Upregulation of Aquaporin-3 Is Involved in Keratinocyte Proliferation and Epidermal Hyperplasia

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Aquaporin-3 (AQP3) is a water/glycerol-transporting protein expressed in keratinocytes of the epidermis. We previously showed that AQP3-mediated transport of water and glycerol is involved in keratinocyte migration and proliferation, respectively. However, the involvement of AQP3 in epidermal hyperplasia in skin diseases, such as atopic dermatitis (AD), is unknown. In this study, we found significantly increased AQP3 transcript and protein expression in the epidermis of human AD lesions. The upregulation of AQP3 expression in human keratinocytes by transfection with human AQP3 DNA plasmid was associated with increased cellular glycerol and ATP, as well as increased cell proliferation. Among several cytokines and chemokines produced in the skin, CCL17, which is highly expressed in AD, was found to be a strong inducer of AQP3 expression and enhanced keratinocyte proliferation. In mouse AD models, AQP3 was strongly overexpressed in the epidermis in wild-type mice. Epidermal hyperplasia was reduced in AQP3-deficient mice, with a decreased number of proliferating keratinocytes. These results suggest the involvement of AQP3 in epidermal hyperplasia by a mechanism involving upregulated AQP3 expression and consequent enhancement of keratinocyte proliferation.

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INTRODUCTION

The aquaporins (AQPs, named AQP0–12) are a family of transmembrane channels that transport water, and in some cases small solutes such as glycerol (Carbrey and Agre, 2009; Verkman, 2009). AQP3 is one such water/glycerol-transporting protein, which is expressed in keratinocytes of the epidermis (Ma *et al.*, 2002). Our previous studies using AQP3 knockout mice and human keratinocytes showed that AQP3-mediated water and glycerol transport is involved in keratinocyte migration and proliferation, respectively, which were implicated to have important roles in cutaneous wound healing and tumorigenesis (Hara-Chikuma and Verkman, 2008a, b). We have also shown that AQP3 deficiency has little effect on differentiation markers in human keratinocytes, suggesting that AQP3 is not involved in keratinocyte differentiation (Hara-Chikuma *et al.*, 2009). Previous conflicting studies had suggested that AQP3 is involved in early differentiation, but not in proliferation, of keratinocytes (Zheng and Bollag, 2003; Bollag *et al.*, 2007).

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Abbreviations: AD, atopic dermatitis; AQP, aquaporin; NHK, normal human keratinocyte; OVA, ovalbumin; Ox, oxazolone; PCNA, proliferating cell nuclear antigen; TARC/CCL17, thymus and activation-regulated chemokine; TEWL, transepidermal water lossTNF-α, tumor necrosis factor-α; WT, wild type

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The development and maintenance of the mature epidermis rely on balanced keratinocyte proliferation and terminal differentiation. Hyperproliferation and disturbed differentiation are associated with certain pathological conditions, such as atopic dermatitis (AD), ichthyosis, and psoriasis (Fuchs and Raghavan, 2002; Jensen *et al.*, 2004). With regard to keratinocyte proliferation, multiple studies have shown that several growth factors and cytokines, including tumor necrosis factor-α (TNF-α), IFN-γ, IL-1, and GM-CSF, are involved in the growth of keratinocyte in skin disorders, such as epidermal barrier disruption and wound healing (Wood *et al.*, 1992; Segre, 2006; Barrientos *et al.*, 2008). Recent studies have revealed that both IL-21 and IL-23 mediate keratinocyte proliferation and epidermal hyperplasia, which was found to be implicated in the pathogenesis of psoriasis (Chan *et al.*, 2006; Caruso *et al.*, 2009).

The aim of this study was to investigate the hypothesis that AQP3 upregulation is involved in keratinocyte proliferation and epidermal hyperplasia in skin disorders. Motivated by the observation that AQP3 expression was increased in the AD skin (Olsson *et al.*, 2006), we used human keratinocytes and murine AD models in AQP3-null mice. We found that upregulation of AQP3 enhanced proliferation of human keratinocytes, which was involved in epidermal hyperplasia during AD development. Our data suggest that AQP3 inhibition by topical agents may be beneficial for the treatment of epidermal hyperplasia in AD.

