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HaCaT Keratinocytes and Primary Epidermal Keratinocytes Have Different Transcriptional Profiles of Cornified Envelope-Associated Genes to T Helper Cell Cytokines

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Abstract

HaCaT cells are the immortalized human keratinocytes and have been extensively used to study the epidermal homeostasis and its pathophysiology. T helper cells play a role in various chronic dermatological conditions and they can affect skin barrier homeostasis. To evaluate whether HaCaT cells can be used as a model cell system to study abnormal skin barrier development in various dermatologic diseases, we analyzed the gene expression profile of epidermal differentiation markers of HaCaT cells in response to major T helper (Th) cell cytokines, such as IFNγ, IL-4, IL-17A and IL-22. The gene transcriptional profile of cornified envelope-associated proteins, such as filaggrin, loricrin, involucrin and keratin 10 (KRT10), in HaCaT cells was generally different from that in normal human keratinocytes (NHKs). This suggests that HaCaT cells have a limitation as a model system to study the pathophysiological mechanism associated with the Th cell cytokine-dependent changes in cornified envelope-associated proteins which are essential for normal skin barrier development. In contrast, the gene transcription profile change of human β2-defensin (HBD2) in response to IFNγ, IL-4 or IL-17A in HaCaT cells was consistent with the expression pattern of NHKs. IFNγ also up-regulated transglutaminase 2 (TGM2) gene transcription in both HaCaT cells and NHKs. As an alternative cell culture system for NHKs, HaCaT cells can be used to study molecular mechanisms associated with abnormal HBD2 and TGM2 expression in response to IFNγ, IL-4 or IL-17A.

Key Words: HaCaT Keratinocytes, Cornified envelope associated genes, HBD2, IFNγ, IL-4, IL-17A

INTRODUCTION

The abnormal skin permeability barrier due to the genetic mutations in cornified envelope-associated proteins, such as filaggrin, loricrin and keratin (KRT) 10, has been recently suggested as one of primary etiological factors to develop various dermatologic diseases (Cork *et al.*, 2006; Hoffjan and Stemmler, 2007; Cork *et al.*, 2009). For instance, the direct linkage of genetic defects in filaggrin with atopic dermatitis has been demonstrated (Hoffjan and Stemmler, 2007; Brown *et al.*, 2012). Therefore, the integrity of the skin permeability barrier is currently regarded as an essential component to understand the pathophysiology of skin diseases (Cork *et al.*, 2006; Proksch *et al.*, 2006; Noh *et al.*, 2010).

Chronic inflammation in various skin diseases can be characterized by their unique and predominant CD4-positive T helper (Th) cell profiles. For example, Th1 cells are predominent in psoriasis, whereas Th2 cells are associated with atopic dermatitis (Nickoloff *et al.*, 2007; Ou and Huang, 2007). Th17 cells have been recently reported to play important roles in psoriasis and vitiligo (Ouyang *et al.*, 2008; Bassiouny and Shaker, 2011).

Previously, we reported the comprehensive analysis on the effect of Th cell cytokines on major epidermal differentiation markers important in the formation of skin permeability barrier (Noh *et al.*, 2010). We found that most of Th cell cytokines, such as interferon (IFN) γ, interleukin (IL)-4, IL-17A, and IL-22 significantly decrease the gene transcription of filaggrin and other cornified envelope-associated proteins in normal human keratinocytes (NHKs) (Noh *et al.*, 2010). In addition, IFNγ increased the gene transcription of transglutaminase (TGM) 1 and TGM2, the essential enzymes to catalyze reactions to

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form stable isopeptide bonds between cornified envelope-associated proteins (Noh *et al.*, 2010). Although the use of primary NHKs is a primary choice to study molecular mechanisms on the regulation of epidermal differentiation markers, immortalized keratinocytes such as HaCaT cells have been used as an alternative model system (Boukamp *et al.* 1988; Lehmann, 1997). For example, vitamin D3 metabolism in human skin can be studied with cultured HaCaT cells (Lehmann, 1997). However, expression profiles of epidermal differentiation markers in HaCaT cells in response to Th cell cytokines has not fully studied in a systematic level.

