1521-0103/384/2/248–253$35.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

dx.doi.org/10.1124/jpet.122.001412
J Pharmacol Exp Ther 384:248–253, February 2023

The Journal of PHARMACOLOGY And Experimental Therapeutics

Human Induced Pluripotent Stem Cell–Derived Keratinocyte-Like Cells for Research on Protease-Activated Receptor 2 in Nonhistaminergic Cascades of Atopic Dermatitis

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Received August 17, 2022; accepted October 6, 2022

ABSTRACT

Keratinocytes are the most abundant cells in the epidermis, and as part of the frontline immunologic defense system, keratinocytes function as a barrier to exogenous attacks. Protease-activated receptor 2 (PAR2) is expressed in human keratinocytes and activated in several inflammatory conditions, such as atopic dermatitis (AD). In this study, we demonstrated the differentiation of human induced pluripotent stem cell into keratinocytes by the improved, robust differentiation procedure and confirmed that human induced pluripotent stem cell–derived keratinocyte-like cells (iKera) express PAR2, which is activated by external addition of the ligand peptide and trypsin. The activation of PAR2 led to the release of calcium from intracellular calcium storage, followed by the release of the proinflammatory cytokine tumor necrosis factor α. Moreover, PAR2 antagonist I-191 (CAS No. 1690172-25-8) inhibited calcium release in a dose-dependent manner. This is the first study to demonstrate that iKera expresses a functional PAR2 protein. Furthermore, our results indicate crosstalk between the PAR2- and IL-4–mediated inflammatory axes in iKera, suggesting that iKera can be used as a platform for a broad range of mechanism-targeted drug screening in AD.

SIGNIFICANCE STATEMENT

This is the first study to provide evidence that human induced pluripotent stem cell–derived keratinocyte-like cells (iKera) express functional protease-activated receptor 2 (PAR2). Furthermore, this study demonstrated in iKera that the IL-4 inflammatory axis can crosstalk with the PAR2-mediated inflammatory axis in keratinocytes. To the best of our knowledge, this is the first report to indicate that iKera can be used for research and as a drug screening platform for atopic dermatitis.

Introduction

Theoretically, human induced pluripotent stem cells (hiPSCs) are an infinite source of somatic cells that can be used in drug development and toxicity studies. Methodologies for keratinocyte differentiation from pluripotent stem cells have been well established (Itoh et al., 2013; Gledhill et al., 2015; Kim et al., 2018). For pharmaceutical studies, the HaCaT cell line, a spontaneously immortalized human keratinocyte, is frequently used for in vitro skin-related research and drug screening systems and has a standard epidermal differentiation ability. However, HaCaT cells have a hypotetraploid karyotype (Nagy et al., 2013) and genetic abnormalities, such as mutations in p53 (Lehman et al., 1993). In this study, we aimed to establish a simple and mechanism-targeted drug screening platform by extensively studying the potential of human induced pluripotent stem cell–derived keratinocyte-like cells (iKera) as an alternative to the HaCaT cell line.

Keratinocytes are the most abundant cell type in the epidermis. They settle on the basement membrane and maintain the keratinized epidermal barrier, which provides protection against exogenous attacks. Keratinocytes not only form a structural barrier but also act as sensors for external assaults, and in response, secrete various proinflammatory cytokines to initiate different biologic responses (Chieosilapatham et al., 2021).

Atopic dermatitis (AD) is characterized by sustained chronic epidermal pruritus caused by an aggravating, vicious cycle of pruritus, scratches, barrier destruction, inflammation, and reinforcement of peripheral sensory nerves (Rerknimitr et al., 2017). The quality of life of patients with AD is considerably disrupted; therefore, it is necessary to identify new therapeutic molecular targets. In a study on humans, local antihistamines alleviated itching caused by mast cell degranulation in controls but not in patients with AD (Steinhoff et al., 2003). This strongly suggests that the

