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Rab25 Deficiency Perturbs Epidermal Differentiation and Skin Barrier Function in Mice

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Abstract

Rab25, a member of the Rab11 small GTPase family, is central to achieving cellular polarity in epithelial tissues. Rab25 is highly expressed in epithelial cells of various tissues including breast, vagina, cervix, the gastrointestinal tract, and skin. Rab25 plays key roles in tumorigenesis, mainly by regulating epithelial differentiation and proliferation. However, its role in skin physiology is relatively unknown. In this study, we demonstrated that Rab25 knock-out (KO) mice show a skin barrier dysfunction with high trans-epidermal water loss and low cutaneous hydration. To examine this observation, we investigated the histology and epidermal differentiation markers of the skin in Rab25 KO mice. Rab25 KO increased cell proliferation at the basal layer of epidermis, whereas the supra-basal layer remained unaffected. Ceramide, which is a critical lipid component for skin barrier function, was not altered by Rab25 KO in its distribution or amount, as determined by immunohistochemistry. Notably, levels of epidermal differentiation markers, including loricrin, involucrin, and keratins (5, 14, 1, and 10) increased prominently in Rab25 KO mice. In line with this, depletion of Rab25 with single hairpin RNA increased the expression of differentiation markers in a human keratinocyte cell line, HaCaT. Transcriptomic analysis of the skin revealed increased expression of genes associated with skin development, epidermal development, and keratinocyte differentiation in Rab25 KO mice. Collectively, these results suggested that Rab25 is involved in the regulation of epidermal differentiation and proliferation.

Key Words: Rab25, Skin, Epidermis, Epidermal differentiation, Skin proliferation

INTRODUCTION

Rab25, a member of the Rab11 small GTPase family, plays a central role in the accomplishment and maintenance of epithelial polarity (Goldenring *et al*., 1993; Wang *et al*., 2000; Welz *et al*., 2014). Along with other Rab small GTPases, it regulates polarized membrane trafficking pathways for recycling of integrins or claudins, a feature critical for the initiation of cellular polarity and basolateral-to-apical differentiation. It is especially involved in the regulation of apical recycling endosomes (Casanova *et al*., 1999). The important roles played by Rab25 in the epithelial tissues have been demonstrated in various reports previously, suggesting its deficiency may possibly augment malignancy of epithelial cancers, such as colon cancer (Nam *et al*., 2010; Goldenring and Nam, 2011), triple negative breast cancer, mammary cancer, head and neck squamous cell carcinoma (Seven *et al*., 2015), and skin squamous cell carcinoma (Jeong *et al*., 2019).

Despite its well-established role in cancer development, little is known about its role in epithelial physiology and function. The epidermis, a major epithelial tissue separating the external milieu from the inner body, is composed of 4 layers: stratum corneum, stratum granulosa, stratum spinosum, and stratum basale (Wickett and Visscher, 2006). The epidermal layer is well organized, with connective tissues and supporting connective tissues, which promote hair growth as well as higher collagen density (Kim *et al*., 2017). The epidermis is composed of keratinocytes, proliferating and differentiating outwards from the basal layer. Corneocytes, at the terminal phase, form a physical and chemical barrier against exoge-

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nous substances (Muroyama and Lechler, 2012). Each layer of the epidermis expresses distinctive markers, such as loricrin, involucrin, keratin family members, filaggrin, and integrins, which are critical for epidermal differentiation and functional maturation of the skin barrier. Meanwhile, integrins α6, β4, and β1 are abundantly expressed in the basolateral side of basal cell layer bordering the dermis, where keratinocyte proliferation is highly active (Rodius *et al*., 2007), thus reflecting the essential roles of integrins in the differentiation and proliferation of keratinocytes, and their interaction with extracellular matrices on basal membrane.

Epidermal marker proteins including keratins, loricrin, involucrin, and filaggrin are richly expressed and tightly regulated in keratinocyte differentiation (Bickenbach *et al*., 1995). They are synthesized as pro-protein and sequestered in keratohyalin granules. They are eventually processed or cross-linked to form structural support of the cornified envelope. Keratins 5 and 15 are expressed at the early differentiation stages in basal cell layer, where cell proliferation is active. On the other hand, keratin 1, keratin 10, involucrin, and loricrin appear at the late differentiation stage from the upper spinous cell layer to the cornified layer. During keratinocyte differentiation, integrin expression is down-regulated and structural matrix proteins are up-regulated. Deletion of β1 integrin results in severe epidermal inflammation (Brakebusch *et al*., 2000) and deficiency of α6 integrin leads to abnormal expression of differentiation genes (Rodius *et al*., 2007), reflecting the crosstalk between integrins and epidermal differentiation marker proteins.