In this study, we analyzed the gene transcription profile of major epidermal differentiation markers, such as filaggrin, loricrin, involucrin, KRT10, TGM1, TGM2 and HBD2, in response to IFNγ, IL-4, IL-17A or IL-22, in cultured HaCaT cells. We found that Th cell-mediated expression profiles in HaCaT cells are different from those in NHKs, suggesting a limitation of the cultured HaCaT model to study Th cell cytokine-mediated changes in epidermal differentiation markers.

MATERIALS AND METHODS

Cell culture and treatment with Th cell cytokines

NHKs from neonatal foreskin were purchased from Lonza (Basel, Switzerland) and cultured in KBM medium containing KGM2 growth supplements containing insulin, human epidermal growth factor, bovine pituitary extract, hydrocortisone, epinephrine, transferrin, and gentamicin/amphotericin B, purchased from Lonza. Cells were serially passaged at 70-80% confluence and then experiments were conducted with subconfluent cells at passage two or three in the proliferation phase. NHKs were starved for 24 hours in keratinocyte KBM medium without hydrocortisone or transferrin before cytokine treatments. The human immortalized keratinocyte cell line, HaCaT, were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% antibiotics (10,000 μg/ml streptomycin and 10,000 units/ml penicillin) at 30°C with 5% CO_2. Cells were serially passaged at 70-80% confluence. When performing experiments, HaCaT cells were grown to 100% confluence and cultured one more week. Then, HaCaT cells were starved for 24 hours in incomplete DMEM without FBS, followed by stimulation with recombinant human IL-17A (50 ng/ml; Sigma Aldrich, St. Louis, MO, USA), IL-4 (50 ng/ml; R&D systems), IL-22 (50 ng/ml; R&D systems), and IFNγ (200 unit/ml; R&D systems) for 24 hours.

RNA extraction and quantitative real time RT-PCR (qRT-PCR)

Total RNA was isolated using Trizol™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically, and the integrity of the RNA was assessed using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Two micrograms of RNA were reverse-transcribed into cDNA using SuperScript®III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and aliquots were stored at −20°C. Quantitative real-time TaqMan RT–PCR technology (Q-RT-PCR) (Applied Biosystems, Foster City, CA, USA) was used to determine the expression level of selected target genes. The cycling conditions included a denaturing step at 95°C for 10 min and 50 cycles of 95°C for 15 sec and 60°C for 1 min. The TaqMan probes (Applied Biosystems) used in the qRT-PCR analysis were: Filaggrin, Hs00856927_g1; Loricrin, Hs01894962_s1; Involucrin, Hs00846307_s1; TGM1, Hs01070316_m1; TGM2, Hs01096681_m1; KRT10, Hs01043110_g1; and HBD2, Hs00175474_m1. Human GAPDH (4333764F, Applied Biosystems) was also amplified to normalize variations in cDNA quantities from different samples. Relative differences in gene expression were calculated from Ct (threshold cycle) values using equations from a mathematical model developed by Pfaffl (Pfaffl *et al.*, 2002).

Statistical analyses

All statistical analyses were performed with MINITAB® software (Minitab Inc. State College, PA, USA). The *p*-values were calculated by using Student's *t*-test. The threshold of significance was set at $p<0.05$.

