IL-21-inducible TRPV6 is reduced in psoriatic skin

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IL-21-inducible TRPV6 is reduced in psoriatic skin

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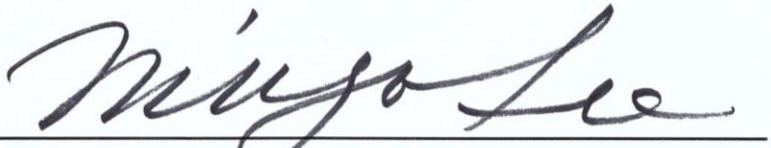
This certifies that the Master's Thesis of

Sung Hee Kim is approved.



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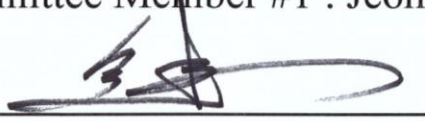

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ABSTRACT

IL-21-inducible TRPV6 is reduced in psoriatic skin

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Psoriasis is characterized by infiltration of inflammatory cells, hyperproliferation of epidermal cells and incomplete keratinocytes (KCs) differentiation (parakeratosis), impaired calcium gradient, and decreased skin barrier function. Interleukin-21 (IL-21), produced predominantly by multiple effector $CD4^+$ T cell types and natural killer T cells, has pleiotropic actions such as induction of KC proliferation. In psoriasis, IL-21 mRNA and protein are highly expressed in the skin and peripheral blood mononuclear cells. IL-21 stimulates proliferation of both normal and psoriatic human KCs. So, a microarray analysis was performed in human KCs treated with IL-21 to find psoriasis-specific molecules, and transient receptor potential vanilloid 6 (TRPV6) was one of the increased genes.

TRPV6 is a highly calcium-selective channel and its expression is mainly confined

to epithelial tissue of different organs such as kidney, testis, digestive tract, ovary and skin. Because psoriatic suprabasal layers display higher calcium concentration than normal skin, highly calcium-selective TRPV6 channel may be related to psoriasis. Moreover, TRPV6 is known to be important for the differentiation of KCs and the proliferation of epithelial cells. Therefore, TRPV6 could involve the defective differentiation and proliferation of psoriatic KCs. Although TRPV6 is known to be important in calcium-induced KC differentiation, its physiological function and mechanisms in the activation of KCs are unclear.

Therefore, this study was investigated to determine whether TRPV6 plays a crucial role in psoriatic KCs.

First, the differentiated model of KCs was established with treatment high calcium (1.2 mM). The RNA and protein levels of differentiation markers and TRPV6 were increased in accordance with differentiation of KCs, detected by real-time PCR and Western blot. However, immunohistochemical staining and real time PCR demonstrated that the mRNA and protein expressions of TRPV6 were decreased in psoriatic skin, suggesting that the decrease of TRPV6 might be related to reduced differentiation of KCs in psoriasis. To evaluate the role of TRPV6 on the proliferation of KCs in psoriasis, the effect of aTRPV6 inhibitor on the proliferation of KCs was evaluated by MTT assay. Contrary to the expectation, the reduced TRPV6 function in HaCaT cells caused by a TRPV6 inhibitor, $LaCl_3$, did not significantly enhance the

proliferation of KCs. In addition, the co-treatment of IL-21 and $LaCl_3$ did not affect the proliferation of KCs. The effect of $LaCl_3$ *in vitro* could be different from the condition of decreased expression of TRPV6 in psoriatic KCs. Besides, the psoriatic KCs might not be fully reflected by the HaCaT KC cell line.

In conclusion, TRPV6 was decreased in psoriatic epidermis, which might play a role in reduced differentiation of psoriatic KCs. However, the inhibition of TRPV6 function did not affect the proliferation of KC cells. Therefore, the role of TRPV6 in psoriatic KCs needs further elucidation.

Key words : psoriasis, IL-21, TRPV6, keratinocytes, differentiation, proliferation

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I. Introduction

Psoriasis is a chronic inflammatory skin disorder affecting approximately 1~3% of the population. Patients with psoriasis frequently have the psychological depressive disorder and decreased quality of life[1,2]. Histologically, psoriasis is characterized by inflammatory cell infiltration, hyperproliferation of epidermal cells, and incomplete differentiation of keratinocytes (parakeratosis)[3]. Although the pathogenic mechanism of psoriasis remains largely unknown, crosstalk between immune cells and keratinocytes (KCs), mediated by various cytokines and chemokines, has been thought to be the major pathologic process in psoriasis[4].

Psoriasis causes defective differentiation and proliferation of epidermal KCs and decreased skin barrier function[5-7]. Psoriatic basal layers contain less extracellular

calcium, favoring exaggerated proliferation. On the other hand, psoriatic suprabasal layers display higher calcium concentration than normal skin, indicating loss of the normal calcium gradient might result in impaired differentiation and proliferation in psoriasis. In spite of high calcium concentration in upper epidermis, the differentiation markers (e.g. involucrin and cytokeratin 10) of KCs are decreased in psoriatic skin compared to normal epidermis[8,9].

Interleukin-21 (IL-21), produced predominantly by multiple effector $CD4^+$ T cell types and natural killer T cells, is a member of the common-γ chain family of cytokines which includes IL-2, IL-4, IL-7, IL-9, and IL-15.[10] It has pleiotropic actions such as induction of KC proliferation, stimulation of natural killer and $CD8^+$ T cells cytotoxicity, and stimulation of B cell differentiation into plasma cells.[11] In psoriasis, IL-21 mRNA and protein levels are increased in the skin and peripheral blood mononuclear cells. IL-21 receptor (IL-21R) is also expressed in KCs isolated from psoriasis[12]. IL-21 stimulates proliferation of both normal and psoriatic human KCs. When IL-21 is intradermally injected into mice, epidermal hyperplasia is accompanied by infiltration of the epidermis and dermis with inflammatory cells.[12] Despite many reports about the role of IL-21 on various cells, the effect of IL-21 on KCs still remains to be elucidated. A microarray analysis was performed to identify psoriasis-specific molecules in human KCs treated with IL-21. The expression of transient receptor potential vanilloid 6 (TRPV6) was found to be elevated as a

candidate of psoriasis-specific molecules. TRPV6 is a subfamily of transient receptor potential (TRP) channels, including TRPA, TRPC, TRPM, TRPML, TRPN, TRPP, and TRPV. TRP channels may be generally described as calcium-permeable cation channels and widely expressed in a large number of different tissues and cell types[13,14]. TRPs are involved in various cutaneous functions, including KCs terminal differentiation, apoptosis and melanocyte pigmentation. They have also been used as pharmacological targets in many inflammatory skin diseases, such as psoriasis, hair disorders, chronic itch and skin cancer[15]. TRPV channels can be divided into the TRPV1-V4 group and TRPV5-V6 group. It is notable that among all TRP channels, TRPV5 and TRPV6 are highly calcium-selective channels and share more than 70% amino acid identity[13]. The TRPV6 expression is mainly confined to the epithelial tissue of different organs like kidney, testis, digestive tract, ovary and skin. Because of its high calcium selectivity over other TRP channels, TRPV6 is shown to participate in regulation of calcium homeostasis in the body[16]. Particularly, TRPV6 is a molecular candidate for the apical calcium entry pathway[15,17]. Moreover, TRPV6 regulates IGF-induced PI3K-PDK1-Akt signaling which results in abnormal epithelial cell proliferation in low extracellular calcium environment[18]. Regarding other TRP channels, reduced TRPC channel expression in psoriatic KCs was reported to be associated with impaired differentiation and enhanced proliferation of KCs[6,19]. So far, there has been no report on the roles of TRPV6 affecting psoriatic KCs, and its

physiological function and mechanisms of activation are unclear. In this study, I evaluated whether TRPV6 was involved in differentiation and proliferation of psoriatic KCs.