Recently, we have demonstrated that Rab25 behaves as a tumor suppressor for skin squamous cell carcinoma development (Jeong *et al*., 2019). Deletion of Rab25 is known to impair the trafficking of integrins, suggesting an important role of Rab25 in the regulation of keratinocyte differentiation and proliferation. Here, we investigated the effects of Rab25 KO on epidermal differentiation and skin barrier function.

MATERIALS AND METHODS

Mice

All animal experiments were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by the IACUC of the Department of Laboratory Animal Resources of Yonsei University College of Medicine, an AAALAC-accredited unit (#001071). Five to nine-week-old 129/J background mice, maintained in specific pathogen-free conditions (SPF), were used for all experiments. We used same age of WT and Rab25 KO mice for all experiment. Rab25 KO mice were genotyped as previously described (Nam *et al*., 2010).

Establishment of Rab25 knockdown cell lines

Recombinant lentiviruses were commercially designed and synthesized using GIPZ lentiviral shRNA vector (Open Biosystems, Huntsville, AL, USA). Lentiviruses were produced by transfection of 293T cells with packaging plasmids PMD2G and psPAX2, using a CalPhos™ Mammalian Transfection Kit (631312, Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Knockdown of Rab25 in HaCaT cells was established by infection with recombinant lentivirus using a polybrene mixture. Stable clones expressing shRNA were selected by further incubation with puromycin (1 μg/ml) and fluorescence of GFP.

Immunohistochemistry

For immunostaining, samples were de-paraffinized and sequentially rehydrated using a descending graded series (100%, 95%, and 70%) of ethanol. Antigen retrieval (S1699, DAKO, Carpinteria, CA, USA) was performed using a pressure cooker. After cooling on ice for at least 1 h, sections were incubated in 3% H_2O_2 for 30 min for blocking endogenous peroxidase. Sections were washed twice with PBS and incubated with protein block serum-free (X0909, DAKO) for 1-2 h at room temperature to reduce non-specific signals. Treatment with M.O.M (BMK-2202, Vector Laboratories, Burlingame, CA, USA) reagent for 1 h was performed with mouse primary antibodies. Primary antibodies were incubated overnight at 4°C. After three washes in PBS, sections were incubated in HRP-conjugated secondary antibody (K4003, DAKO) (K4001, DAKO) for 15 min at room temperature. For immunohistochemistry, DAB (K3468, DAKO) was used for the development of antibodies, and Mayer's hematoxylin (S3309, DAKO) was used for counterstaining. Each experiment was performed using an identical time for DAB development. For immunofluorescence, primary antibodies were detected with Cy3-conjugated anti-mouse IgG and Alexa488-conjugated anti-rabbit IgG. Immunofluorescence images were taken with an EVOS-FL.

For BrdU assay, BrdU (B5002, Sigma, St. Louis, MO, USA) was dissolved in PBS (20 mg/ml) at room temperature and immediately administered to wild-type (WT) and Rab25 knockout (KO) mice by intraperitoneal injection (4 mg/20 g). After 48 h, the mice were sacrificed, and their skin samples were fixed in 4% paraformaldehyde. BrdU staining was performed following the immunohistochemistry protocol detailed above.

Antibodies

The following primary antibodies were commercially purchased: Rab25 (3F12, Novus, Rockford, IL, USA, 1:10K for WB and IHC), Kertin1 (ab185628, Abcam, Cambridge, Cambs, UK, 1:1K for WB and IHC), Keratin 5 (ab52635, Abcam, 1:500 for WB and IHC), Keratin 10 (ab76318, Abcam, 1:3K for WB and IHC), Keratin 14 (ab1851595, Abcam, 1:4K for WB and IHC), BrdU (033990, Novex, Frederick, MD, USA, 1:2K for IF), Involucrin (MA5-11803, Invitrogen, Waltham, MA, USA, 1:100 for WB and IHC), Loricrin (ab24722, Abcam, 1:500 for WB and IHC), Ceramide (ALX-804-196, Enzo, Farmingdale, NY, USA, 1:100 for IHC), and GAPDH (ab181602, Abcam, 1:10K for WB).

