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Sophora japonica extracts accelerates keratinocyte differentiation through miR-181a

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Abstract

Background: The *Sophora japonica* extracts contain flavonol triglycoside, isoflavonol, coumarone chromone, saponin, triterpene glucoside, phospholipids, alkaloids, amino acids, polysaccharides, and fatty acids. These components have physiological effects such as anti-infertility and anti-cancer activities. This study investigated the regulation of keratinocyte differentiation upon treatment with the *S. japonica* extracts in keratinocyte and the molecular cell biological mechanism involved.

Methods: To determine whether the *S. japonica* extracts or troxerutin, which is its main component, regulates keratinocyte differentiation, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on keratinocyte differentiation markers such as keratin 1 (K1), keratin 10 (K10), involucrin, and filaggrin after treatment with the *S. japonica* extracts. miR-181a knockdown confirmed that keratinocyte differentiation was regulated by increased miR-181a expression upon treatment with the *S. japonica* extracts or troxerutin.

Results: The expression of keratinocyte differentiation markers such as K1, K10, involucrin, and filaggrin increased upon treatment with the *S. japonica* extracts and troxerutin. Furthermore, miR-181a expression, which is known to increase during keratinocyte differentiation, increased upon treatment with the *S. japonica* extracts and troxerutin. When miR-181a was knocked down, the increased expression of keratinocyte differentiation markers upon treatment with the *S. japonica* extracts and troxerutin decreased again. Finally, it was confirmed that miR-181a directly regulated and reduced the expression of Notch2, which reduces keratinocyte differentiation, and that the decrease in Notch2 expression by miR-181a regulated keratinocyte differentiation.

Conclusions: These results suggest that the *S. japonica* extracts or troxerutin accelerates keratinocyte differentiation through miR-181a. This accelerated keratinocyte differentiation was confirmed to have resulted from the regulation of Notch2 expression by miR-181a. The results of this study provide an opportunity to confirm the molecular cell biological mechanism of *S. japonica* extracts or troxerutin on skin keratinization, and we expected that this study contribute to develop a moisturizing cosmetic material that can strengthen the skin barrier through regulating keratinocyte differentiation.

Keywords: miR-181a, microRNA, Keratinocyte differentiation, *Sophora japonica*, Troxerutin

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Background

The skin plays a role in protecting the body from external stimuli such as microbes and ultraviolet radiation (Chuong et al., 2002). These skin defense functions are mainly functions of the epidermis composed of keratinocytes and melanocytes. Among these cells, keratinocytes construct complicated structure by keratinocyte differentiation (Kalinin et al., 2002; Rice & Green, 1977). Basal layer keratinocyte was differentiated to corneocytes that exist in the outer layer of the epidermis, resulting in gene expression regulating differentiation, cell cycle, and cell morphology. Among these genes, transglutaminases 1 and 3, involucrin, cornifin, loricrin, filaggrin, and small proline-rich protein (SPR) are specifically regulated by keratinocyte differentiation (Fuchs, 1993; Steinert & Marekov, 1995). Factors affecting keratinocyte differentiation are known as calcium, retinoic acid, vitamin D, and 12-o-tetradecanoylphorbol-13-acetate (TPA). Among these, calcium is well known for promoting keratinocyte differentiation, which is known to increase the density of the upper epidermis (Menon et al., 1985; Hennings et al., 1980; Yuspa et al., 1989).

When keratinocyte differentiation proceeds, transglutaminase binds structural proteins, such as involucrin, cornifin, loricrin, and SPR, inside the cell membrane to form a cornified cell envelope, which is a solid structure that does not dissolve in water (Candi et al., 2005; Ishida-Yamamoto & Iizuka, 1998). When abnormal keratinocyte differentiation occurs, the skin barrier becomes defective, which causes dry skin, resulting in chronic skin diseases such as atopy. Therefore, many attempts have been made to control keratinocyte differentiation using chemical compounds or the extracts (Tsuchisaka et al., 2014).

Sophora japonica flowers and blooms are well-known traditional Chinese medicinal herbs (Ma & Lou, 2006; Lo et al., 2009) and have anti-infertility and anti-cancer effects (Ma & Lou, 2006; Wang et al., 2001). *S. japonica* components include flavonol triglycoside, isoflavonol, coumarone chromone, saponin, triterpene glucoside, phospholipids, alkaloids, amino acids, polysaccharides, and fatty acids (Grupp et al., 2001). The *S. japonica* extracts are commonly used to treat bleeding-related disorders such as hematochezia, hemorrhoidal bleeding, and uterine bleeding as well as diarrhea (Zhao, 2004). However, the effects of the *S. japonica* extracts on keratinocytes have yet to be extensively studied; therefore, this study was conducted to confirm that the *S. japonica* extracts regulate keratinocyte differentiation and to investigate the molecular cell biological mechanism involved.