II. MATERIALS AND METHODS

1. Microarray analysis and establishment of KCs differentiation model

A. KCs culture

KCs (Modern Cell & Tissue Technologies, Seoul, Korea) were cultured in keratinocyte growth medium (KGM) (LONZA, Walkersville, MD, USA) supplemented with IGF-1, bovine pituitary extract, epidermal growth factor(EGF), hydrocortisone, gentamycin and amphotericin B (LONZA) at 37^{o}C under 5% CO_2 incubator. KCs at the third to fourth passage were used for experiments. Human immortalized KCs (HaCaT) were grown in Dulbecco's modified Eagle's medium (DMEM) (LONZA) supplemented with 10% fetal bovine serum and 1% antibiotics at 37^{o}C under 5% CO_2 incubator. When KCs were grown to 70-80% confluency, cells were seeded in multi plates for experiments.

B. Microarray analysis

Microarray data analyzed in this thesis were published previously[20]. For the micro-array, KCs were grown to 70-80% confluency and treated with or without 100 ng/ml human recombinant IL-21 (PeproTECH. Rocky Hill, NJ, USA) for 24 hrs. Total RNA was extracted using Trizol reagent[21] (Ambion, Carlsbad, CA, USA) in accordance with manufacturer's protocol. RNA quality was evaluated with Agilent's 2100 Bioanalyzer

(Agilent Technologies, Santa Clara, CA, USA), and RNA was labeled using Agilent's Low RNA Input Linear Amplification kit PLUS. Amplified RNA was hybridized using Agilent's Gene Expression Hybridization kit on the Agilent Human GE 4 X 44K (V2). Data analysis was conducted by using Agilent's GeneSpring software.

C. Calcium-induced differentiation model of KCs

To induce differentiation of KCs, cells were cultured in serum-free KGM containing 1.2 mM calcium [22] during 0, 1, and 3 days for real-time PCR and 0, 1, 3, 7 and 10 days for Western blotting. The medium was changed every other day. After incubation in the high calcium condition (1.2 mM), cells were analyzed by real-time PCR and Western blotting with differentiation markers, such as involucrin, and cytokeratin10.

2. *In vitro* analysis of TRPV6 expression on KCs

A. RNA extraction from differentiated KCs

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's protocol. In brief, cells were added with RLT buffer to disrupt the cells. 10 µl β-mercaptoenthanol were added to buffer RLT Plus beforehand. Cells were transferred to a gDNA eliminator spin column and centrifuged for 30 seconds at ≥8000 x g. After adding 1 volume of 70% ethanol, transferred the sample to an RNeasy spin column and centrifuged. After discarding the flow-through,

700 µl Buffer RW1 and 500 µl Buffer RPE were added respectively to the RNeasy spin column, and centrifuged again. 30–50 µl RNase-free water were added directly, and centrifuged to elute the RNA.

B. Real-time PCR

cDNA was synthesized using a reverse-transcription kit (Takara, Shiga Japan). Real-time PCR was performed using SYBR master mix (Life Technologies, Grand Island, NY, USA) on real-time PCR machine (ABI, Foster City, CA, USA). The Primer sequences used in the study were specified in Table 1. The cycling condition were for 10 min at 95 ^{o}C followed by 45 cycles of 15 seconds at 95 ^{o}C and 1 minute at 60 ^{o}C, and 15 seconds at 95 ^{o}C, and the melting curve extension from 60 to 95 ^{o}C. The expression level of gene transcript was normalized by the β-actin and relative quantitation of gene expression was used using comparative C_T method (ΔΔCt).

C. Western blotting

Differentiated KCs were cultured at 0, 1, 3, 7 and 10 days and cells were treated with lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM ethylenediaminetetraactic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, a complete EDTA-free protease inhibitor, and 1% Nonidet P-40 mixture from (Elpisbio, Seoul, Korea) and centrifuged at 4 ^{o}C (10 min, at 12000xg). Proteins diluted in sample buffer were boiled for 5 min at 95 ^{o}C and electrophoretically separated on 8% SDS-PAGE. Proteins were blotted onto polyvinylidene difluoride (PVDF) membrane (GE

Table 1. Primer sequences used in real-time PCR

Name	**Forward (5'→3')**	**Reverse (5'→3')**
β-actin	GAGCACAGAGCCTCGCCTTT	TCATCATCCATGGTGAGCTGG
TRPV6	ATGGTGATGCGGCTCATCAGTG	GTAGAAGTGGCCTAGCTCCTCG
Involucrin	GAAACAGCCAACTCCACTGC	CAGGCAGTCCCTTTACAGCA
IL-21R	AGCTCTTTGGGAAGAGACGC	CTGGTCTTGCCAGGTAAGGG
MMP10	TCTTGCATTCCTTGTGCTGTTG	ATTGCTGGGCAAGATCCTTGT
SOX4	CAGAAGGGAGGGGGAAACATA	GAATCGGCACTAAGGAGTTGGT
CD58	CCAATGCATGATACCAGAGCAT	CCAATGCATGATACCAGAGCAT
CD276	ACAGGGCAGCCTATCACATT	TGATCTTTCTCCAGCACACG

healthcare, Buckinghamshire, UK) for 1 hr. After blocking in 5% skim milk, the membranes were incubated with rabbit polyclonal anti-TRPV6 (Abcam, Cambridge, UK) anti-cytokeratin 10 antibody (Abcam), anti-involucrin (Abcam), and anti-GAPDH (Cell signaling, Danvers, MA, USA) for overnight at 4 ^{o}C. The protein bands were detected by Luminescent image analyzer system (LAS 4000, GE healthcare) and analyzed by densitometry.

3. *In vivo* analysis of TRPV6 expression in human skin

A. Immunohistochemical staining

To stain the psoriasis and normal tissue, we used Envision TM G2 double stain sytem (Dako, Glostrup, Denmark) according to manufacturer's protocol. In brief, paraffin-embedded normal (n=4) and psoriatic patients (n=8) sections were subjected to deparaffinization followed by antigen retrieval using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 95 ^{o}C water bath for 20min. After blocking endogenous enzyme, anti-TRPV6 antibody (Abcam) and anti-involucrin (Abcam) were applied on tissue at 4 ^{o}C for overnight. After washing with PBST (1x PBS, 0.1% Tween 20) twice, incubated tissue sections were applied with polymer-HRP for 30 min. Diaminobenzidine (DAB) solution and hematoxlyin solution were applied 1 min and 30 seconds, respectively. After repeated washing, mounted the cover slips onto slides using

mounting solution, and slide have been left to air-dry completely.

B. RNA extraction from normal and psoriatic skin

Normal and psoriasis human skin samples were obtained from Department of Dermatology. After specimens were sterilized in 70% ethanol and washed with PBS, tissues were incubated with 2 U/m^{-1} dispase Ⅱ (Roche, Penzberg, Germany) at 37 ^{o}C for 1 hr. RNA from epidermis was extracted using Trizol reagent[21] in accordance with manufacturer's protocol. In brief, 1 ml Trizol was added for lysing the cells. After shortly vorteing, 200 μl chloroform was added. After centrifuging at 12,000x g for 15 min, transferred the upper clear phase to a new tube. 0.5x isopropanol was added and centrifuged again. Supernatant was discarded, 75% ethanol was added in DEPC-treated water.

