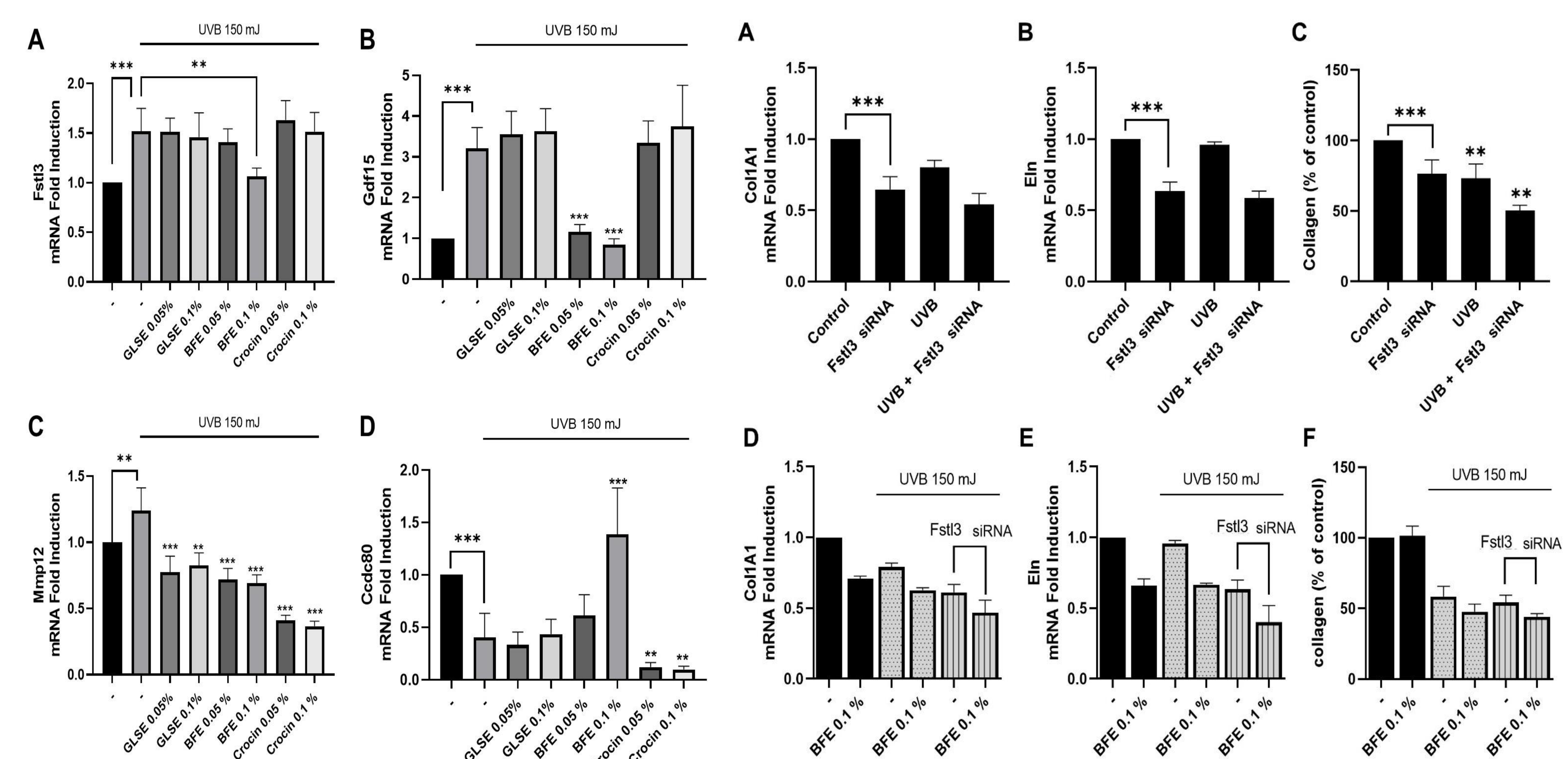


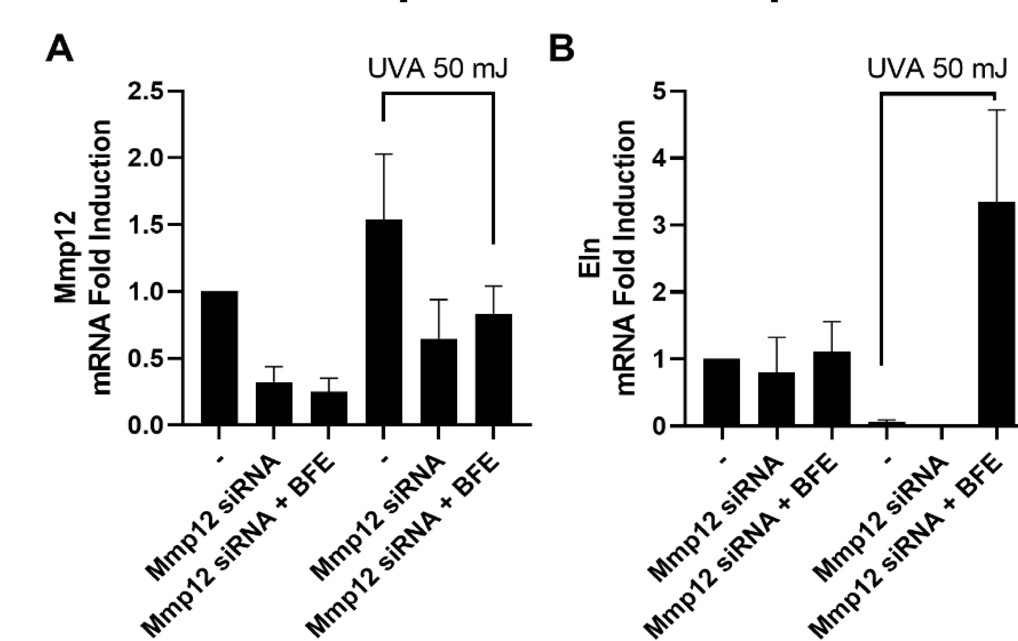
Fig3. Effects of the skincare ingredients on age-related gene expression (A) *Fstl3* gene (B) *Gdf15* gene (C) *Mmp12* gene (D) *Ccdc80* gene. Data are shown as the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to the control.

Fig4. Effects of *Fstl3* on Collagen and Elastin expression. Expression of (A) *Col1A1* gene (B) *Eln* gene and (C) Collagen synthesis level in the *Fstl3* Knockdown condition of the UVB applied model. Effects of BFE on (D) *Col1A1* gene (E) *Eln* gene and (F) Collagen synthesis level in the *Fstl3* Knockdown condition of the UVB applied model. Data are shown as the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 compared to the control

4. Effects of *Fstl3* on Collagen and Elastin expression

Col1A1 and *Eln* gene expression levels and collagen synthesis decreased when the *Fstl3* gene expression was decreased. However, even when the *Fstl3* gene expression was increased by UVB irradiation, *Col1A1* gene expression and collagen synthesis were decreased. Even when UVB-induced *Fstl3* gene expression was suppressed using siRNA, the *Col1A1* and *Eln* mRNA levels and the production of collagen proteins were also reduced (Fig.4A, B, C). BFE not only reduces the *Fstl3* gene expression but also suppresses *Col1A1* and *Eln* gene expression, and this effect is stronger under the *Fstl3* Knockdown conditions (Fig.4D, E). When the synthesis of collagen was evaluated, there was no change in the collagen synthesis when the cells were only treated with BFE, but when the cells were treated with BFE under UVB irradiation conditions, the collagen production decreased. Even when siRNA was used to knockdown *Fstl3*, there was no difference between time and the result (Fig.4F).