This study was supported by a grant from the KOSÉ Cosmetology Research Foundation [Grant No. J-18-29].
No author has an actual or perceived conflict of interest with the contents of this article.
dx.doi.org/10.1124/jpet.122.001412.
This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: AD, atopic dermatitis; hiPSC, human induced pluripotent stem cell; iKera, induced pluripotent stem cell–derived keratinocytes-like cell; IL, interleukin; PAR2, protease-activated receptor 2; qPCR, quantitative polymerase chain reaction; Th2, helper T cell; TNFα, tumor necrosis factor α; TSLP, thymic stromal lymphopoietin.

inefficacy of the histaminergic cascade in alleviating itching in AD patients is due to the presence of other nonhistaminergic targets. Furthermore, the administration of codeine to patients with AD increased endogenous tryptase by fourfold compared with that in healthy controls. Interestingly, intracutaneous injection of endogenous protease-activated receptor 2 (PAR2) agonists provoked enhanced and prolonged itching in patients with AD (Steinhoff et al., 2003).

PAR2 is a G-protein–coupled receptor activated by cleavage of its own blocking N-terminal peptide by endogenous tryptases (Saifeddine et al., 1996). Mice with epithelial PAR2 overexpression showed AD-like skin inflammation, scratching bouts, and skin remodeling (Smith et al., 2019), with an increased density of nerve fibers (Buhl et al., 2020) when sensitized to house dust. Kawagoe et al. (2002) developed PAR2-deficient mice and showed that PAR2 deficiency palliates pathologic skin remodeling caused by a topical application of picryl chloride or oxazolone. These reports strongly suggest a potential role for keratinocyte PAR2 in AD.

Recently, the interleukin (IL)-4/IL-13 receptor blockade using antibodies was found to be an effective therapeutic strategy for the treatment of AD (Kim et al., 2022). IL-4 and IL-13 are primarily secreted by helper T cells (Th2) and mast cells and directly affect keratinocyte phenotypes. To date, the crosstalk between IL-4/IL-13–related responses and PAR2 remains unclear.

From the results of this study, we suggest the use of iKera as a potentially useful platform that can be applied to drug screening systems focusing on the vicious PAR2-mediated inflammatory cycle involving crosstalk with the Th2 cytokine axis.

Materials and Methods

hiPSCs. The hiPSC lines (RIKEN-2F and 253G1) were obtained from the Laboratory for Pluripotent Cell Studies, RIKEN Center for Developmental Biology, Tukuba-city, Ibaragi, Japan.

Maintenance and Keratinocyte Differentiation of hiPSCs. The hiPSC lines were maintained on plastic dishes (Corning Inc., NY) coated with 0.5 μg/cm^2 iMatrix511 Silk (Nippi Inc., Tokyo, Japan), using StemFit AK02N (Ajinomoto, Tokyo, Japan) as the culture medium. The hiPSCs were differentiated into keratinocytes based on the previously reported methods with modifications (Guenou et al., 2009; Kogut et al., 2014). Undifferentiated hiPSCs were seeded at a density of 15,000 cells per cm^2 on a 6-well plate coated with 0.5 μg/cm^2 iMatrix511 Silk, using StemFit AK02N supplemented with 10 μM ρ kinase inhibitor Y-27632 (Sellec Inc., Tokyo, Japan) 2 days before the first day of differentiation (day 1). Upon initiation from epiblast to ectodermal-differentiating cells, the medium was changed to N2B27 medium consisted with Dulbecco's modified Eagle medium/F12 (Fuji Film Wako Chemical Inc., Miyazaki, Japan) and Neurobasal medium (1:1; Thermo Fisher Scientific, Waltham, MA) supplemented with 0.1 mM nonessential amino acids, 1 mM glutamine, 55 μM 2-mercaptoethanol, 1% N-2 supplement (Thermo Fisher Scientific), 2% B-27 supplement (Thermo Fisher Scientific), 50 μg/mL ascorbic acid 2-phosphate (Merck KGaA, Darmstadt, Germany), 0.05% bovine serum albumin (Merck), and 100 ng/mL FGF-basic (Nacalai Tesque Inc., Kyoto, Japan). This process was reported by Kogut et al. (2014). Here, we prolonged this process from the originally reported 24 hours to 48 hours. We observed that most of the central-part of the colony of human iPSCs seems to keep their pluripotency, but the edge of the colonies slightly enlarge their cytoplasm. We found this process stabilized their future direction in the further ectodermal differentiation processes in avoidance of unexpected sudden death. Therefore, we think that this process might change the iPSCs internally from pluripotent epiblast state to ectodermally directed state. Depending on internal states of iPSC, this process should be optimized between 48 and 72 hours. On day 3, the medium was changed to Defined Keratinocyte-SFM (without adding the attached supplement) supplemented with the epitherial ectodermal cell induing mixture consisting of 0.5 μg/mL hydrocortisone, 1 μM all-trans-retinoic acid, 25 ng/mL hBMP-4 (Thermo Fisher Scientific), 2.4 μg/mL adenine, 1.37 ng/mL triiodothyronine, 0.3 mM ascorbic acid 2-phosphate, and 2 μM forskolin. On day 5 and where after, the medium was changed to Defined Keratinocyte-SFM (with adding the attached supplement) supplemented with the epitherial ectodermal cell induing mixture every two days. The above set of supplements was reported by Guenou et al. (2009). Here, we used similar supplement compositions except originally used cholera toxin was replaced by forskolin Keratinocyte-SFM. We confirmed that this method can differentiate RIKEN-2F and 253G1 cell lines to keratinocyte-like cells with high reproducibly. Please see the time table shown in Supplemental Fig. 1.