Western blotting

For immunoblot using the cell line, cells were harvested and incubated in protein lysis buffer (20 mM Tris (pH 7.4), 0.15 M NaCl, 2.5 mM EDTA, and 1% Triton X-100) for 40 min. For immunoblot using mouse skin, mice were shaved and euthanized in a CO_2 chamber. The skin was carefully cut and collected in 1.5-ml microtubes. Proteins were extracted from the sections using protein lysis buffer (20 mM HEPES (pH 7.0), 0.15 M NaCl, 10% Glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 10 mM β-phosphoglycerate) along with protease and phosphatase inhibitor cocktails (Thermo, MA, USA). All lysates were collected by centrifugation (13,000 rpm, 15 min) and boiled in 1× SDS-PAGE sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10%

glycerol, and 0.01% bromophenol blue) after measurement of protein concentration. Approximately 20 μg protein samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, USA). The membranes were incubated with primary antibodies overnight at 4°C. Signal intensities were measured using the image analysis software Image J (National Institutes of Health, MD, USA).

Quantitative real-time PCR

RNA was extracted from tissues using TRIzol (Life Technologies, Carlsbad, CA, USA). cDNAs were synthesized from 1-μg samples after treatment with DNase (Takara, Kusatsu, Shiga, Japan). It used the ImProm-II™ reverse transcription system (Promega, Madison, WI, USA). POWER SYBR Green Master Mix from Applied Biosystems (4367659, Life Technologies) was used to perform real-time PCR. The specific sequences of primers were as follows:

Rab25;

Fw: CTTAAAAGCTGAGAGTTG, Rv: CTCGCCGATCAGCACCAC

Dlx3;

Fw: GTGCCTTAGGGGTAAGGCTGTCAG, Rv: GGGACCTGCTTCTCTTGGTTGCTC

Elf5;

Fw: GTGGCATCAAGAGTCAAGACTGTC, Rv: CTCAGCTTCTCGTACGTCATCCTG

Foxn1;

Fw: GGCCCTCAATCCTTCCAAAATCGAC, Rv: GCTGGATGCATTGGGTGCAGAGG

Measurement of skin physiology

The dorsal skin of mice was carefully shaven, without any injury, just 1 day before the experiment. TEWL and moisture content were measured by a vapometer (Delfin technologies, Kuopio, Finland) and moisture meter (Delfin technologies, Kuopio, Finland), respectively. Measurement was performed in SPF condition with identical humidity and temperature to reduce variability, and the calculator was perfectly attached to the dorsal skin of mice.

Affymetrix microarray

Mouse skin was shaved, and specimens were collected, with a biopsy punch (Kai medical, BP-40F), in a 1.5-ml conical tube. RNAlater (AM7024, Invitrogen, Waltham, MA, USA) was supplemented in the conical tube immediately to stabilize the RNA, followed by 24-h incubation in the cold room. Total RNA was extracted with TRIzol (Life Technologies) as described above. GeneChip® Mouse Gene 2.0 ST Array was used for platform, and microarray was conducted by Macrogen, Inc (Seoul, Korea). The acquired data were processed by Affymetrix® GeneChip Command Console® Software (AGCC, Thermo).

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined using unpaired Student's *t*-test or one-way ANOVA with Dunnett's multiple comparison. $p<0.05$ was considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RESULTS

Rab25 KO mice have normal skin morphology, but exhibit skin barrier dysfunction

To investigate the expression of Rab25 on mouse skin, we conducted immunohistochemistry staining of the skin of 5-week old mice. As shown in Fig. 1A, Rab25 expression in WT mice was exclusively detected in the epidermis of skin; however, its expression was absent in the skin dermis or subcutis. As expected, Rab25 expression was absent in the epidermis of Rab25 knock-out (Rab25 KO) mice. In addition, the mRNA level of Rab25 in skin specimen was significantly reduced in Rab25 KO mice compared to that in WT mice (Fig. 1B). Interestingly, deficiency of Rab25 did not induce notable histopathological differences in the skin, and Rab25 KO mice exhibited relatively normal skin structure, with intact hair follicles (HF) and sebaceous glands (SG), compared to that in the skin of WT mice (Fig. 1C). Although abnormal features of epidermis were not apparent in Rab25 KO mice, moisture content and trans-epidermal water loss (TEWL) in the dorsal skin of Rab25 KO mice were altered significantly, revealing that Rab25 KO mice might have a dysfunctional skin barrier (Fig. 1D). Increased TEWL, with accompanying low skin hydration, can be observed in dry skin diseases, like diabetes mellitus (Park *et al*., 2011; Kim *et al*., 2019) or atopic dermatitis (Joo *et al*., 2015), reflecting that Rab25 KO mice might have xerotic skin.