RESULTS

The effects of IFNγ on the gene transcription of epidermal differentiation markers in HaCaT cells

To determine the effect of IFNγ, a representative cyto-

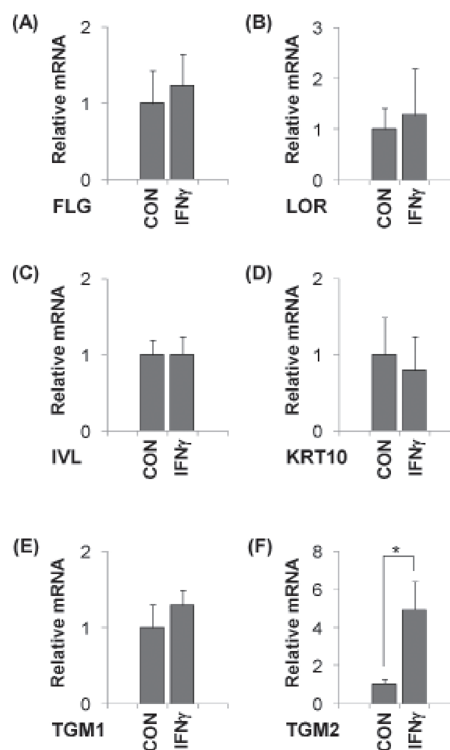

Fig. 1. Effects of IFNγ on mRNA expressions of cornified envelope-associated genes in HaCaT cells. Confluent HaCaT cells were were maintained at least 7 days. Before HaCaT cells were treated with 200 IU of IFNγ in DMEM with 10% FBS, the cells were synchronized by treating with DMEM without FBS for 24 hours. HaCaT cells were treated with 200 IU of IFNγ in DMEM with 10% FBS. At 24 hour after the IFNγ treatment. Total RNA was extracted, and qRT-PCR was performed for (A) filaggrin (FLG), (B) loricrin (LOR), (C) involucrin (IVL), (D) KRT10, (E) TGM1 and (F) TGM2. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression (n=3), *$p<0.05$.

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kine for Th1 immune responses, on epidermal differentiation marker gene transcription, first, we measured the mRNA expression levels of cornified envelope-associated genes, such as filaggrin, loricrin, involucrin, KRT10, TGM1 and TGM2 by quantitative RT-PCR (qRT-PCR) (Fig. 1). In order to compare gene expression profiles between HaCaT cells and NHKs, we treated 200 IU of IFNγ for 24 hours in HaCaT cells, which is the identical condition for evaluating the Th cell cytokine-dependent transcription profile changes in NHKs (Noh *et al.*, 2010). In HaCaT cells, IFNγ significantly increased the TGM2 gene transcription (Fig. 1F). However, mRNA levels of filaggrin, loricrin, involucrin, KRT10 and TGM1 were unaffected in HaCaT cells after treating with IFNγ (Fig. 1).

The effects of IL-4 on epidermal differentiation marker gene transcription in HaCaT cells

Next, we evaluated the effect of IL-4, a predominant cytokine produced in Th2 cells, on epidermal differentiation marker gene transcription in HaCaT cells (Fig. 2). Although IL-4 down-regulated mRNA levels of filaggrin in NHKs (Noh *et al.*, 2010), IL-4 significantly increased filaggrin gene transcription in HaCaT cells (Fig. 2A). In contrast, mRNA levels of two cornified envelope-associated proteins, KRT10 and involucrin, were down-regulated in HaCaT cells by IL-4 (Fig. 2). Similar to IFNγ, IL-4 increased TGM2 gene transcription two folds (Fig. 2F). However, the gene transcriptions of both loricrin and TGM1 in HaCaT cells were unaffected by IL-4.

The effects of Th17 cell cytokines, IL-17A, and IL-22, on epidermal differentiation marker gene transcription in HaCaT cells

The cytokine production profile of Th17 cells is characterized by IL-17A, IL-17F and IL-22. In HaCaT cells, IL-17A had no effect on gene transcription of filaggrin, loricrin, involucrin, KRT10, TGM1 and TGM2 (Fig. 3). Similar to IL-17A, IL-17F did not change mRNA levels of epidermal differentiation markers we measured (data not shown). In contrast to IL-17A and IL-17F, IL-22 decreased KRT10 gene transcription in HaCaT cells (Fig. 4D). Like IFNγ and IL-4, IL-22 also increased TGM2 mRNA level in HaCaT cells (Fig. 4F). However, IL-22 had no effect on mRNA levels of filaggrin, loricrin, involucrin and TGM1 (Fig. 4).