RESULTS

Increased epidermal AQP3 expression in human AD

A previous report showed increased AQP3 transcript expression in the whole skin affected by atopic eczema (Olsson

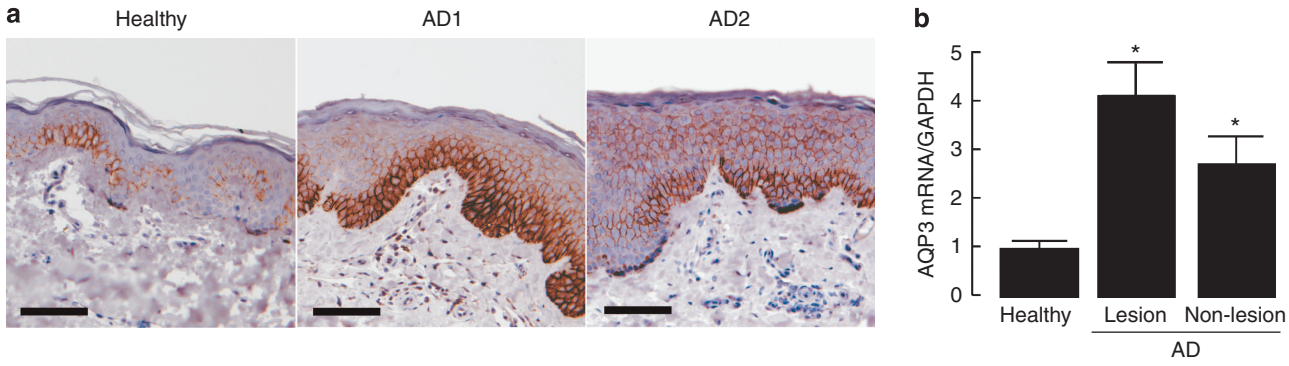


Figure 1. AQP3 expression in the epidermis of AD lesions and healthy subjects. (**a**) Skin specimens were immunostained with anti-AQP3. Left, healthy subject; right, lesion from AD patients. Bar = 50 μm. (**b**) AQP3 mRNA expression in the epidermis from AD patients (lesion, $n = 7$; non-lesion, $n = 4$) and healthy subjects ($n = 6$) by quantitative RT-PCR. Data are expressed as AQP3/GAPDH ratio (mean ± SE). *$P < 0.01$. AD, atopic dermatitis; AQP, aquaporin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR.

et al., 2006). To verify AQP3 protein expression in the epidermis of AD lesions, we performed immunostaining with anti-AQP3 antibody. Figure 1a shows remarkably greater AQP3 protein expression on the plasma membrane of keratinocytes in AD lesions than in the healthy epidermis. AQP3 was broadly distributed throughout the AD lesions. To quantify AQP3 transcript expression, we isolated the epidermis from the AD skin (lesion and non-lesion) and assayed AQP3 mRNA by quantitative reverse transcription-PCR. Figure 1b shows approximately four-fold increased AQP3 transcript expression in the epidermis of AD lesions versus controls.

Greater AQP3 expression enhances keratinocyte proliferation

We determined whether increased AQP3 expression could enhance keratinocyte proliferation. Normal human keratinocytes (NHKs) were transfected with either empty vector or plasmid expressing human AQP3. Figure 2a shows that transfection of AQP3 plasmid produced at least 3.2-fold increase in AQP3 mRNA. Immunoblot analysis showed an approximately six-fold increase in AQP3 protein expression, with the expected molecular size of 28 kDa (Figure 2a, right). Keratins 5 and 14, markers of basal proliferating cells, were significantly increased in AQP3-overexpressing NHKs compared with empty vector-transfected cells (Figure 2b). We found no significant differences in keratins 1 and 10, markers of early differentiation. Measurement of cell growth using the modified MTT assay showed that AQP3 upregulation enhanced cell growth (Figure 2c). We have previously proposed that AQP3-facilitated glycerol transport is an important determinant of keratinocyte proliferation and cellular ATP generation (Hara-Chikuma and Verkman, 2008a, b). Levels of cellular glycerol and ATP were increased in NHKs with upregulated AQP3 expression (Figure 2d and e). These findings show that increased AQP3 expression enhances keratinocyte proliferation.