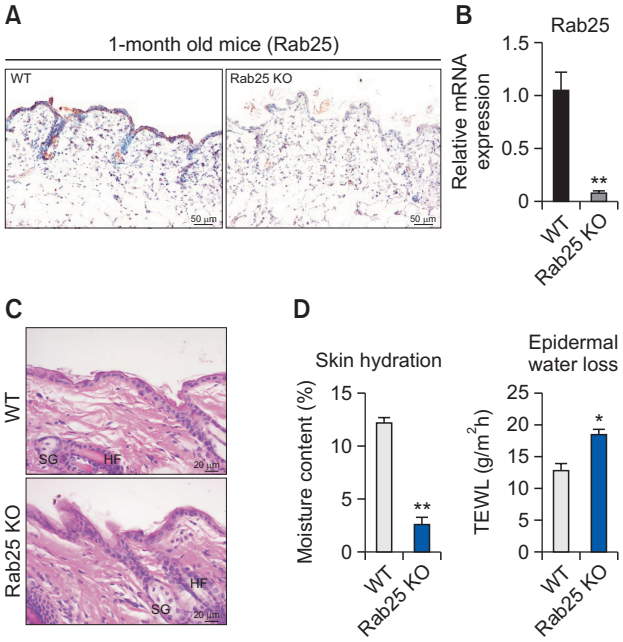

Fig. 1. Histopathological and physiological features of skin in Rab25 KO mice. (A) Representative immunohistochemistry images for Rab25 in 5-week old WT and Rab25 KO mice. (B) mRNA expression of Rab25 was normalized to that of GAPDH. Graphs represent mean ± SEM (Unpaired student's *t*-test, n=4, **$p<0.01$). (C) Representative hematoxylin and eosin (H&E) staining images of WT and Rab25 KO mouse skin (HF: hair follicle, SG: sebaceous gland). (D) Skin hydration and epidermal water loss were measured by close attachment of Delfin's vapometer and moisturemeter on the dorsal skin of 9-week old mice. Graphs represent mean ± SEM (Unpaired student's *t*-test, *$p<0.05$, **$p<0.01$).

Rab25 KO mice show increased keratinocyte proliferation

To identify the reason behind skin barrier dysfunction in Rab25 KO mice, we first investigated the proliferation of epidermal cells in Rab25 KO mice. Bromodeoxyuridine (BrdU), an S-phase proliferation marker, was pre-injected into Rab25 KO mice 2 h before sacrifice, and BrdU-positive cells were detected in the epidermis by immunofluorescence staining. BrdU-positive cells in the skin epidermis were significantly increased in Rab25 KO mice compared to that in control (Fig. 2A, 2B). The majority of BrdU-positive proliferating cells were progenitor cells of the basal cell layer, where keratins 5 and 14 were highly positive. As shown in Fig. 2C and 2D, the number of BrdU- and keratin 5 (K5)-double positive cells was remarkably increased in Rab25 KO mice. Taken together, loss of Rab25 promoted proliferation of keratinocyte but did not induced alteration of its distribution on skin epidermis.

Rab25 KO globally increases the expression of keratinocyte differentiation markers

Intercellular lipids in stratum corneum play a critical role in skin barrier function, for the prevention of epidermal water loss and invasion of pathogen. Patients with defective skin barrier functions, like atopic dermatitis, show altered ceramide composition in the skin (Choi and Maibach, 2005; Park *et al*., 2012; Joo *et al*., 2015). To explore the relation between ceramide composition and barrier defects in Rab25 KO mice, we performed immunohistochemistry staining for ceramide in WT and Rab25 KO mice. Surprisingly, the staining intensity of ceramide in the epidermis barely changed in Rab25 KO mice, and its expression and distribution were indistinguishable from that in WT mice (Fig. 3A).

We investigated other components of stratum corneum, such as, epidermal differentiation markers in KO mice. Indeed, aberrant or increased expression of early differentiation markers like keratins 5 and 14 (Fig. 3B), as well as of late differentiation markers like keratin 1, keratin 10, involucrin, and loricrin (Fig. 3C), was found in the epidermis of Rab25 KO mice. Interestingly, although these markers increased significantly in content, their distribution was not different from that in WT mice.