4. The effect of TRPV6 on the proliferation of KCs

A. MTT cell proliferation assay

We performed MTT assay to confirm the effect of TRPV6 on KCs using TRPV6 inhibitor, $LaCl_3$ (Sigma-Aldrich, St. Louis, MO, USA).[23] HaCaT cells are a immortalized human KCs line that has been widely used for studies of skin biology[24]. HaCaT cells were seeded $1x10^4$ cells in a 96-well plate, pretreated with human recombinant IL-21 (PeproTECH) or 0.01, 1 mM $LaCl_3$ and co-treated with $LaCl_3$ and

IL-21. Experiment was performed in both calcium-treated and calcium-free condition for 24 and 72 hrs. After treatment, cells were incubated with thiazolyl blue tetra-zolium bromide **[**3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide**]** (MTT) (Sigma-Aldrich) at a final concentration of 0.5 mg/ml for 3 hrs at 37 ^{o}C in the dark. Then, the solutions were removed, and the formazan crystals were solubilized by 200 µl dimethyl sulfoxide (Sigma-Aldrich). The absorbance of sample was measured at 570 nm with a microplate reader (Molecular devices, California, CA, USA).

B. Detection of apoptotic rate

Apoptosis-mediated cell death was examined in HaCaT cells using FITC annexin V/ propidium iodide (PI) apoptosis detection kit (BD Biosciences, San Jose, CA, USA) in accordance with manufacturer's protocols. In brief, after treatment with different concentration of $LaCl_3$ (Sigma), cells were washed in PBS and resuspended in a 1x binding buffer. 5 µl FITC-conjugated annexin V and 10 µl PI were added together, and incubated for 15 min at room temperature in the dark. After adding 400 µl of 1x binding buffer to each tube, apoptotic rate was analyzed by flow cytometry (BD FACS verse, San Jose, CA, USA) within 1hr.

C. Statistical analysis

In addition to Microsoft Office Excel, GraphPad PRISM™ (version 5.0) was used for statistical analysis and to create the graphs. The data was expressed as the means ± S.D.

III. RESULTS

1. Microarray analysis

Microarray data from IL-21 treated KC[20] were re-analyzed for this thesis, and total of 1,588 genes were statistically different between IL-21-treated and non-treated KCs (Figure 1). Among those genes, we selected genes which might have relevance to psoriasis such as CD276, CD58, MMP10, SOX4 and TRPV6 (Table 2). The expression of selected genes was confirmed using real-time PCR treated with IL-21 in KCs to validate microarray results. As shown Fiugure 2, those genes were significantly increased in KCs treated with IL-21 confirmed by real-time PCR.

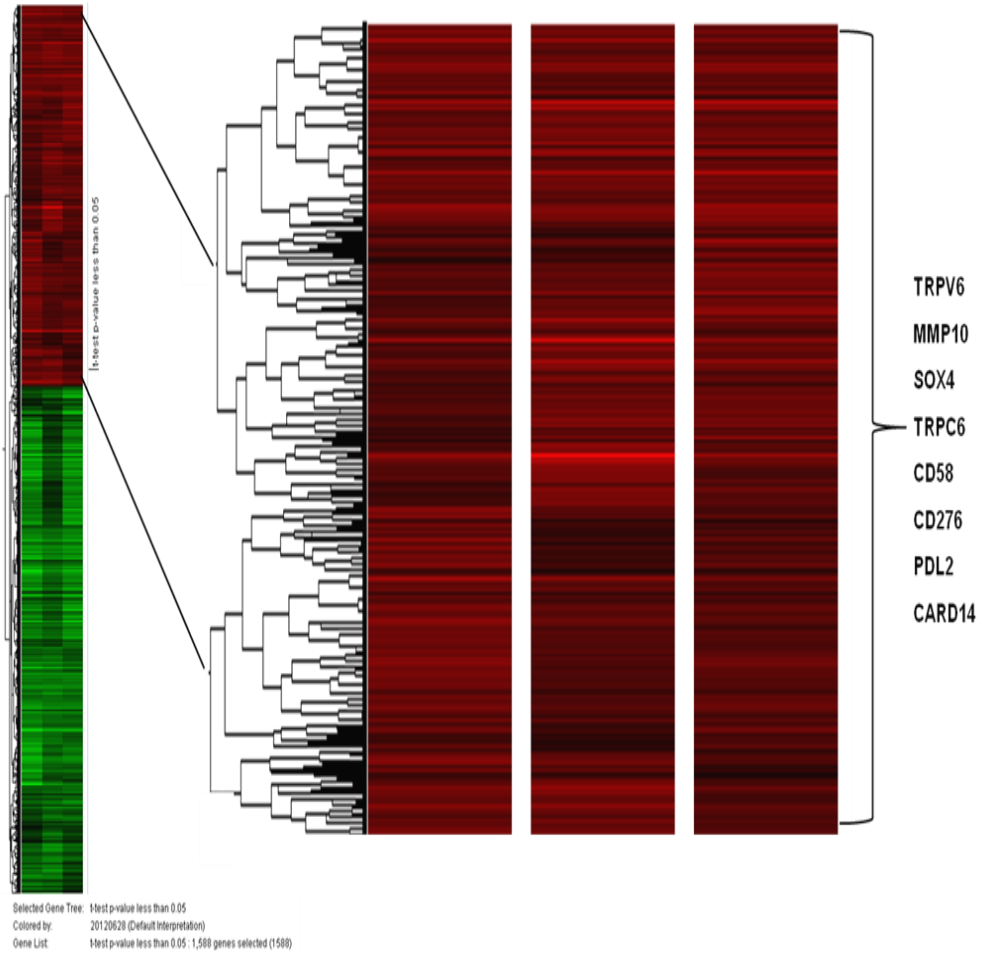


Figure 1. Microarray analysis. (A) Heatmap of 1,588 genes that were statistically different between IL-21- treated and non-treated KCs.

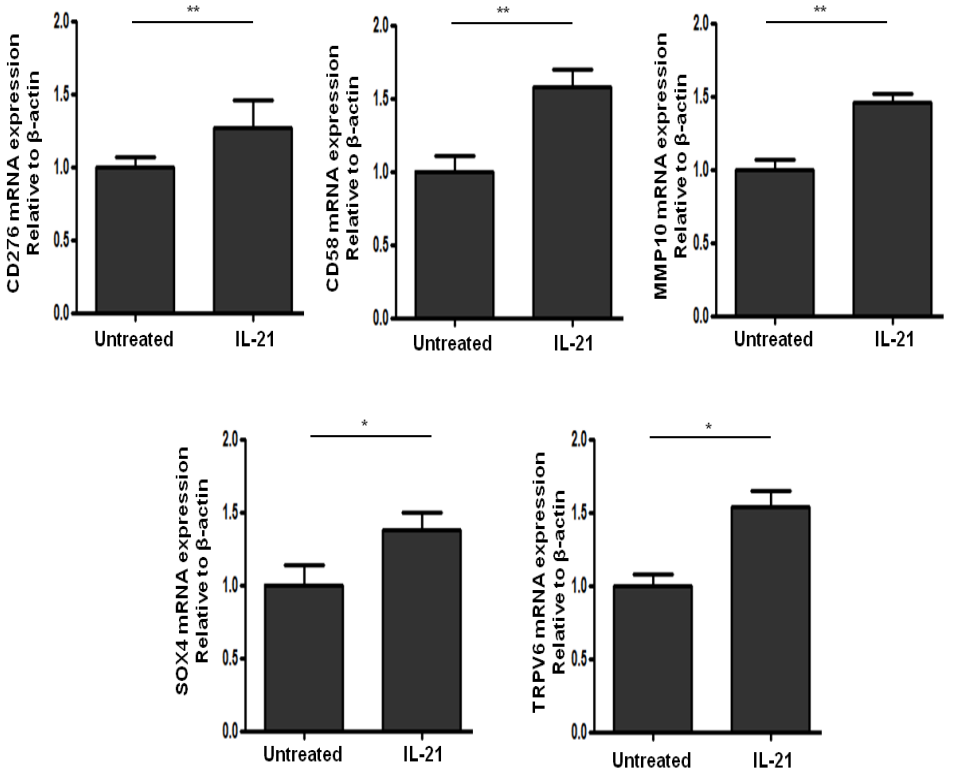


Figure 2. Confirmation of microarray results by real-time PCR. The mRNA expression levels of selected genes including TRPV6 were significantly increased in KCs treated with IL-21 compared to untreated KCs (*$p < 0.05$, **$p < 0.01$) .