TABLE 1
Primer pairs for qPCR

Primers for qPCR	Sequence
Gene name	
par2	
Fw	TGCCTGAGCAGCTCTTGGTGGGAGA
Rv	AGGCAGAGGCTGTGAGGAAGGCTGG
kertin 1	
Fw	GCATCACTAACAAATATGCTTGGC
Rv	AAACTTCATTGGGAAACAGCAGAAA
keratin 10	
Fw	AGTCTCCCTTCACACAGACCATTAT
Rv	TCCATAGACCATCAAGACAGAAGTGT
keratin 14	
Fw	GAGATGCAGATTGAGAGCCTGAAG
Rv	CCTCTGTCTTGGTGAAGAACCATTC
18s ribosomal RNA	
Fw	TCAACTTTCGATGGTAGTCGCCGT
Rv	TCCTTGGATGTGGTAGCCGTTTCT
sema-3a	
Fw	ACCCAACTATCAATGGGTGCCTTA
Rv	AACACTGGATTGTACATGGCTGGA
tnf α	
Fw	CCGAGGCAGTCAGATCATCTT
Rv	AGCTGCCCCTCAGCTTGA
tslp	
Fw	TAGCAATCGGCCACATTGCC
Rv	CTGAGTTTCCGAATAGCCTG
ngf	
Fw	TGTGGGTTGGGGATAAGACCA
Rv	GCTGTCAACGGGATTTGGGT

Quantitative Polymerase Chain Reaction Analysis. Total RNA was extracted from the cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of 50 ng of RNA was performed using ReverTra Ace (Toyobo, Osaka, Japan) with oligo (dT) primers. To investigate keratinocyte differentiation and PAR2 ligand- and IL-4–stimulated induction of *tumor necrosis factor α* (*tnfα*) gene and other gene expression, a quantitative polymerase chain reaction was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) with gene-specific primer sets designed using Primer3. All experiments were performed using independently-prepared three samples. All gene expression levels were normalized to internal 18S ribosomal RNA expression levels. Primer pair sequences are listed in Table 1.

ELISA. Quantification of TNFα in the medium was performed using the TNF alpha ELISA Kit (#88-7346-22; Thermo Fisher Scientific), following the manufacturer's instructions. Quantification of thymic stromal lymphopoietin (TSLP) in the medium was performed using Human TSLP ELISA Kit (ab155444, Abcam, Cambridge, UK), following the manufacturer's instructions.