Rab25 KO is associated with remarkable alteration of multiple biological processes in the skin of mice and in human keratinocyte cell line HaCaT

To clarify further the alteration of keratinocyte differentiation in Rab25 KO mice, we conducted western blot analysis for the differentiation markers in skin. As shown in Fig. 4A, early and late differentiation markers were all up-regulated in the skin of Rab25 KO mice at the protein level, which was in line with the immunohistochemistry findings (Fig. 3B, 3C). To identify whether this event can occur in human skin as well, Rab25 was knocked down using single hairpin RNA (shRNA)

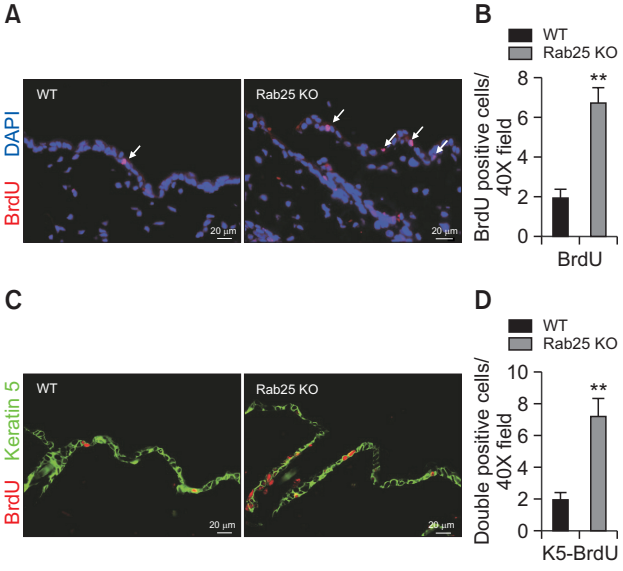

Fig. 2. Increment of proliferating cells in 9-week old Rab25 KO mouse skin. (A) Representative immunofluorescence images of BrdU-positive cells in the skin epidermis of WT and Rab25 KO. White arrow indicated BrdU-positive cells. (B) Quantitation of BrdU-positive proliferating cells in 40×-magnification field of view. Graphs represent mean ± SEM (Unpaired student's *t*-test, $^{**}p<0.01$). (C) Dual immunofluorescence images of BrdU and keratin 5 (K5) in the epidermis of WT and Rab25 KO mice. (D) Quantitation of K5 and BrdU double-positive cells in 40×-magnification field of view. Graphs represent mean ± SEM (Unpaired student's *t*-test, $^{**}p<0.01$).

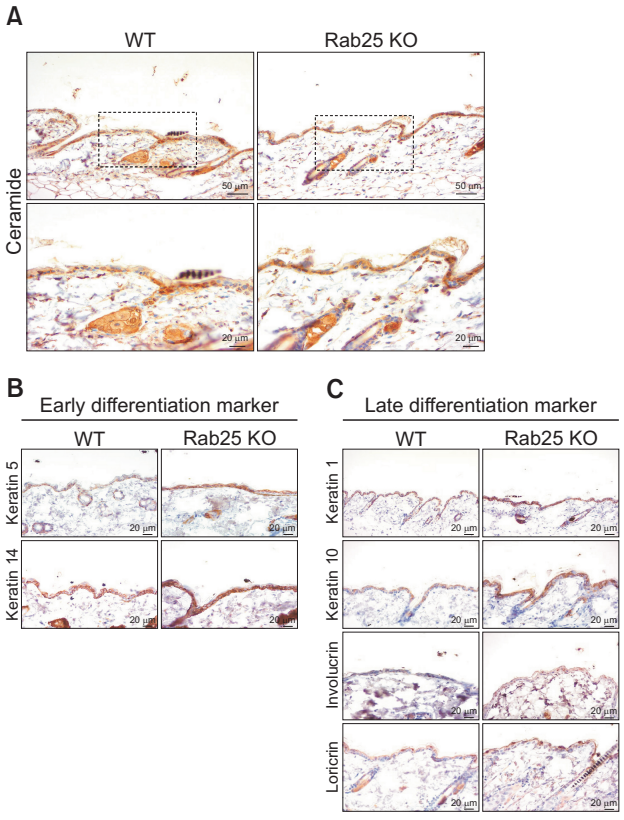

Fig. 3. Characteristics of skin differentiation markers in 9-week old Rab25 KO mice. (A) Representative immunohistochemistry images of ceramide. Lower panels indicate higher magnification of epidermis. (B, C) Immunohistochemistry images of representative keratinocyte differentiation markers. Both (B) early differentiation markers (kerain 5 and keratin 14) and (C) late differentiation markers (kerain 1, keratin 10, involucrin, and loricrin) were exclusively stained in the epidermis of WT and Rab25 KO mice.