CCL17 increases AQP3 expression in human keratinocytes

It has been reported that various Th1 and Th2 cytokines/chemokines are altered in the epidermis in AD, which are proposed to be involved in epidermal hyperplasia and AD pathogenesis (Novak *et al.*, 2003). We asked whether cytokines/chemokines could affect AQP3 expression in keratinocytes during the development of AD. Human keratinocytes (HaCaT) were used in this study to examine the effect of cytokines/chemokines on AQP3 expression, because the expressions of cytokine/chemokine receptors are more stable in HaCaT than in NHKs. Cells were incubated with cytokines/chemokines for 2 days, and AQP3 expression was quantified by immunoblotting. We found that TARC (thymus and activation-regulated chemokine)/CCL17, a Th2 chemotactic chemokine, increased AQP3 expression (Figure 3a). TNF-α significantly decreased AQP3 expression, which is consistent with previous data in a human squamous cell carcinoma cell line (DJM-1) (Horie *et al.*, 2009). Figure 3b shows that CCL17 increased AQP3 expression in a dose-dependent manner.

CCL17 is produced by dendritic cells, T cells, and keratinocytes (Reiss *et al.*, 2001). It has been reported that stimulation with IFN-γ and TNF-α synergistically induced CCL17 production in HaCaT cells (Vestergaard *et al.*, 2000). To elucidate the mechanism of CCL17-facilitated AQP3 upregulation, HaCaT keratinocytes were incubated with TNF-α and INF-γ, and assayed for CCL17 and AQP3 mRNA expression. Figure 3c shows that addition of TNF-α/INF-γ increased intrinsic CCL17 in keratinocytes approximately nine-fold, without effect on AQP3 expression. In contrast, incubation with CCL17 increased AQP3 expression, whereas intrinsic CCL17 expression was not altered. These results suggest that exogenous CCL17 affects AQP3 expression.

We next studied whether exogenous CCL17 could affect cell signaling, resulting in increased AQP3 expression. Cells were treated with various cell signaling inhibitors following incubation with CCL17 for 1 day, after which AQP3 mRNA expression was assessed. Figure 3d shows that mitogen-activated protein kinase and protein kinase C inhibitors (U0126 and R03-2432) suppressed CCL17-facilitated AQP3 upregulation, suggesting the involvement of CCL17-dependent mitogen-activated protein kinase and/or protein kinase C cell signaling in increased AQP3 expression.

CCL17 enhances keratinocyte proliferation

We determined the effect of CCL17 on keratinocyte proliferation. HaCaT keratinocytes were starved for 1 day,