The effects of IFNγ, IL-4, IL-17A and IL-22 on mRNA levels of anti-microbial peptides in HaCaT cells and NHKs

In contrast to NHKs (Noh *et al.*, 2010), the changes in cornified envelope-associated gene transcription in HaCaT cells were virtually unaffected (Fig. 1-4). Next, we evaluated that the gene transcription of epidermal anti-microbial peptides, LL37 and HBD2, was analyzed due to its role in skin barrier function and to its significance of skin diseases (Schröder and Harder, 1999). IFNγ significantly decreased mRNA levels of LL37 in NHKs whereas it did not affect LL37 levels in HaCaT cells (Fig. 5A and 5B). In contrast, IFNγ increased HBD2 gene transcription in both HaCaT cells and NHKs (Fig. 5A and 5B).

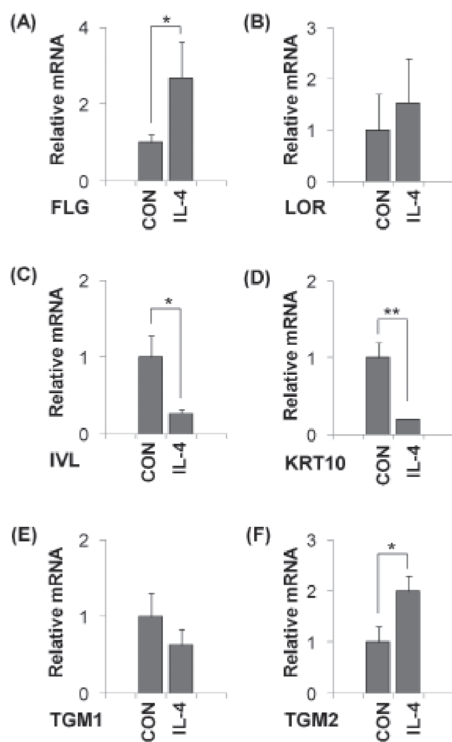

Fig. 2. Effects of IL-4 on mRNA expressions of cornified envelope-associated genes in HaCaT cells. HaCaT cells were treated with 50 ng/ml of IL-4. Total RNA was extracted, and qRT-PCR was performed for (A) FLG, (B) LOR, (C) IVL, (D) KRT10, (E) TGM1 and (F) TGM2. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression (n=3) *p<0.05 and **p<0.01.

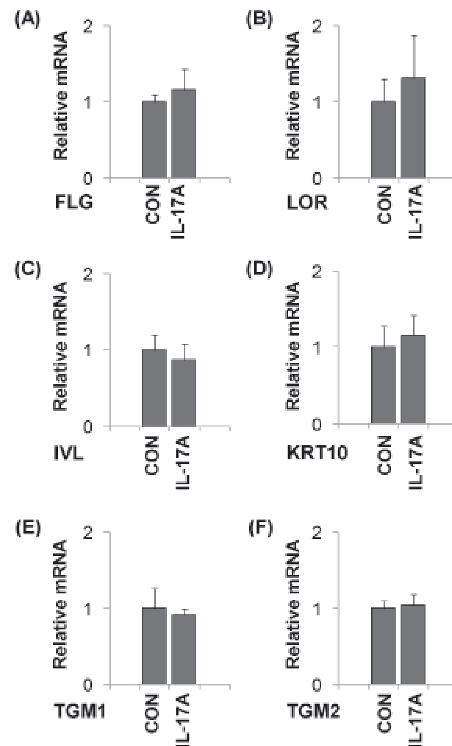

Fig. 3. Effects of IL-17A on mRNA expressions of cornified envelope-associated genes in HaCaT cells. HaCaT cells were treated with 50 ng/ml of IL-17A. Total RNA was extracted, and qRT-PCR was performed for (A) FLG, (B) LOR, (C) IVL, (D) KRT10, (E) TGM1 and (F) TGM2. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression (n=3).