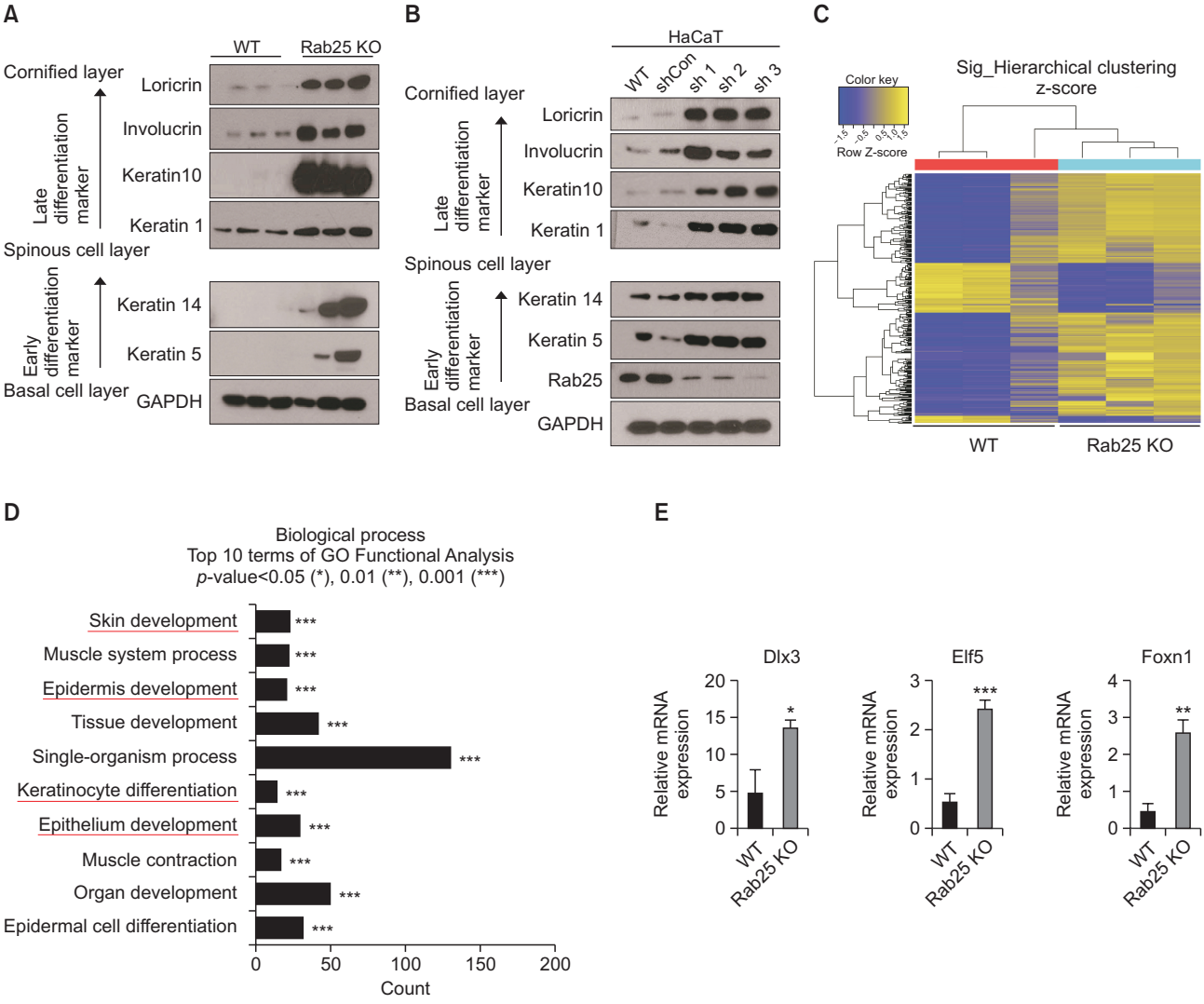

Fig. 4. Alteration of skin differentiation in 9-week old Rab25 KO mice. (A) Images of western blots for representative keratinocyte differentiation markers using skin specimens from WT and Rab25 KO mice. GAPDH was used for housekeeping control. (B) Images of western blots for representative keratinocyte differentiation marker. Knockdown-HaCaT cell (sh1, sh2, and sh3) line was constructed by infection with the recombinant pGIPZ vector, using polybrene mixture. Expression level of the knockdown cell was compared to that of non-infected cell (WT) and vector-conveying cell (shCon). GAPDH was used for housekeeping control. (C) Schematic image of cluster analysis-hierarchical clustering heat map acquired from Affymetrix GeneChip® Mouse Gene 2.0 ST Array (Thermo) using skin specimen of WT and Rab25 KO mice. The heat map was represented and sorted by the normalized value of expression level using each probe. (D) Bar graph indicated top 10 biological processes with the highest *p*-value. GO functional analysis was based on gomap_stat. (E) mRNA expression was normalized with GAPDH. Graphs represent mean ± SEM (Unpaired student's *t*-test, n=4, **p*<0.05, ***p*<0.01, ****p*<0.001).

in HaCaT cells, a human keratinocyte cell line. Consistent with the findings in Rab25 KO mice, all skin markers, including early differentiation and late differentiation markers, were found to be up-regulated overall (Fig. 4B).