Table 2. List of genes that were elevated more than one fold in KCs treated with IL-21 compared to untreated KCs ($p < 0.05$).

Systematic	Gene Symbol	Gene Name
A_23_P13094	MMP10	matrix metallopeptidase 10 (stromelysin 2)
A_33_P3228322	IL18BP	interleukin 18 binding protein
A_24_P911676	SOX4	SRY (sex determining region Y)-box 4
A_23_P49975	KRT10	keratin 10
A_33_P3364854	TRPV6	transient receptor potential cation channel, subfamily V, member 6
A_33_P3337627	TRPC6	transient receptor potential cation channel, subfamily C, member 6
A_33_P3217393	CD276	CD276 molecule
A_23_P100795	STAT3	signal transducer and activator of transcription 3

2. Calcium- induced differentiation in KCs

To elucidate the mechanism of KC differentiation, we cultured KCs and HaCaT cells as established models. We initially studied the differentiation level of KCs cultured in KGM containing 1.2 mM high calcium. Analysis of cell morphology showed that KCs became flattened after 3 days of incubation, suggesting a differentiated phenotype (Figure 3). To confirm differentiation status, real-time PCR was performed to assess the expression of a differentiation marker, involucrin. The expression of involucrin was increased as the cells underwent differentiation (Figure 4A). The induction of cytokeratin 10 was also detected by western blot from 7 days after calcium treatment (Figure 4B).

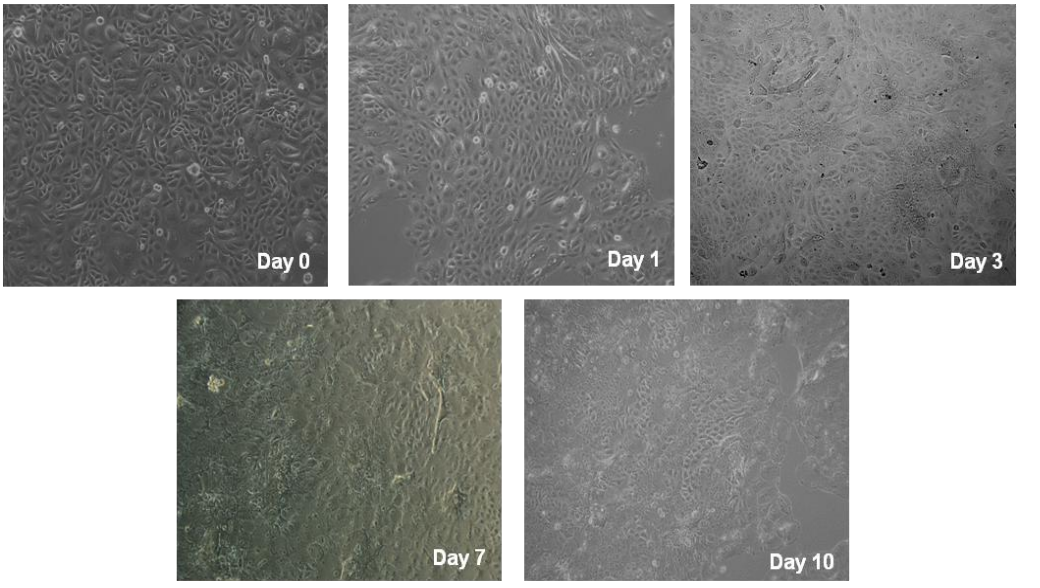


Figure 3. Calcium-induced differentiation of KCs. Phase contrast microscopy of KCs in basal, low calcium maintained (Day 0) and 1.2 mM high calcium maintained (Days 1, 3, 7, and 10). KCs became flattened after 3 days of incubation in high calcium environment.

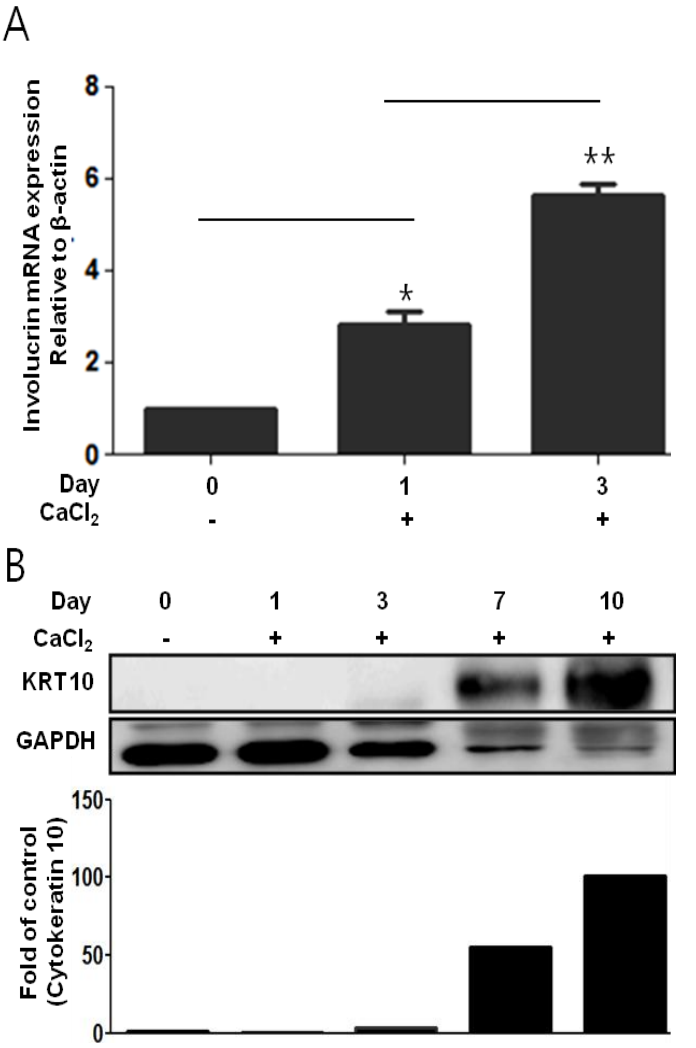


Figure 4. The expression of mRNA and protein of differentiation marker were increased in accordance with differentiation. (A) The mRNA level of involucrin and (B) protein level of cytokeratin 10 were increased in calcium-induced differentiated KCs by real-time PCR and western blot, respectively. The levels of expression were normalized to the controls (*$p < 0.05$, **$p < 0.01$).

3. TRPV6 mRNA and protein expression in differentiated KCs treated with calcium

TRPV6 mRNA and protein expression were analyzed in the differentiated KCs treated with calcium. The mRNA level of TRPV6 was significantly increased in accordance with the differentiation of KCs (Figure 5A). Expression level of TRPV6 protein was increased from 7 days after calcium treatment (Figure 5B). These results demonstrate that the expressions of TRPV6 mRNA and protein in KCs are apparently differentiation-dependent (Figures 4 and 5).