Immunofluorescent Staining. Cells were fixed in 4% paraformaldehyde for 5 minutes at 25°C. The cells were then washed twice

TABLE 2
Antibody list

First Antibody		
Keratin 10	Poly 19054	Biolegend
Keratin 14	SC-53253	Santacruz
PAR-2	SC-13504	Santacruz
b-Actin	SC-1615	Santacruz
Second Antibody		
Donkey anti-mouse IgG(H+L) Alexafluo488	A-21202	ThermoFisher Scientific
Donkey anti-rabbit IgG(H+L) Alexafluo546	A-10040	ThermoFisher Scientific
Goat anti-mouse IgG(H+L)-HRP	SC-2005	Santacruz
Donkey anti-goat IgG(H+L)-HRP	SC-2020	Santacruz

with Tris-buffered saline containing 0.2% Tween-20 and subsequently treated with a blocking solution (Nacalai Tesque) for 30 minutes at room temperature. The first antibody-containing blocking agent was added and incubated overnight at 4°C with paraffin sealing to prevent evaporation. The cells were then washed thrice with Tris-buffered saline containing 0.2% Tween-20 and immersed in the second antibody-containing blocking agent for 1 hour at room temperature. After washing thrice, fluorescent signals were observed using a fluorescence microscope (Nikon Instruments, Tokyo, Japan). The primary and secondary antibodies used are listed in Table 2.

Observation of PAR2 Stimulation and Intracellular Calcium Release. Calcium imaging was performed using a Nikon Eclipse Ti2 inverted microscope (Nikon Instruments) with a Fluo-4 AM Ester (Biotium Inc., Fremont, CA). Fluo-4 AM was loaded onto the iKera for 30 minutes at 37°C. After washing with a prewarmed Defined Keratinocyte-SFM medium with the addition of the attached supplement, we started time-lapse recordings and added the PAR2 ligand peptide or trypsin. The strength of fluorescence in randomly selected cells ($n = 8$) was evaluated using NIS Elements software (Nikon Instruments).

IL-4 Stimulation and PAR2 Protein Upregulation. IL-4 (10 or 50 ng/mL) was added to the culture medium to treat iKera on the differentiation day 30 for 3 days. The cells were washed once with PBS(-) and lysed using RIPA buffer with a protease inhibitor cocktail (#08714; Nacalai Tesque). SDS-PAGE using 1.0-mm thick 4%–12% gradient gel with Bolt electrophoresis System (Thermo Fisher Scientific) was used. Proteins were transferred onto a polyvinylidene fluoride membrane (Thermo Fisher Scientific). The primary and secondary antibodies used are listed in Table 2. The chemiluminescent reaction was induced by the addition of horseradish peroxidase substrates (Cytive, Tokyo, Japan) and visualized and quantified using Fusion Solo S (M&S Instruments Inc., Osaka, Japan).

Statistical Analysis. Statistical analyses were performed using EZR software (Jichi Medical University, Japan) (Kanda, 2013). For multiple comparisons, significant differences were determined using one-way ANOVA, followed by post hoc testing using the Tukey-Kramer test. A Student's t test was performed for comparisons between the two samples. Statistical significance was set at $P < 0.05$.

Results

Confirmation of Keratinocyte Differentiation. Keratinocyte differentiation was confirmed using fluorescent immunostaining of keratin 10 and 14 proteins and mRNA expression of *keratin* 1, 10, and 14 using the primers listed in Table 1. Between differentiation days 30 and 40, the cells were fixed, and fluorescent immunostaining was performed (Fig. 1A) using the antibodies listed in Table 2. Furthermore, we observed that iKera expressed PAR2 (Fig. 1B). The expression of *par2* and *keratin 1, 10, and 14* mRNA in iKera was also confirmed, as shown in Fig. 1C.