Rab25 appeared to be a key mediator of skin differentiation, as shown above. To clarify this finding, we performed transcriptomic analysis using gene-chip microarray for full dorsal skin specimens of Rab25 KO and WT mice. As presented in Fig. 4C, considerable differences in gene expression patterns and hierarchical clusters were seen. Moreover, significant up-regulation of the genes involved in processes such as skin development, epidermis development, keratinocyte differentiation, and epithelium development was observed (Fig. 4D, Table 1), confirming the key role played by Rab25 in epidermal differentiation. For the validation of expression data from gene-chip microarray, we investigated the mRNA level of representative transcription factors, which were involved in keratinocyte differentiation and epithelium development (Fig. 4D, Table 1). Importantly, we observed a significant up-regulation of *Dlx3*, *Elf5*, and *Foxn1* expression in Rab25 KO mice compared to skin from WT mice (Fig. 4E).

DISCUSSION

This study demonstrated that Rab25 deficiency may lead

Table 1. Fold change of expression level on Affymetrix gene array

Classification of gomap_genes					
OutID	GOID	Term	Gene count	*p*-value	Rab25 KO/ WT.fc
Prr9	GO:0043588	Skin development	23	1.354E-19	23.195826
Alox8					3.990096
Col1a1					–2.366208
Col1a2					–2.561034
Foxn1					4.624904
Hoxc13					3.283733
Igfbp5					–5.097948
Krt16					4.779589
Krt36					3.299925
Krt27					14.471341
Krt84					16.987127
Krt6a					9.439672
Dsg4					12.462400
Krtap21-1					42.104232
Msx2					3.291838
Ryr1					–4.751830
Sprr1a					5.974784
Sprr1b					11.170615
Sprr2h					10.017172
Tgm3					5.976207
Sprr4					12.113076
Krt71					10.671418
Krt25					8.066111
Prr9	GO:0008544	Epidermis development	20	4.825E-15	23.195826
Alox8					3.990096
Foxn1					4.624904
Hoxc13					3.283733
Igfbp5					–5.097948
Krt16					4.779589
Krt36					3.299925
Krt27					14.471341
Krt84					16.987127
Krt6a					9.439672
Dsg4					12.462400
Krtap21-1					42.104232
Msx2					3.291838
Sprr1a					5.974784
Sprr1b					11.170615
Sprr2h					10.017172
Tgm3					5.976207
Sprr4					12.113076
Krt71					10.671418
Krt25					8.066111
Prr9	GO:0030216	Keratinocyte differentiation	14	3.665E-13	23.195826
Alox8					3.990096
Foxn1					4.624904
Krt16					4.779589
Krt36					3.299925
Krt84					16.987127
Krt6a					9.439672
Dsg4					12.462400
Msx2					3.291838
Sprr1a					5.974784
Sprr1b					11.170615
Sprr2h					10.017172

Table 1. Continued

Classification of gomap_genes					
OutID	GOID	Term	Gene count	*p*-value	Rab25 KO/ WT.fc
Tgm3					5.976207
Sprr4					12.113076
Prr9	GO:0060429	Epithelium development	30	6.836E-13	23.195826
Alox8					3.990096
Car2					3.634902
Dlx3					3.746855
Elf5					7.438697
Foxn1					4.624904
Hoxc13					3.283733
Igfbp5					–5.097948
Krt16					4.779589
Krt36					3.299925
Krt27					14.471341
Krt84					16.987127
Krt6a					9.439672
Dsg4					12.462400
Krtap21-1					42.104232
Mef2c					–3.516759
Foxc1					2.852506
Msx2					3.291838
Myf6					–3.946508
Sprr1a					5.974784
Sprr1b					11.170615
Sprr2h					10.017172
Stox1					11.114799
Tgm3					5.976207
Sprr4					12.113076
Slc40a1					4.670423
Krt71					10.671418
Tmem100					–2.873409
Krt25					8.066111
Plet1					2.866307

to skin barrier dysfunction in KO mice, as determined by higher trans-epidermal water loss and lower cutaneous hydration than in wild type mice. While the skin morphology was observed to be relatively normal, aberrantly increased expression of epidermal differentiation markers such as loricrin, involucrin, and keratins 5, 14, 1, and 10 was observed in Rab25 KO mice, which might cause perturbation of epidermal physiology. In line with this, depletion of Rab25 with shRNA led to increased expression of differentiation markers in the human keratinocyte cell line, HaCaT, reflecting the critical role played by Rab25 in epidermal differentiation of human skin. Transcriptomic analysis of the skin revealed that Rab25 KO to have increased the expression of genes associated with skin development, epidermal development, and keratinocyte differentiation, thus suggesting that Rab25 is involved in the regulation of epidermal differentiation and proliferation.