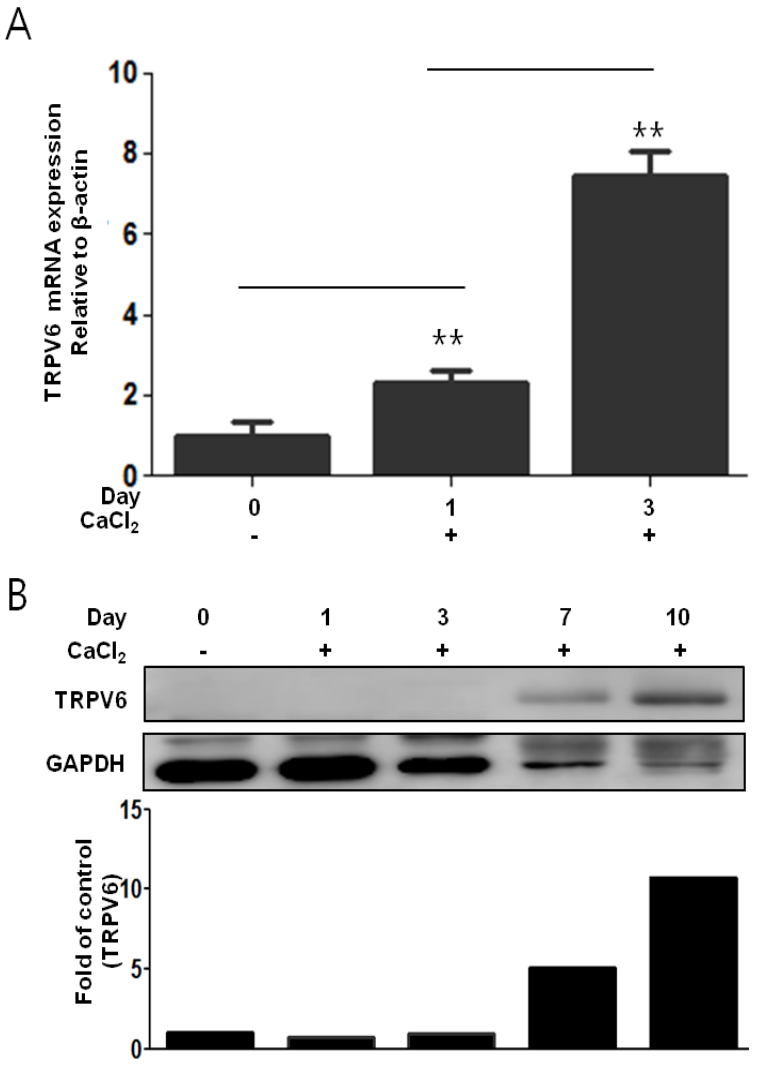


Figure 5. The expressions of mRNA and protein of TRPV6 were increased in accordance with the differentiation of KCs. (A) TRPV6 mRNA levels were increased in accordance with differentiation. (B) Expression levels of TRPV6 protein by western blot were increased from 7 days after calcium treatment. (**$p < 0.01$)

4. The TRPV6 expression in normal and psoriatic skins

To evaluate the pattern of TRPV6 expression in skin tissue, immunohistochemical staining of TRPV6 was performed in normal (n = 4) and psoriatic skins (n = 8). In addition to TRPV6, skin was stained for a differentiation marker, involucrin. Involucrin is well known to be a marker of terminally differentiated KCs. The involucrin expression was lower in psoriatic skin compared to normal skin (Figure 4A). TRPV6 was mainly expressed in stratum spinosum of the epidermis, and the expression of TRPV6 was lower in psoriatic skin than normal one (Figure 4B). Detection of TRPV6 expression by immunohistochemical staining in human skins revealed a gradient of TRPV6 expression correlated with the differentiation marker, involucrin. We performed real-time PCR using normal (n = 1) and psoriatic (n = 2) epidermis. The mRNA level of TRPV6 was significantly lower in psoriasis compared to normal epidermis (Figure 4C). Altogether, the levels of TRPV6 mRNA and protein are reduced in psoriasis, suggesting that the reduced TRPV6 is related to KC differentiation within the skin.

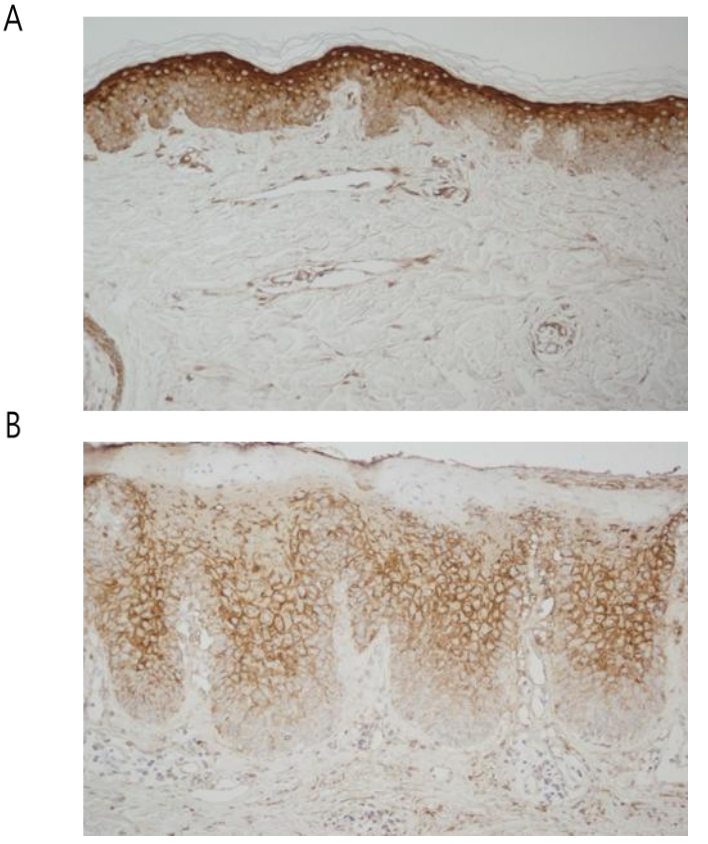


Figure 6. The expression of differentiation marker was reduced in psoriatic skin. The protein level of involucrin was reduced in immunohistochemical staining of (A) psoriatic skin in comparison with (B) normal skin (n = 1).