PAR2 Agonist-Induced Intracellular Calcium Spark and Its Chemical Inhibition. We observed intracellular calcium sparks following the addition of 100 μM of the PAR2 agonist peptide SLIGKV using two iPSC lines, RIKEN2F (Fig. 2A; Supplemental Video 1) and 253G1 (Fig. 2B; Supplemental Video 2). We also observed the immediate elevation of intracellular calcium concentration by the addition of 0.25 mg/mL trypsin into the culture medium using RIKEN2F (Fig. 2C; Supplemental Video 3). The calcium sparks induced by the addition of SLIGKV in 253G1

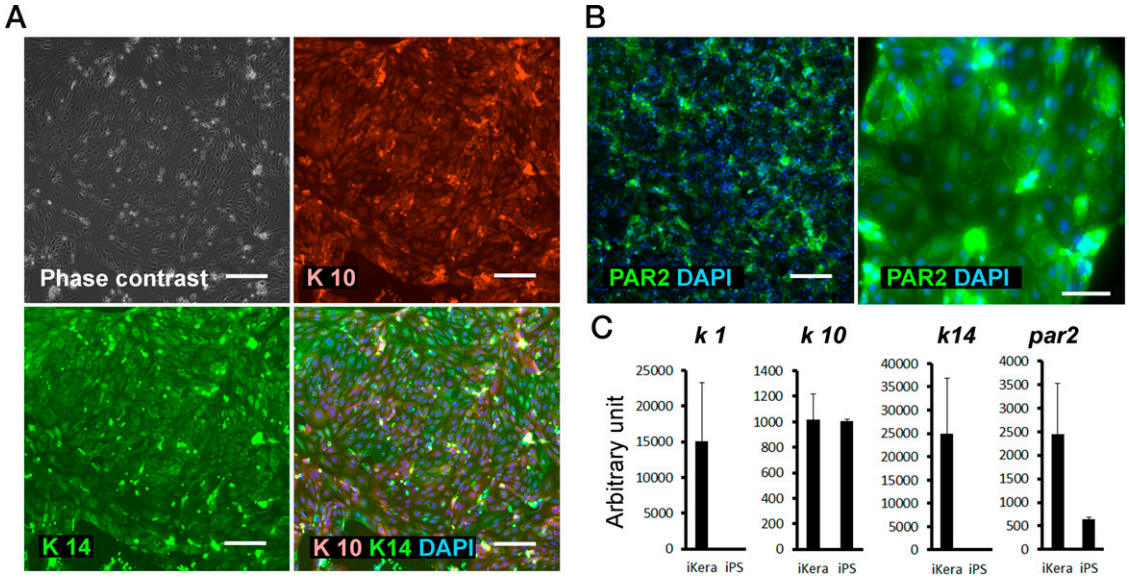

Fig. 1. Confirmation of keratinocyte differentiation. (A) Phase-contrast image of human iKera; immunohistochemical images for Keratin 10 (K10: red), Keratin 14 (K14: green), and merged image with nuclear staining with DAPI (blue). (B) Immunohistochemical staining for PAR2 in lower (left) and higher (right) magnification images. Scale bars, 200 μM in (A) and (B) (left panel); (B) (right panel), 100 μM. (C) Comparison of mRNA levels of *keratin 1* (*k1*), *10* (*k10*), *14* (*k14*), and *par2* with undifferentiated human iPSCs (RIKEN2F). DAPI, 4',6-diamidino-2-phenylindole.