Interestingly, Rab25 is linked with the activation of AKT/PI3K pathway by binding and activating AKT (Cheng *et al*., 2012; Fan *et al*., 2015), which promotes the resistance of cancer cells against metabolic stress through enhancing ATP generation and glycogen synthesis. AKT/PI3K pathways are also known to be pivotal in keratinocyte differentiation (Calautti *et al*., 2005). During keratinocyte differentiation, the activation of PI3K pathway, which depends on the activity of EGFR, Fyn/Src kinases, and E-cadherin-mediated adhesion, actually initiates AKT activation, which in turn, promotes the growth arrest and differentiation of keratinocytes, as evidenced by increased expression of filaggrin, loricrin, keratin 1, and keratin 5. Inhibition of PI3K activity with wortmannin or Ly294002 results in the suppression of these differentiation markers and results in cell death. In this regard, our results, showing that Rab25 KO leads to increased expression of differentiation markers, contradict the existing mechanisms underlying epidermal differentiation. It would therefore be worth examining the PI3K/AKT pathway in the skin of Rab25 KO mice to clarify this conflict.

Rab25 facilitates the transport of integrins to the plasma membrane and is critical in membrane recycling along with other Rab11 family proteins (Welz *et al*., 2014), including integrins or receptor tyrosine kinases like EGFR (Dozynkiewicz *et al*., 2012; De Franceschi *et al*., 2015). In line with this, we had previously demonstrated that Rab25 KO leads to the depletion of integrins, β1, β4, and α6 in the skin of mice (Jeong *et al*., 2019), thereby indicating that Rab25 deficiency leads to dysregulation of integrins in keratinocytes. Rodius *et al*. (2007)

reported that deletion of α6 integrin in keratinocytes increases the expression of keratins 1, 10, and 14, and loricrin, involucrin, and filaggrin, which is in good agreement with our findings. Rodius *et al*. (2007) also reported that increased levels of c-Jun, c-Fos, and phospho-Jun, which ultimately activate AP-1 transcription factor, are attributable to the increased differentiation markers in α6 integrin-deficient keratinocytes.

Not only AP-1, but also other transcription factors have been reported to play a critical role in regulating skin homeostasis and keratinocyte differentiation (Park and Morasso, 1999; Tummala and Sinha, 2006; Hwang *et al*., 2011). Previous reports had shown higher expression of ELF5 and DLX3 to occur during Ca^{2+}-induced mouse keratinocyte differentiation *in vitro* (Park and Morasso, 1999; Tummala and Sinha, 2006). Moreover, Hwang *et al*. (2011) had discovered that deletion of Dlx3 using K14-Cre ($Dlx3^{Kin/f}$) mice leads to abnormal keratinocyte differentiation. Simialrly, activation of FOXN1, a transcription factor expressed in the suprabasal layer, promoted the expression of keratinocyte differentiation markers like involucrin and keratin 10, whereas inhibition of PI3K prevented FOXN1-induced differentiation (Janes *et al*., 2004). Moreover, Prowse *et al*. (1999) constructed a transgenic mouse, expressing FOXN1 under the involucrin promoter, and revealed that proliferation of basal keratinocyte was promoted in the transgenic mouse. These findings on FOXN1 were also consistent with our findings in Rab25 KO mice. In our genechip microarray, we found significant changes in the mRNA of transcription factors such as *Dlx3*, *Elf5*, *Foxn1*, *Hoxc13*, while there was no alteration for c-Jun or c-Fos. Indeed, we verified the increased mRNA expression of these transcription factors in the epidermis of KO mice by q-RT PCR. Interestingly, these transcription factors are critical in the hair follicle cycle, especially in the period of anagen (Lin *et al*., 2004), and gene ontology analysis revealed their strong association with epithelial development. Although further studies are required to clarify the underlying mechanism, it would be interesting to investigate the involvement of transcription factors in abnormal epidermal differentiation in the skin of Rab25 KO mice.

Collectively, we demonstrated that Rab25 deficiency perturbs epidermal differentiation and skin barrier function, leading to high trans-epidermal water loss (TEWL) and low skin moisture. It is notable that although the expression of epidermal differentiation markers increased, skin barrier function was significantly impaired in Rab25 KO mice, implying other factors are accountable for the barrier dysfunction. Further studies in this regard are recommended in the future.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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