Methods

Cell line

HaCaT cells were cultured in Dulbecco' s modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, USA) added with 1% penicillin/streptomycin (P/S; Gibco) and 10% fetal bovine serum (FBS; Gibco, USA) at 37 °C, 5% CO_2 incubator.

Cytotoxicity

Cytotoxicity was determined by EZ-cytox enhanced cell viability kit (Daeil Lab, Korea) based on water-soluble tetrazolium salt (WST). 5×10^4 cells/well and 1×10^5 cells/well HaCaT were plated in each 96-well plate and 24-well plate. And then the cells were incubated for 24 h. After 24 h, the cells were then treated at indicated concentrations of *Sophora japonica* extracts. At 24 h, EZ-cytox enhanced cell viability was added in each well. The cells were incubated for 1 h, and then absorbance was measured at 450 nm using a spectrophotometer (Bio-Rad, USA).

RNA isolation and qRT-PCR (quantitative real-time polymerase chain reaction)

RNA was isolated from HaCaT using the Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA quality was assessed with nanodrop spectrophotometry (Maestrogen, Taiwan). cDNA was prepared using the M-MLV reverse transcriptase (Enzynomics, Korea) according to the manufacturer's instructions. All qPCR analyses were performed on a Linegene K (BioER, China) with HOT FIREPol EvaGreen PCR Mix Plus (Solis BioDyne). Sequences of forward and reverse primers used in real-time PCR reactions are listed in Table 1.

miRNA transfection

For miR-181a and Anti-miR-340 (Bioneer, Korea) transfection, HaCaT cells were plated into 60-mm culture plates and allowed to grow overnight. Then the cells were transiently transfected with negative scramble miRNA or miR-181a or Anti-miR-340 for 48 h using RNAi max (Invitrogen, USA) according to the manufacturer's instructions.

Table 1 The primers used in qRT-PCR

Gene	Primer sequence
K1	F: 5′-CAGCATCATTGCTGAGGTCAAGG-3′ R: 5′-CATGTCTGCCAGCAGTGATCTG-3′
K10	F: 5′-TGGTTCAATGAAAAGAGCAAGGA-3′ R: 5′-GGGATTGTTTCAAGGCCAGTT-3′
Involucrin	F: 5′-CAAAGAACCTGGAGCAGGAG-3′ R: 5′-CAGGGCTGGTTGAATGTCTT-3′
Filaggrin	F: 5′-GCAAGGTCAAGTCCAGGAGAA-3′ R: 5′-CCCTCGGTTTCCACTGTCTC-3′
β: 5′-C	F: 5′-GGATTCCTATGTGGGCGACGA-3′ R: 5′-CGCTCGGTGAGGATCTTCATG-3′

Statistical analyses

Statistical comparisons were performed with Microsoft Excel (Microsoft, USA), using Student's *t* test for two data sets.

Results

S. japonica extracts regulated keratinocyte differentiation in HaCaT cells

The WST assay was performed to determine the cytotoxicity of the *S. japonica* extracts and troxerutin, which is its main component, to keratinocytes and to determine the effective concentration range for experiments. According to the measurement of cytotoxicity on HaCaT cells, the viability of these cells was found to be 90% or more at an *S. japonica* extract concentration of 200 μg/mL or less and over 90% at a troxerutin concentration of 100 μM (data not shown). To evaluate whether the *S. japonica* extracts regulate keratinocyte differentiation, mRNA levels of keratinocyte differentiation markers, such as keratin 1 (K1), keratin 10 (K10), involucrin, and filaggrin, were measured using keratinocytes. According to the mRNA levels of K1, K10, involucrin, and filaggrin measured from collected cells treated with 200 μg/mL of the *S. japonica* extract, the increased expression of keratinocyte differentiation markers due to calcium was further increased upon treatment with the *S. japonica* extracts (Fig. 1). In addition, troxerutin, a representative component of the *S. japonica* extract, further increased the expression of keratinocyte differentiation markers increased by calcium (Fig. 2).