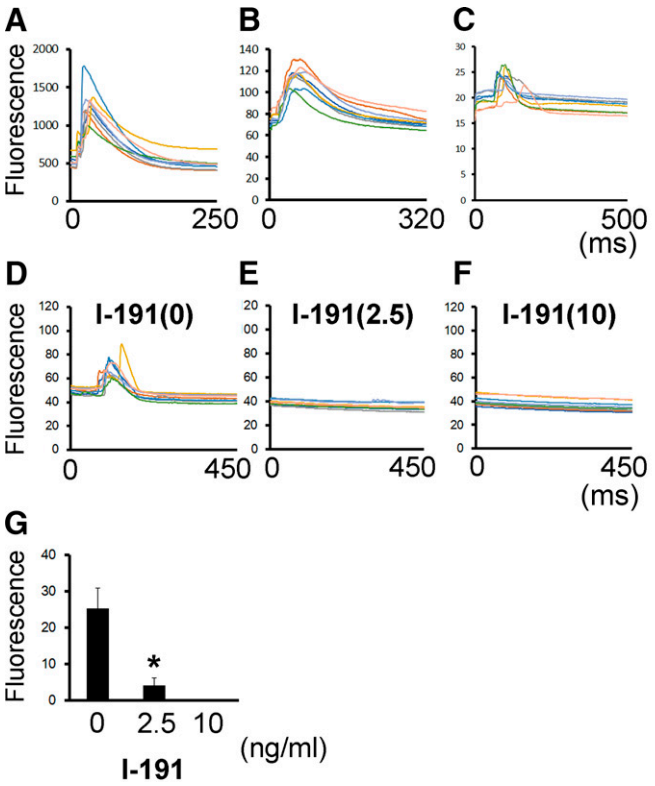

Fig. 2. Calcium sparks induced by PAR2 activation in iKera. (A) RIKEN2F-derived iKera stimulated by PAR2 agonistic peptide SLIGKV. (B) 253G1-derived iKera stimulated by SLIGKV. (C) 253G1-derived iKera stimulated by trypsin. (D–F) 253G1-derived iKera stimulated by SLIGKV in the presence of 0, 2.5, and 10 ng/mL PAR2 antagonist I-191, respectively. (G) Inhibitory effects of I-191 against PAR2-agonistic stimulation measured by the fluorescence change from the base line. $^*P < 0.05$, versus no I-191 treatment.

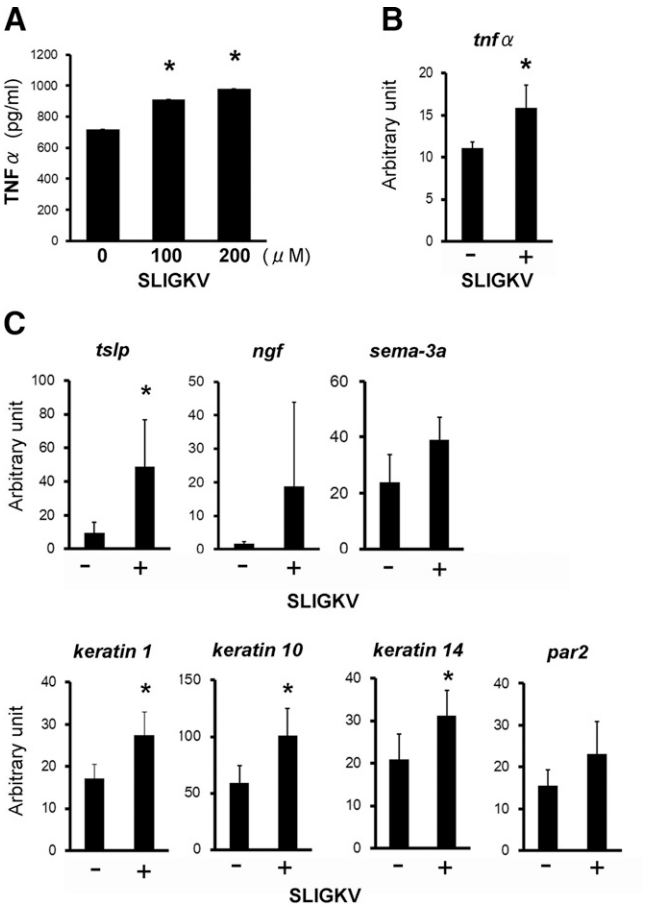

Fig. 3. Effect of PAR2-agonistic peptide treatment in iKera. (A) TNFα secretions from iKera measured by ELISA assay. (B) Elevation of mRNA expression levels of TNFα. (C) The other significant gene expression levels under PAR2 stimulation. $^*P < 0.05$, versus no SLIGKV treatment.

cells were dose-dependently inhibited by I-191 (CAS No. 1690172-25-8) (Fig. 2, D–G).

Induction of TNFα Expression and Release via PAR2 Stimulation. We challenged iKera with a 100- and 200-μM PAR2 agonistic peptide and investigated the resulting TNFα secretion and mRNA expression. We found that TNFα concentration was significantly increased by PAR2 stimulation (Fig. 3A); quantitative polymerase chain reaction (qPCR) analysis quantified the significant increase in *tnfα* mRNA levels using the primers listed in Table 1 (Fig. 3B).