IL-4 decreased mRNA levels of HBD2 in both HaCaT cells and NHKs (Fig. 5C and 5D). LL37 expression was unchanged in both NHKs and HaCaT cells at 24 hours after the treatment of IL-4. IL-17A significantly up-regulated HBD2 gene transcription in both NHKs and HaCaT cells (Fig. 5E and 5F). Although LL37 was significantly up-regulated in NHKs by IL-17A, there was no change in LL37 mRNA levels of HaCaT cells (Fig. 5F). IL-22 up-regulated the mRNA level of HBD2 in NHKs (Fig. 5G) and no effect was observed in HaCaT cells (Fig. 5H). Except IL-22, HBD2 transcriptional profiles in response to IFNγ, IL-4 or IL-17A were similar between HaCaT cells and NHKs. However, the LL-37 transcriptional response profile of HaCaT cells to Th cell cytokines was different from that of NHKs.

DISCUSSION

Cornified envelope-associated proteins, such as filaggrin, loricrin and KRT10, are important in the development of normal skin permeability barrier (Cork *et al.*, 2006; Cork *et al.*, 2009; Brown *et al.*, 2012). Currently, abnormal skin barrier development is regarded as a primary etiologic factor to cause various dermatologic conditions like atopic dermatitis. We previously analyzed mRNA expression profiles of cornified envelope-associated genes in NHKs after the treatments of Th cell cytokines such as IFNγ, IL-4, IL-17A and IL-22 (Noh *et al.*, 2010). Although NHKs are the cell of choice to study molecular mechanisms of epidermal differentiation in two dimensional cell culture, the use of primary cells like NHKs is practically difficult when developing high-throughput assay systems for drug discovery. As an alternative system, the immortalized keratinocyte cell lines like HaCaT cells have been widely used in epidermal functions of NHKs (Boukamp *et al.*, 1988; Ryle *et al.*, 1989; Breitkreutz *et al.*, 1998).

In the present study, we evaluated whether Th cell cytokines affected cornified envelope-associated genes in HaCaT cells as they did in NHKs (Noh *et al.*, 2010) and compared the transcriptional profiles between HaCaT cells and NHKs. As shown in Table 1, HaCaT cells and NHKs have distinctive

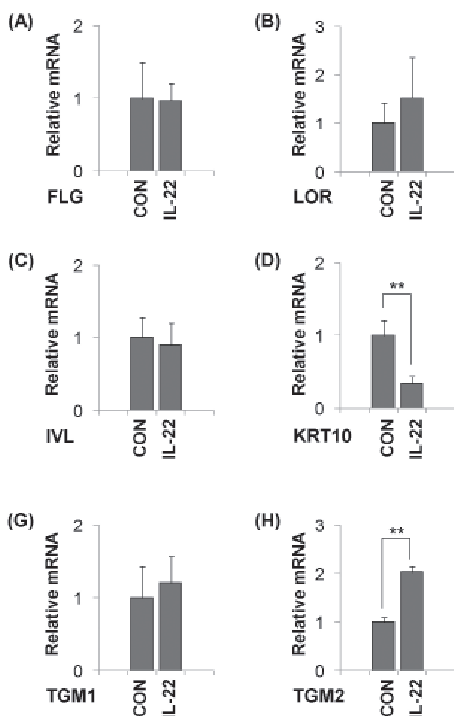

Fig. 4. Effects of IL-22 on mRNA expressions of cornified envelope-associated genes in HaCaT cells. HaCaT cells were treated with 50 ng/ml of IL-22. Total RNA was extracted, and qRT-PCR was performed for (A) FLG, (B) LOR, (C) IVL, (D) KRT10, (E) TGM1 and (F) TGM2. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression (n=3), ***p*<0.01.