S. japonica extracts regulated miR-181a expression in HaCaT cells

To determine whether keratinocyte differentiation upon treatment with the *S. japonica* extracts is regulated through miR-181a, the change in the expression of miR-181a associated with treatment with these extracts was measured. The results showed that the expression of miR-181a increased upon treatment with the *S. japonica* extracts (Fig. 3a). Additionally, the expression of miR-181a increased upon treatment with troxerutin (Fig. 3b).

Troxerutin, a major ingredient of *S. japonica* extracts, regulated keratinocyte differentiation and miR-181a expression in HaCaT cells

Because the expression of miR-181a, which is known to be closely related to keratinocyte differentiation due to its increased expression in a keratinocyte differentiation model, is regulated upon treatment with the *S. japonica* extracts and troxerutin (Hildebrand et al., 2011), this study specifically investigated whether keratinocyte differentiation regulated by treatment with the *S. japonica* extracts and troxerutin occurs through miR-181a. To achieve this, anti-miR-181a was used with the aim of

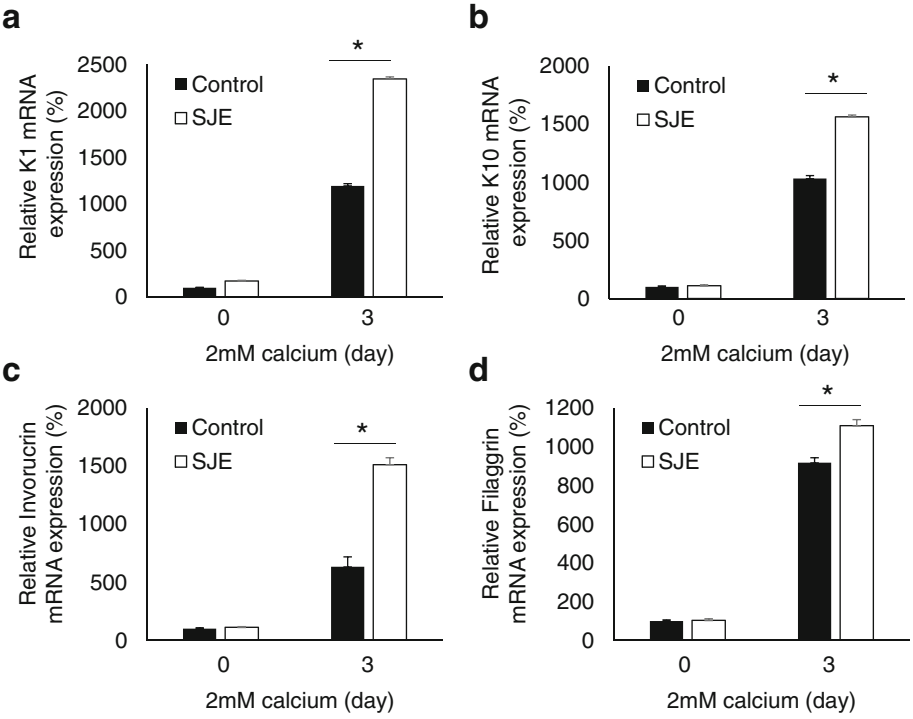

Fig. 1 *S. japonica* extracts regulated marker of keratinocyte differentiation in HaCaT cells. **a** *K1* mRNA expression. **b** *K10* mRNA expression. **c** *Involucrin* mRNA expression. **d** *Filaggrin* mRNA expression. SJE is *S. japonica* extracts. *<0.05