Alteration of Other mRNA Expression Levels by PAR2 Stimulation. We also investigated other significant mRNA expression levels by qPCR using the primers listed in Table 1. Strong itch-mediating cytokines, thymic stromal lymphopoietin (*tslp*), and the principal structural proteins of skin, *keratin 1, 10,* and *14*, were significantly increased (Fig. 3C). The attractant and repellant of peripheral sensory nerves, nerve growth factor (ngf), and semaphorin-3A (sema-3a) showed an each increasing tendency of mRNA level (Fig. 3C) by PAR2 stimulation.

Upregulation of PAR2 Protein and mRNA Expression Levels by IL-4 Treatment. We conducted IL-4 treatment in iKera and investigated PAR2 protein levels by western blotting and found a significant upregulation. We also measured *par2* mRNA expression levels by qPCR and found a significant dose-dependent increase in expression (Fig. 4A).

Alteration of Other mRNA Expression Levels by IL-4 Stimulation. Other AD-related mRNA expression levels were investigated by the use of qPCR, and significant increases in the mRNA levels of *ngf* and *sema-3a* in a dose-dependent manner were found (Fig. 4B).

Costimulation of PAR2 and IL-4/IL-13 Receptor Increased Secretion of TSLP from iKera. We challenged 200 μM PAR2 agonistic peptide to iKera for 5 days; however, we failed to detect any change of TSLP secretion. On the other hand, each 50-ng/ml IL-4 and IL-13 treatment significantly enhanced TSLP secretion. Interestingly, we found that costimulation of PAR2 and IL-4/IL-13 receptor further enhanced the TSLP secretion than the single-stimulation of IL-4/IL-13 receptor (Fig. 4C).

Discussion

AD is sustained chronic inflammation of the epidermis caused by an aggravating, vicious cycle consisting of scratches, barrier destruction, inflammation, and peripheral sensory nerve remodeling (Rerknimitr et al., 2017). In this study, we focused on the significance of keratinocytes in this cycle as an upper acceptor of scratching effects and an amplifier of cell-cell interactions. Various layered, culture-based skin models