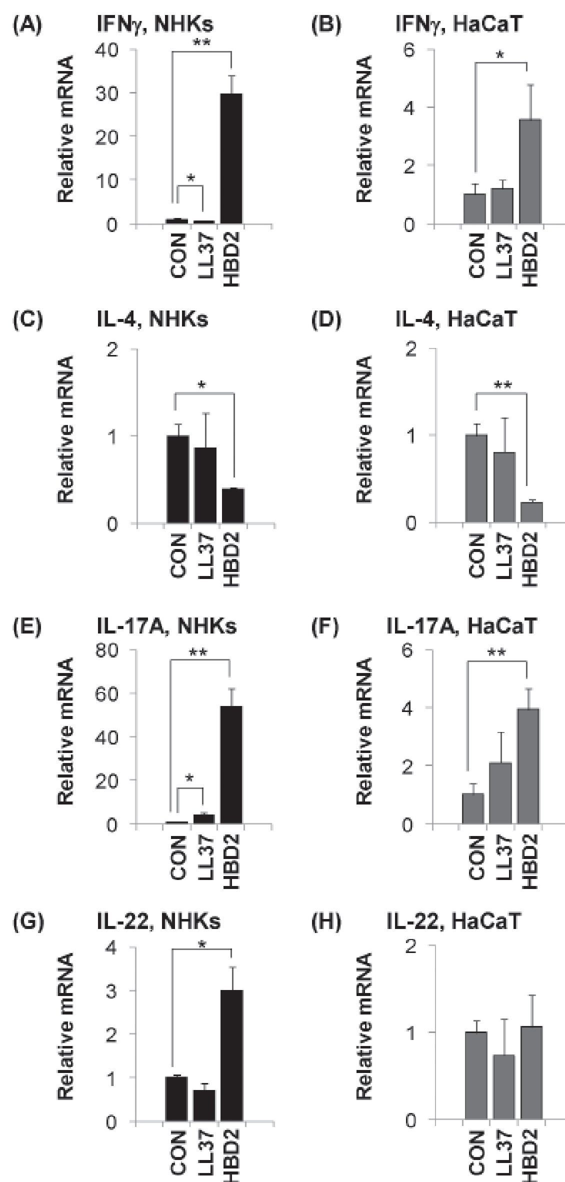

Fig. 5. Effects of IFNγ, IL-4, IL-17A and IL-22 on LL37 and HBD2 gene transcription in NHKs and HaCaT cells. NHKs and HaCaT cells were cultured described in Materials and Methods. INFγ, IL-4, IL-17A and IL-22 were treated in NHKs or HaCaT cells for 24 hours. Total RNA was extracted, and qRT-PCR was performed for LL37 and HBD2. Values represent the mean expression ± SE of the mRNA of the various genes relative to human GAPDH expression (n=3) **p*<0.05 and ***p*<0.01.

Table 1. Summary of transcriptional profiles of epidermal differentiation marker genes in HaCaT cells and NHKs in response to Th cell cytokines

	IFNγ		IL-4		IL-17A		IL-22	
	HaCaT	NHKs	HaCaT	NHKs	HaCaT	NHKs	HaCaT	NHKs
FLG	NC	↓↓↓	↑	↓↓	NC	↓↓	NC	↓
LOR	NC	↓↓	NC	↓	NC	↓↓	NC	↓
IVL	NC	NC	↓↓	NC	NC	NC	NC	NC
KRT10	NC	↓↓↓	↓↓	↓↓↓	NC	↓↓	↓↓	↓↓
TGM1	NC	↑↑↑	NC	NC	NC	↑	NC	NC
TGM2	↑↑	↑↑↑	↑	NC	NC	NC	↑	NC
LL37	NC	↓	NC	NC	↑↑	NC	NC	NC
HBD2	↑↑↑	↑↑↑	↓↓	↓↓	↑↑↑	↑↑↑	NC	↑↑