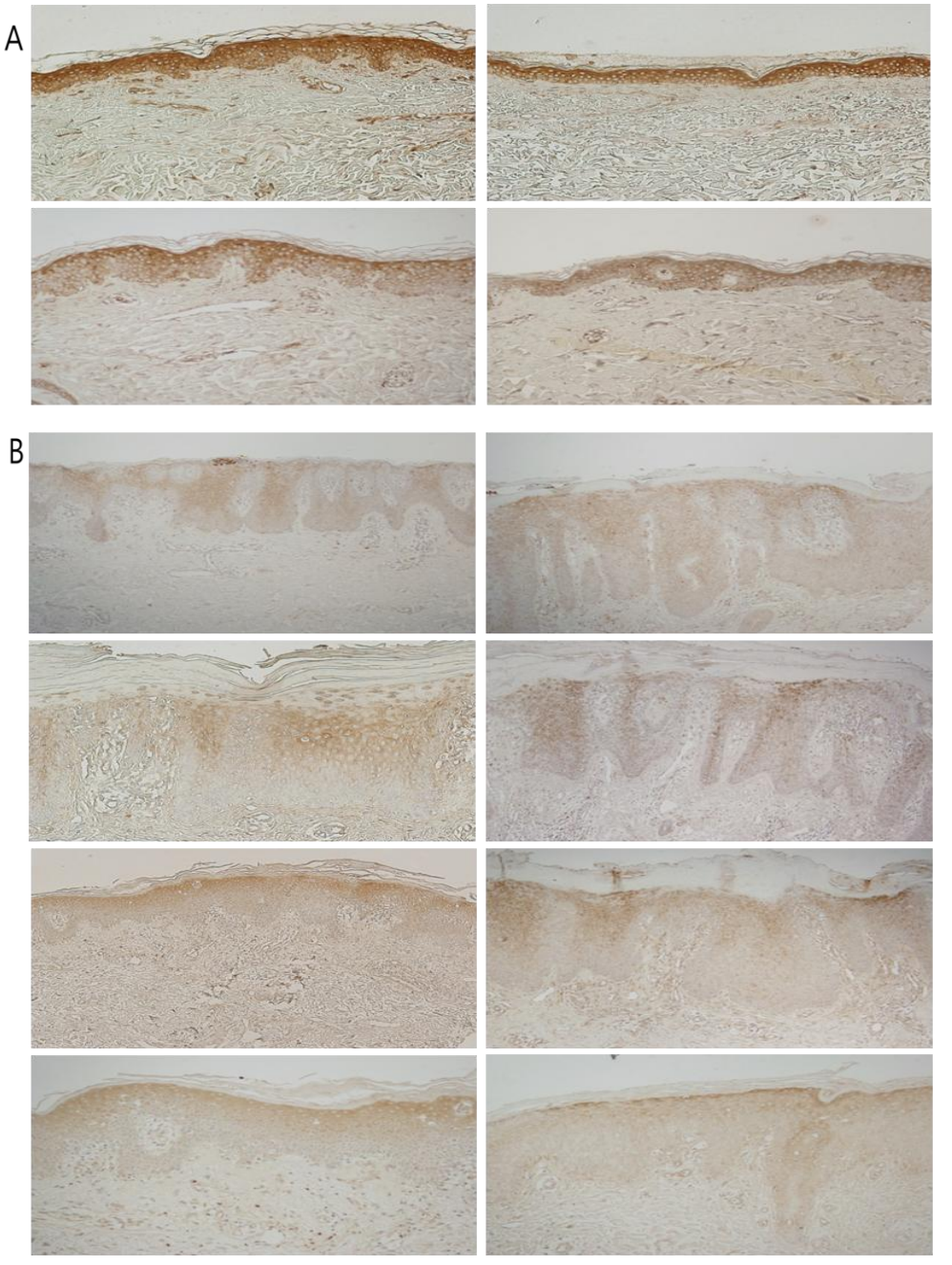

A
B

Figure 7. The expression of TRPV6 was reduced in psoriatic skin. Paraffin-embbeded psoriatic (n = 8) and normal skin (n = 4) were stained for expression of TRPV6. Compared to (A) normal skin, the expressions of TRPV6 was lower in immunohistochemical staining of (B) psoriatic skin.

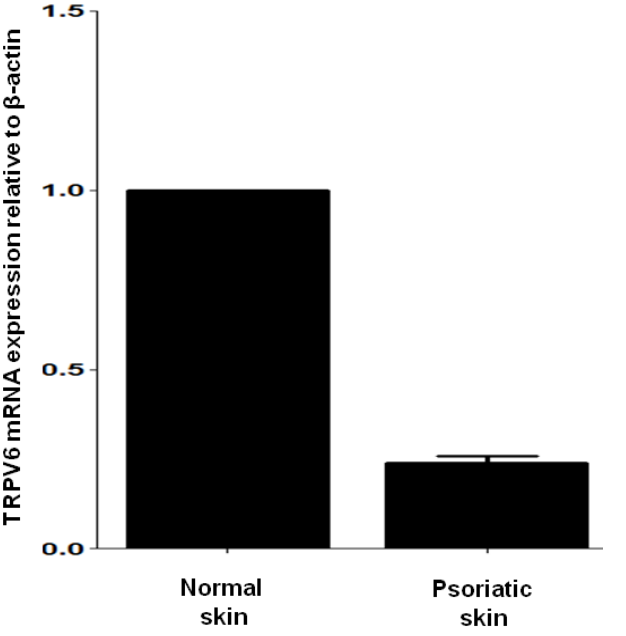


Figure 8. TRPV6 mRNA expression was reduced in real-time PCR of psoriatic epidermis. The mRNA level of TRPV6 was reduced in psoriatic epidermis in comparison with normal epidermis.

5. Proliferation of HaCaT cells treated with TRPV6 inhibitor, $LaCl_3$ and IL-21

To investigate whether reduced TRPV6 is related to the abnormal KC proliferation in psoriasis, MTT assay was performed to confirm the effect of a TRPV6 inhibitor ($LaCl_3$) on cell proliferation. The induction of TRPV6 expression was confirmed by western blot in calcium-induced differentiated HaCaT cells. Expression of TRPV6 protein was increased in accordance with differentiation (Figure 9). In high calcium environment, mRNA level of IL-21 receptor (IL-21R) was increased according to the differentiation of cells (Figure 10). The proliferation of HaCaT cells was observed in low calcium environment until after 72 hrs of incubation. In high calcium environment, however, HaCaT cell proliferation was stopped after 48 hrs of incubation (Figure 11A). After the treatment of a $LaCl_3$ (0.01 and 1 mM), the proliferation of HaCaT cells in low calcium environment was reduced in a dose-dependent manner, but the changes were not statistically significant (Figure 11B). Co-treatment with IL-21 and $LaCl_3$ reduced HaCaT cells proliferation, slightly increased with treatment of $LaCl_3$ and IL-21, respectively (Figure. 11C). Apoptotic rates among all groups showed no significant changes in high calcium environment (Figure 12). Although the HaCaT cells demonstrated a tendency of decreased proliferation with TRPV6 inhibitor and IL-21 treatment, the changed in both low and high calcium environments was not statistically significant. The data suggest that despite its crucial role in differentiation of KCs, TRPV6 might not affect proliferation of KCs.

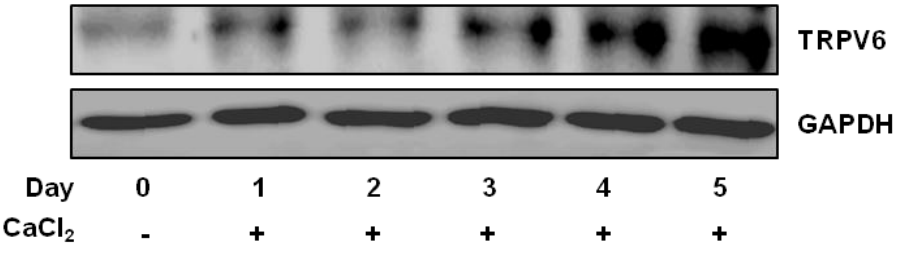


Figure 9. Calcium-induced differentiation in HaCaT cells. The protein levels of TRPV6 were increased in accordance with differentiation in western blot of HaCaT cells .

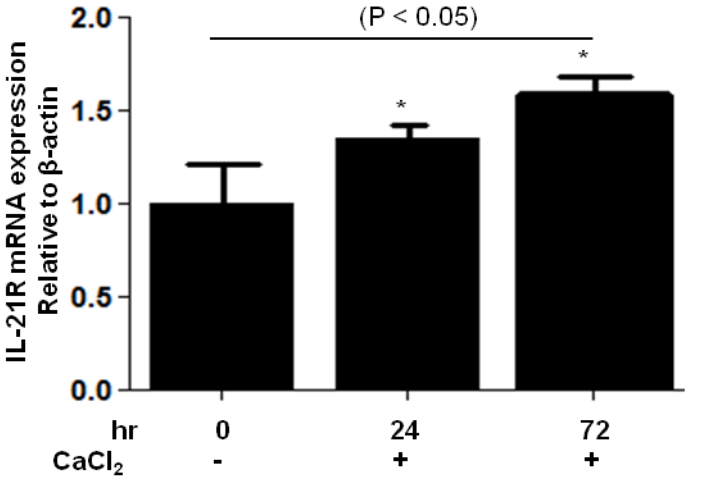
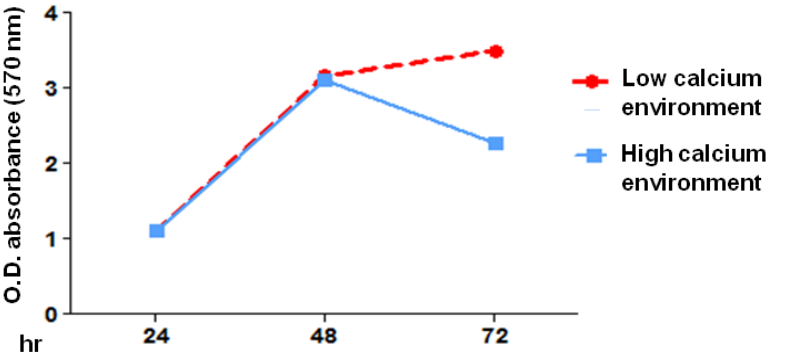
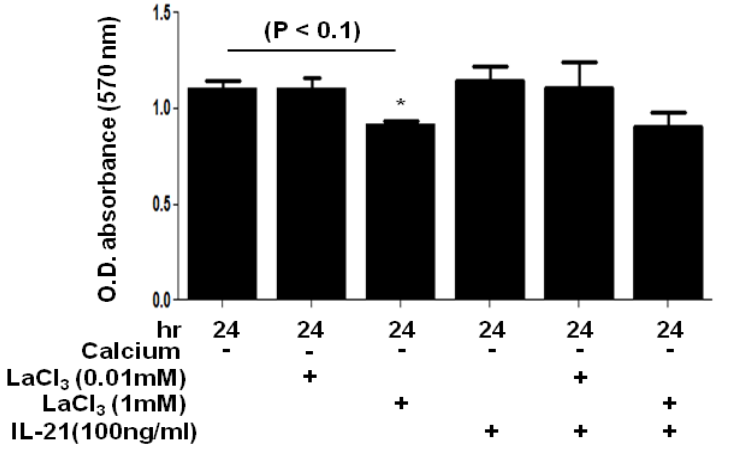