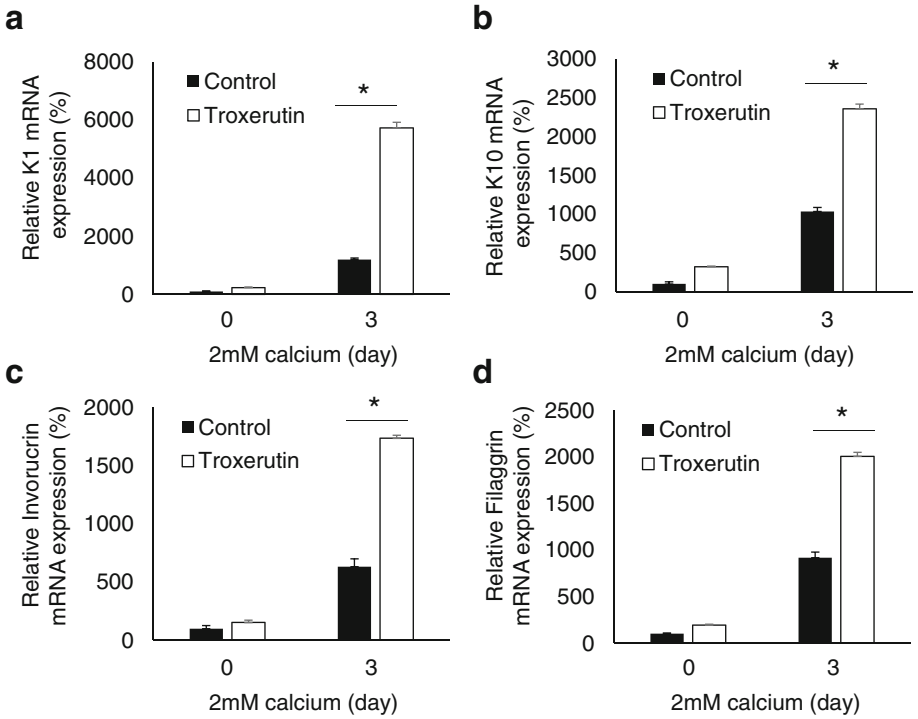

Fig. 2 Troxerutin regulated marker of keratinocyte differentiation in HaCaT cells. **a** *K1* mRNA expression. **b** *K10* mRNA expression. **c** *Involucrin* mRNA expression. **d** *Filaggrin* mRNA expression. *<0.05

inhibiting the acceleration of keratinocyte differentiation by the *S. japonica* extracts and troxerutin. The experimental results showed that keratinocyte differentiation was regulated upon treatment with the *S. japonica* extracts and troxerutin through miR-181a (Fig. 4).

Discussion

The stratum corneum is formed by keratinocytes in the epidermis and maintains the moisture of the epidermis and blocks external pollutants. The differentiation of keratinocytes can be easily confirmed using keratinocyte differentiation markers such as K1, K10, involucrin, and filaggrin.

The skin is the primary barrier to the external environment and plays a major role in protecting individuals. Keratinocytes are the main cells that constitute the epidermis of the skin and play a role in forming the skin barrier through division and differentiation. In case of skin diseases such as atopic dermatitis, a normal skin barrier is not produced and abnormal keratinocyte differentiation occurs; therefore, in such diseases, keratinocyte differentiation can be promoted to aid in the normal formation of the skin barrier (Leung et al., 2007).

The *S. japonica* extracts contain flavonol triglycoside, isoflavonol, coumarone chromone, saponin, triterpene glucoside, phospholipids, alkaloids, amino acids, polysaccharides, and fatty acids. These components have

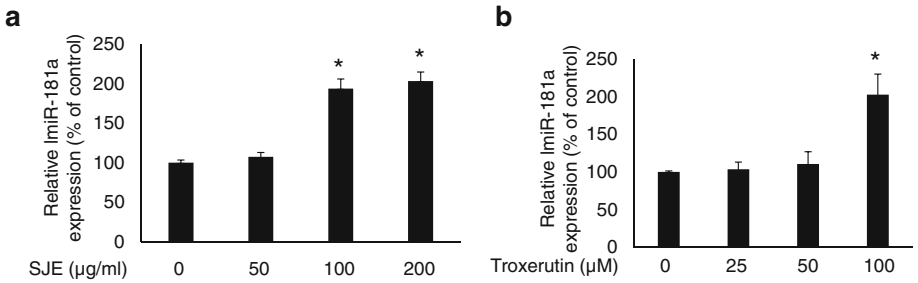

Fig. 3 *S. japonica* extracts and troxerutin regulated miR-181a expression in HaCaT cells. **a** *S. japonica* extract-mediated miRNA-181a expression change. SJE is *S. japonica* extracts. **b** Troxerutin-mediated miRNA-181a expression change. *< 0.05