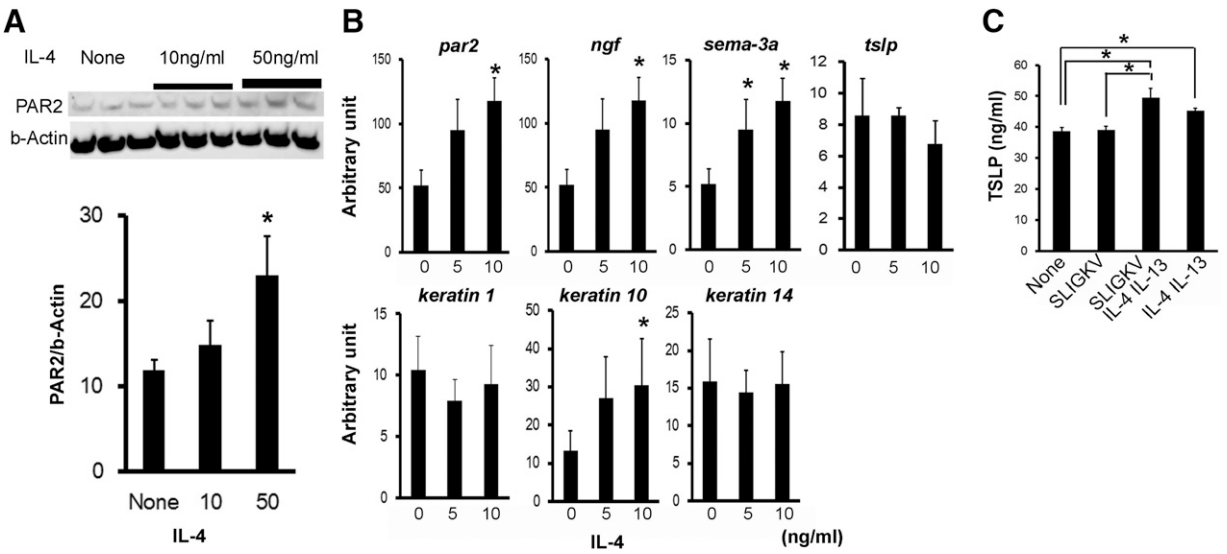

Fig. 4. Effect of IL-4 treatment in iKera. (A) PAR2 protein expression levels were increased by IL-4 dose dependently. (B) *par2* and other significant gene expression levels under IL-4 stimulation. (C) TSLP secretions from iKera measured by Elisa assay (n = 3). The cells were treated with 200 μM SLIGKV and/or 50 ng/ml each IL-4 and IL-13 for 5 days.

using human pluripotent stem cells have been reported; however, they involve complex processes and require more time before they can be applied in testing. We believe that a highly reproducible screening system would require a simple platform. In this study, we demonstrated that iKera is a potential platform that can be applied for high-throughput screening to target PAR2-based mechanisms in AD.

To date, PAR2 expression in iKera has not yet been reported. In this study, we demonstrated that administration of the PAR2 agonist peptide, as well as trypsin treatment, induced intracellular calcium sparks, suggesting that iKera expresses functional PAR2 protein and the corresponding downstream signal transducers. To the best of our knowledge, this study is the first to demonstrate this relationship.

Stimulation of PAR2 induces iKera to release TNFα, suggesting that iKera have a functional PAR2-mediated inflammatory response. Furthermore, IL-4 treatment augmented *par2* mRNA and protein levels in iKera cells. These results suggest that PAR2 plays an important role as an enhancer in the aggravating cycle of AD via crosstalk with Th2 cytokines.

Skin hypersensitivity to physical stimulation causes an unrelenting urge to scratch. This is a significant contributing factor to the development and aggravation of AD. Keratinocytes secrete NGF and semaphorin-3A, which attract and repel itch-sensing neurons called type-C fibers, respectively, which are believed to cause sensory neuron remodeling (Tominaga et al., 2009; Tominaga and Takamori, 2014). However, there are no reports indicating a relationship between PAR2 stimulation or IL-4 treatment and the above peripheral sensory nerve regulating factors. In our study, IL-4 treatment significantly augmented and PAR2 agonistic peptide treatment tended to augment the mRNA expression levels of ngf and semaphorin-3A in iKera, suggesting the possible existence of cross-talk in signaling cascades between PAR2 and the Th2 cytokine-axis in keratinocytes.

So far, no report showed that iKera can secrete TSLP. We showed iKera can secrete TSLP by IL-4/IL-13 receptor activation. Furthermore, PAR2 and IL-4/IL-13 receptor costimulation further enhanced TSLP secretion. This strongly suggests that the PAR2-mediated inflammatory and itchy axis is a coplayer with Th2 cytokine axis in the vicious cycle of AD pathology.

Damaged barrier function in AD skin lesions is sustained by skin remodeling, which is partly due to alterations in keratinocyte phenotypes (Totsuka et al., 2017). Keratinocytes alter the expression of keratin subtype genes in accordance with the symptoms of AD. In our study, each iKera transiently stimulated with a PAR2 agonist or IL-4 altered the mRNA expression levels of different keratin subtype genes. However, most of these gene regulations are not the same as those observed in primary keratinocytes from AD lesions (Totsuka et al., 2017); further studies using iKera are required to mimic AD pathology.

Some studies suggested that keratinocyte PAR2 plays a role in the development and aggravation of AD. However, few studies have been conducted to determine its role in human pathology and pathologic significance. Therefore, further investigations using various chemical inhibitors of keratinocyte PAR2 as research tools are required to explore new drugs for the treatment of AD. In this study, we proposed a simple and efficient drug screening system that targets keratinocytes and PAR2. We believe that PAR2-targeted drugs could be potential breakthroughs in AD treatment.

Authorship Contributions

Participated in research design: Hattori.
Conducted experiments: Nishimoto, Kodama, Yamashita, Hattori.
Performed data analysis: Nishimoto, Kodama, Yamashita, Hattori.
Wrote or contributed to the writing of the manuscript: Hattori.

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