Figure 10. The correlation between IL-21R and differentiation in HaCaT cells. The mRNA level of IL-21 receptor (IL-21R) was increased in accordance with differentiation of HaCaT cells.

A


O.D. absorbance (570 nm)
4
3
2
1
0
hr
24
48
72
Low calcium environment
High calcium environment

B


O.D. absorbance (570 nm)
1.5
1.0
0.5
0.0
(P < 0.1)
*
hr 24 24 24 24 24 24
Calcium - - - - - -
LaCl3 (0.01mM) + +
LaCl3 (1mM) + +
IL-21(100ng/ml) + + +

C

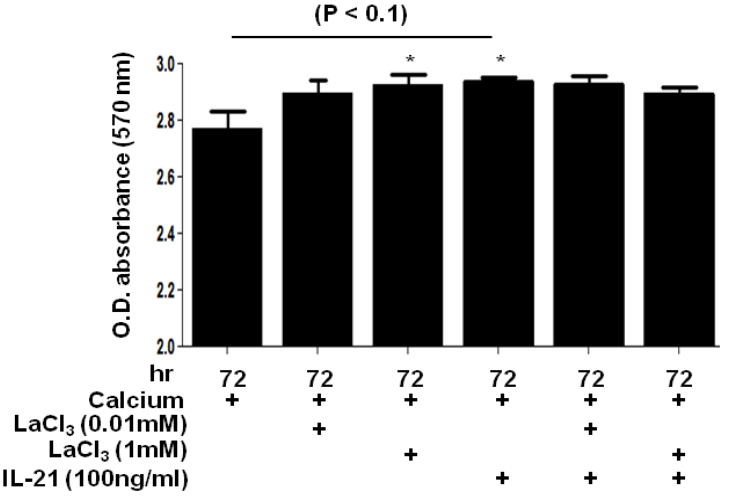

O.D. absorbance (570 nm)
3.0
2.8
2.6
2.4
2.2
2.0
(P < 0.1)
* *
hr 72 72 72 72 72 72
Calcium + + + + + +
LaCl3 (0.01mM) + +
LaCl3 (1mM) + +
IL-21 (100ng/ml) + + +

Figure 11. TRPV6 did not significantly affect HaCaT cell proliferation. MTT assay was performed to confirm the effect of a TRPV6 inhibitor ($LaCl_3$) on cell proliferation. (A) The proliferation of HaCaT cells was increased in a time-dependent manner. In high calcium environment, the proliferation of HaCaT cells was reduced after 48 hrs incubation. (B, C) After treatment of $LaCl_3$ (0.01 and 1 mM), proliferation was changed in a dose-dependent manner in low and high calcium environments ($P < 0.1$). $LaCl_3$ inhibited IL-21-induced proliferation of HaCaT cells, but the changes were not statistically significant differences.

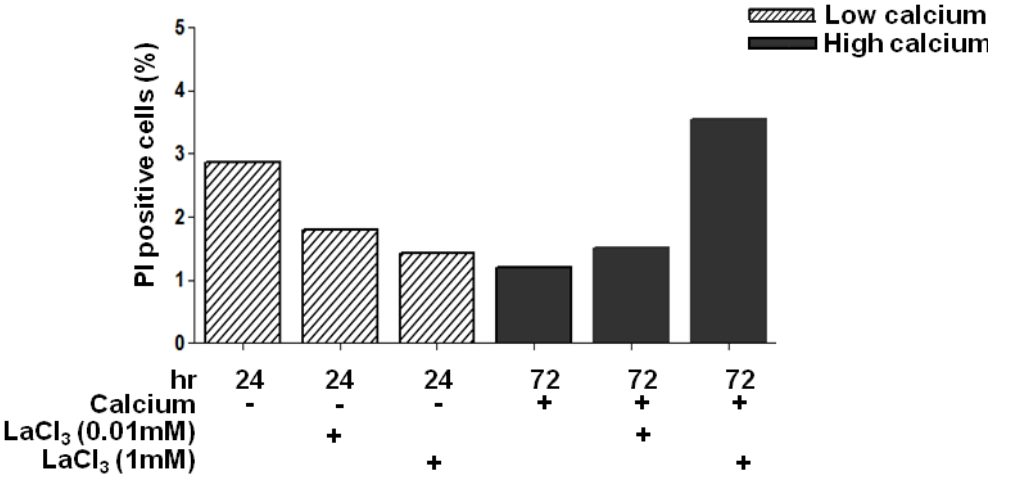


Figure 12. The changes of proliferation induced by $LaCl_3$ were not due to dead cells. Apoptotic rates (PI positive cells) demonstrated no differences in annexin V/ PI staining in low and high calcium environments (PI positive cells $\leq$ 3.54%).

IV. DISCUSSION

Psoriasis is an inflammatory skin disease mediated by the cells and molecules of both the innate and adaptive immunity[25]. The characteristics of psoriatic KCs are defective differentiation and proliferation, and impaired calcium homeostasis[8,9]. Calcium in the epidermis is associated with the skin homeostasis involving terminal differentiation of KCs and permeability barrier function[26]. Defective calcium gradients in the epidermis have been observed in many cutaneous diseases known to be characterized by impaired barrier function, such as psoriasis and atopic dermatitis[7,27]. Moreover, it has already been reported that epidermal damage status and pathology of skin diseases are reflected in the features of calcium distribution in cultured KCs[28,29]. Therefore, investigating the mechanisms of calcium distribution in the epidermal KCs will provide us with better understanding and novel therapeutic candidate of psoriasis.