5. Effects of *Mmp1* on Elastin expression



The *Eln* gene expression levels increased when the *Mmp12* gene expression was decreased. And, when the cells were treated with BFE, the *Eln* gene expression increased. Under the UVA irradiation condition, the expression of *Mmp12* expression increased and the expression expression of *Eln* expression decreased. However, the expression of *Eln* gene was not increased in *Mmp12* knockdown condition, but, treatment with BFE dramatically increased the expression of *Eln* expression (Fig5.B).

Fig5. Effects of *Mmp12* on Elastin expression. Expression of (A) *Mmp12* gene (B) *Eln* gene in the *Mmp12* Knockdown condition of the UVA applied model. Data are shown as the mean ± S.D. of three experiments.

Conclusions

Skin aging is the most important field in cosmetics. In this study, we screened for skin aging markers associated with aging peaks in the human life span and discovered their functions to develop new targets for the development of new anti-aging technologies and screening systems for the development of new anti-aging ingredients

References

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2. Lehallier B, Gate D, Schaum N, Nanasi T, Lee SE, Yousef H, Moran Losada P, Berdnik D, Keller A, Verghese J, Sathyan S, Franceschi C, Milman S, Barzilai N & Wyss-Coray T (2019) Undulating changes in human plasma proteome profiles across the lifespan. Nat Med 25: 1843-1850. doi:10.1038/s41591-019-0673-2