The mRNA levels of epidermal differentiation markers in HaCaT cells in response to Th cell cytokines were compared with those in NHKs which we previously reported (Noh *et al.*, 2010). The symbols ↑ and ↓ represent up-regulation and down-regulation, respectively. NC denotes 'no significant change in mRNA expression'.

transcriptional profiles of cornified envelope-associated genes when T helper cell cytokines were treated for 24 hours. For example, the mRNA expression of filaggrin and loricrin in HaCaT keratinocytes after the treatment of INFγ, IL-17A or IL-22 were unchanged, although the changes in NHKs were significant (Noh *et al.*, 2010). Interestingly, IL-4 increased filaggrin gene transcription in HaCaT cells whereas it significantly decreased in NHKs (Table 1). Therefore, even though HaCaT cells have been widely used for the study of epidermal differentiation and proliferation, we found a clear difference between HaCaT cells and NHKs in regards to the expression patterns of cornified envelope-associated genes after treating cells with INFγ, IL-17A or IL-22.

Although the gene expression pattern of cornified envelope-associated proteins in HaCaT cells is different from that in NHKs, HBD2 gene transcription in response to INFγ, IL-4 and IL-17A was similar between HaCaT cells and NHKs (Fig. 5, Table 1). In addition, the up-regulation of TGM2 gene transcription was observed when both HaCaT cells and NHKs were treated with IFNγ. In NHKs, IFNγ increased TGM1 gene transcription, but TGM1 was not responsive to IFNγ in HaCaT cells (Table 1). In spite of minor differences in the responses for IL-17A and IL-22 between HaCaT cells and NHKs, we can conclude that HBD2 and TGM2 gene expression responses are similar between HaCaT cells and NHKs.

HaCaT cells are the immortalized keratinocyte line (Boukamp *et al.*, 1988). Because HaCaT cells have a high differentiation potential in cell culture based on the expression of various epidermal differentiation markers, this cell line has been widely used as an alternative for NHKs (Grabbe *et al.*, 1996; Lehmann, 1997). However, the differences in Th cell cytokine-dependent gene transcription profiles of cornified envelope-associated proteins between HaCaT cells and NHKs suggest that HaCaT cell system in culture has a limitation to study the relationship between skin barrier disruption and related skin diseases where Th cells play a predominant role in pathophysiological changes. Recently, we reported that membrane associated protein 17 (MAP17), also known as PDZK1IP1, regulates Th cell cytokine-dependent suppression of filaggrin gene transcription (Noh *et al.*, 2010). In HaCaT cells, MAP17 is not expressed or expressed at a lower level (Guijarro *et al.*, 2007). Therefore, the down-regulated MAP17 expression in HaCaT cells may explain the difference in the gene transcription of cornified envelope-associated proteins like filaggrin in NHKs at a molecular level. Although the IFNγ-dependent changes in HBD2 and TGM2 are similarly working in both HaCaT cells and NHKs, it is currently unknown for common cellular mechanisms to explain the IFNγ-dependent response in both NHKs and HaCaT cells. In order to define the strength and limitation of HaCaT cells to study molecular and cellular mechanism to regulate epidermal functions, further studies will be required.

In conclusion, HaCaT cells and NHKs have the different Th cell cytokine-dependent transcriptional profiles of epidermal differentiation markers, which is important in the skin permeability barrier formation. In contrast, the regulation of HBD2 gene transcription by INFγ, IL-4 and IL-17A, which are representative cytokines for Th1, Th2 and Th17 responses, respectively, are similar between HaCaT cells and NHKs. These results suggest that the careful interpretation may require to exploit the results from HaCaT cell systems for the extrapolation to *in vivo* epidermal functions, especially in pathological conditions involved in the abnormal cornified envelope-associated gene expression.

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