TRPV6 is a highly calcium-selective channel, which mediates differentiation of KCs[30,31]. TRP channels may be generally described as calcium-permeable cation channels with polymodal activation properties and they have been emerging as molecular targets for disease-modifying drugs[32]. TRPV6 is presents in differentiated KCs at comparatively high levels. As mentioned before, some groups reported that TRPV6 is expressed and plays an important role in the epidermis where the role of

calcium is pivotal for skin differentiation. In this regards, TRPV6 is a molecular candidate for the apical calcium entry pathway[16,30]. Up-regulation of TRPV6 led to a significant increase in calcium uptake in both differentiated and undifferentiated KCs[15,30]. These data demonstrated that TRPV6 mRNA and protein were increased in accordance with KCs differentiation. Taken together, TRPV6 seems to play a crucial role in human KCs as a functional calcium-mediated channel with an expression pattern dependent on differentiation state of KCs. The expression and function of other TRP channel in psoriatic KCs had already been reported[19,33]. TRPC channels including TRPC1, TRPC4, or TRPC6, for example, were related to impaired calcium homeostasis in psoriatic KCs and were reduced in psoriatic skin[6]. However, the role of TRPV6 in psoriatic KCs is still unknown. Real-time PCR data verified that the mRNA expression of TRPV6 was significantly reduced in psoriatic epidermis. Immunohistochemical staining confirmed the reduced TRPV6 expression in psoriatic lesional skin compared with normal skin. The gradient of TRPV6 expression was correlated with the gradient of differentiation markers, such as involucrin. These results suggest that the involvement of the TRPV6 expression may be related to KCs differentiation within the skin. Defective calcium homeostasis in psoriatic KCs with impaired differentiation is also related to reduced expression of TRPV6. Because TRPV6 is a prerequisite for KCs entry into differentiation[30], reduced TRPV6 expression in psoriasis could affect defective differentiation of psoriatic KCs. There

are some reports suggesting that low calcium concentration in psoriatic basal layer resulted in hyperproliferation features of psoriasis[7], and TRPV6 regulated abnormal epithelial cell proliferation in low extracellular calcium environment[18]. Together with TRPV6, some cytokines related to proliferation of KCs in psoriasis were searched. Several cytokines produced by inflammatory cells contribute to the induction of the proliferation of KCs in psoriasis. IL-21 is one of such cytokines[2]. IL-21 has been reported to be an inducer of KCs proliferation, and IL-21R is highly expressed in the skin of individuals with psoriasis[12]. IL-21R was increased in accordance with differentiation of KCs. Despite many reports about the role of IL-21 on various cells, the effect of IL-21 on KCs still remains to be elucidated. So, we performed microarray analysis to identify psoriasis-specific molecules in IL-21- treated human KCs, and selected TRPV6 as one of the psoriatic specific molecules. The effect of TRPV6 on KCs proliferation was checked using TRPV6 inhibitor, $LaCl_3$ in low and high calcium environment. TRPV6 was not significantly associated with enhanced proliferation of KCs ($p < 0.1$). And co-treatment with IL-21 and $LaCl_3$ was also not associated with proliferation of KCs. These data indicated that the reduced TRPV6 might be related to KC differentiation. However, TRPV6 did not affect KC proliferation. Several explanations are possible about the discrepancy of the results between *in vitro* and *in vivo* data. The effect of TRP6 inhibitor *in vitro* study did not demonstrate the same condition of decreased expression of TRPV6 on psoriatic KCs,

which could be caused by several factors. And the characteristics of the psoriatic KCs might not be fully reflected in KCs cell line (HaCaT cells), therefore the change of HaCaT cells to psoriatic KCs may be necessary to confirm the effect of TRPV6 on the proliferation of psoriatic KCs. The other possibility is that even though the expression of TRPV6 was decreased on psoriatic KCs, TRPV6 may not have an important role in KCs than my initial expectation. Although TRPV6 might exert a supplementary effect on KCs differentiation and proliferation, other key mediators, such as IL-17, IL-22, IL-23, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ), could countervail the effects of TRPV6. And further studies need to confirm the induction of differentiation after treatment with $LaCl_3$ and siTRPV6 in KCs.

In conclusion, we confirmed TRPV6 was decreased in psoriatic epidermis and reduced TRPV6 in psoriasis could be involved in decreased differentiation of psoriatic KCs. However, in terms of the proliferation of psoriatic KCs, the role of TRPV6 needs to be disclosed.

V. CONCLUSION

To elucidate whether TRPV6 plays a crucial role in psoriasis, real-time PCR, Western blotting, and MTT assay were performed in calcium-induced differentiated KCs and human psoriatic skin. TRPV6 was decreased in psoriatic epidermis and reduced TRPV6 in psoriasis could be involved in decreased differentiation of psoriatic KCs. However, the role of TRPV6 in the proliferation of psoriatic KCs still needs to be elucidated.

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ABSTRACT (IN KOREAN)

건선 피부염에서 IL-21이 유도하는 TRPV6의 감소

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김성희

건선은 표피 각질세포의 분화 감소와 과도한 증식으로 인한 표피비대증이 특징인 피부질환이다. IL-21은 활성화된 CD4 T 세포와 자연살해세포에서 주로 생성되며, 각질형성세포의 증식, CD8 T 세포독성에 관여한다. 최근 건선에서 IL-21 발현이 증가 되어있고 세포증식에도 관여한다고 보고되었다. 그래서 본 연구실에서 인간 각질형성세포에 IL-21을 처리한 후 microarray로 건선관련 물질을 찾고자 하였다, 이 중 TRPV6의 발현 증가를 관찰하였고, TRPV6가 건선에서 중요한 역할을 하는지 확인하고자 하였다.

TRPV6는 주요 칼슘 수용체 중 하나로 콩팥, 소화기관, 고환, 난소, 피부 등의 상피조직에 주로 발현한다. 건선 표피에서 정상 조직에 비교해서 칼슘농도의 변화가 많으므로, 칼슘 수용체 중 하나인 TRPV6가 건선에 관여할 가능성이 높다. 또한 TRPV6는 칼슘으로 유도된 각질세포 분화와 상피세포 증식에 중요하다고 알려져 있으므로 각질세포의 분화와 증식에 이상 있는 건선에서도 TRPV6가 중요한 역할을 할 수 있으리라 생각한다. 그러나 건선에서 TRPV6의 발현에 대한 보고는 없으므로, 건선에서 TRPV6 발현과 그 역할에 대해 알아보고자 하였다.

칼슘 농도가 증가되어 있는 건선 표피와 비슷한 조건을 만들기 위해 정상각질형성세포에 고농도(1.2 mM)의 칼슘을 처리하였다. 각질형성세포의 분화 마커인 involucrin과 cytokertin 10이 증가하였고, 이 때 TRPV6 mRNA와 단백 발현이 증가함을 real-time PCR과 Western blot으로 확인하였다. 그러나 건선 환자의 조직에서는 TRPV6 mRNA와 단백발현이 감소함을 면역화학염색법과 real-time PCR으로 확인하였다. 건선환자에서 TRPV6가 감소함은 건선 각질형성세포의 분화 감소에 TRPV6가 관여할 수 있음을 의미한다. 건선의 또 다른 특징인 표피증식에 TRPV6의 영향을 확인하기 위해 각질형성세포에 저농도(정상배양조건)와 고농도(1.2 mM)의 칼슘으로 배양하면서 TRPV6 억제제를 처리하여 MTT assay를 수행 하였다. TRPV6 억제제 처리시 세포의 증식에는 큰 영향을 미치지 않았으며, 각질형성세포 증식에 관여하는 IL-21을 억제제와 함께 세포에 처리한

경우에도 증식에는 영향을 미치지 않았다. 이러한 결과는 *in vitro* 상에서 억제제를 통한 TRPV6 감소가 실제 건선 조직에서의 TRPV6 감소와 동일한 조건이 아닐 수 있으며, 실험에 사용한 HaCaT 세포와 건선 각질형성세포의 차이 때문일 수도 있을 것으로 생각한다. 그리고 TRPV6가 생각과 달리 건선에서 중요한 작용을 하지 않을 가능성도 배제하기 어렵다.

결론적으로, 본 연구에서 건선 병변에서 TRPV6의 발현이 감소되어 있음을 확인하였고, 이는 표피 각질세포의 분화 감소와 관련 있을 것으로 생각할 수 있으나, 각질세포의 증식에서의 역할은 확실치 않았다.

핵심이 되는 말 : 건선, IL-21, 각질형성세포, TRPV6 , 분화, 증식