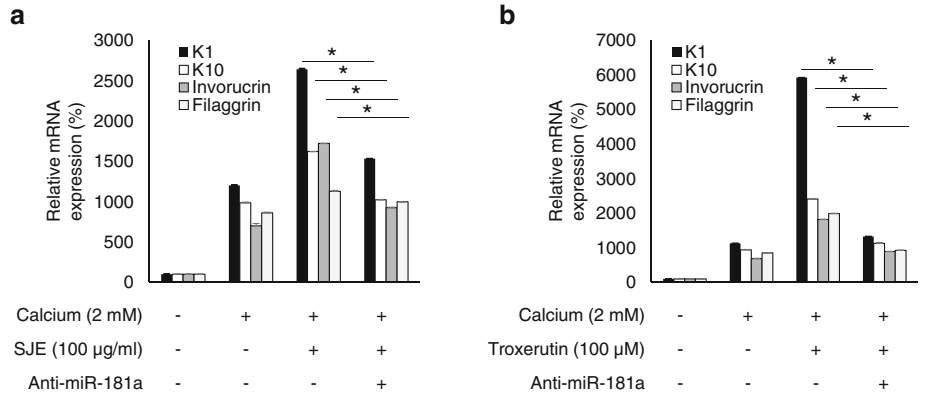

Fig. 4 *S. japonica* extracts and troxerutin regulated the marker of keratinocyte differentiation through miR-181a in HaCaT cells. **a** Anti-miRNA-181a effect to expression of keratinocyte differentiation markers in *S. japonica* extract-treated HaCaT cells. SJE is *S. japonica* extracts. **b** Anti-miRNA-181a effect to expression of keratinocyte differentiation markers in troxerutin-treated HaCaT cells. *< 0.05 compared with each calcium and *S. japonica* extracts or troxerutin-treated condition

physiological effects such as anti-infertility and anti-cancer activities. This study investigated the regulation of keratinocyte differentiation upon treatment with the *S. japonica* extracts and the molecular cell biological mechanism involved.

The expression of early differentiation markers such as K1, K10, and involucrin significantly increased upon treatment of cultured keratinocytes with the *S. japonica* extracts and troxerutin. In addition, the expression of late differentiation markers such filaggrin and loricrin significantly increased (Figs. 1 and 2). These results suggest that the *S. japonica* extracts and troxerutin activate the early and late signal transduction systems of keratinocyte differentiation. It is well known that keratinocyte differentiation plays an important role in activating the intracellular signal transduction system by delivering external stimuli. For example, keratinocyte differentiation-promoting factors, such as calcium, are known to activate protein kinase C and affect downstream signaling systems (Matsui et al., 1992; Dlugosz & Yuspa, 1993; Bollag et al., 1993; O'Driscoll et al., 1994). The activation of mitogen-activated protein kinases, such as ERK1/2 and p38, has also been shown to play a pivotal role in keratinocyte differentiation (Efimova et al., 2003; Eckert et al., 2002). These intracellular signal transduction activities increase the gene expression of keratinocyte differentiation markers by activating transcriptional regulatory factors such as AP-1, SP-1, and Ets (Nakamura et al., 2007; Jang & Steinert, 2002; Lee et al., 1996).

In the present study, the expression levels of early and late differentiation markers increased upon treatment with the *S. japonica* extracts and troxerutin; therefore, this study suggested that the effect of promoting keratinocyte differentiation was more related to the intracellular signal transduction system than to the increase in expression of a specific differentiation marker gene. In the future, it will be necessary to study the effect of colostrum on the intracellular signal transduction system of keratinocytes.

Conclusions

In this study, keratinocyte differentiation markers were identified to investigate the possibility of the *S. japonica* extracts and troxerutin to be regulators of keratinocyte differentiation. The expression of keratinocyte differentiation markers K1, K10, involucrin, and filaggrin increased upon treatment with the *S. japonica* extracts and troxerutin. Therefore, the *S. japonica* extracts and troxerutin showed keratinocyte differentiation-regulating effects. In addition, the *S. japonica* extracts and troxerutin regulated miR-181a, which targets the keratinocyte differentiation regulator Notch2, thereby regulating keratinocyte differentiation. Through such regulation of keratinocyte differentiation, it is possible to control the skin barrier formed by keratinocyte differentiation in the epidermal layer, enhancing the moisturizing ability of the skin. Therefore, this study confirmed that the *S. japonica* extracts and troxerutin strengthen the skin barrier of the epidermis and enhance the moisturizing ability of the skin.

Abbreviations

DMEM: Dulbecco' s modified Eagle's medium; K1: Keratin 1; K10: Keratin 10; qRT-PCR: Quantitative real-time polymerase chain reaction; *S. japonica*: *Sophora japonica*; SPR: Proline-rich protein; TPA: 12-o-Tetradecanoylphorbol-13-acetate; WST: Water-soluble tetrazolium salt

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Availability of data and materials

Not applicable

Authors' contributions
KK, HJC, DJ, and SJC performed the experiments. ISA and SA were involved in experimental design and advising. KK and HJC analyzed data and wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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