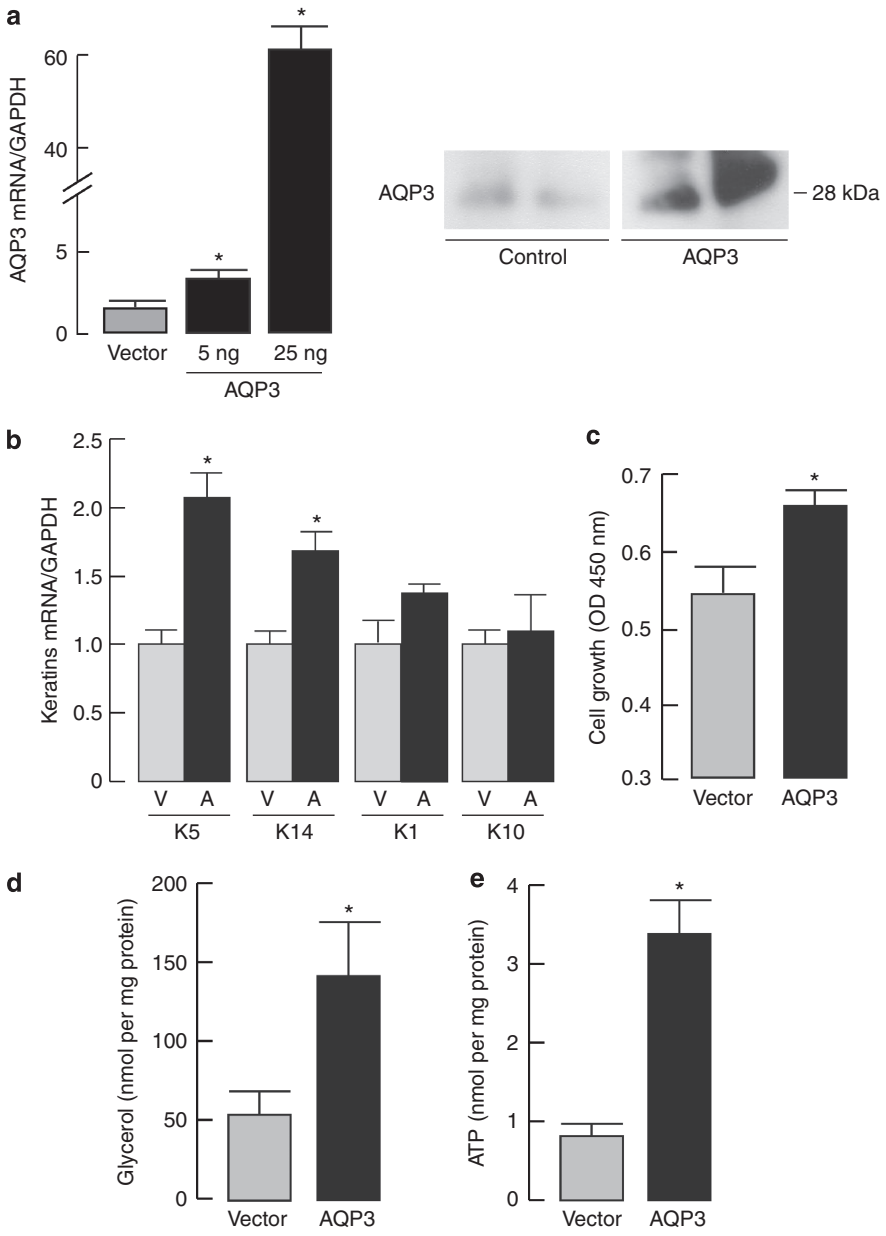


Figure 2. Effect of AQP3 upregulation in human keratinocytes. NHKs were transfected with empty vector (pCMV6-XL4) or plasmid expressing human AQP3 (5–25 ng per 8×10^3 cells). (**a**) (Left) The relative mRNA expression of AQP3/GAPDH (SE, $n = 4$–5, $^*P < 0.01$). (Right) Immunoblot of cell homogenates with anti-AQP3 (15 μg protein per lane, 100 ng per 2×10^5cells). (**b**) The relative mRNA expressions of keratins 5, 14, 1, and 10 in NHKs transfected with empty vector (V) or AQP3 plasmid (A). Data are expressed as keratins/GAPDH ratio (mean ± SE) ($n = 3$) $^*P < 0.01$. (**c**) Cell proliferation was assessed by modified MTT assay (SE, $n = 5$, $^*P < 0.01$, 5 ng per 8×10^3 cells). (**d**) Cellular glycerol content and (**e**) ATP content (SE, $n = 5$, $^*P < 0.01$, 5 ng per 8×10^3 cells). AQP, aquaporin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NHK, normal human keratinocyte.

and treated with CCL17 for 6 hours. AQP3 mRNA expression was increased 2.6-fold in CCL17-treated cells (Figure 4a, left). CCL17 also significantly increased the expressions of proliferation markers, keratins 5 and 14, but not those of differentiation markers, keratins 1 and 10 (Figure 4a, right). To examine the effect of CCL17 on cell growth, cells were treated with CCL17 for 2 days in the starved medium, after which cell proliferation was induced by replacing the medium with 0.1 or 2% fetal bovine serum. As shown in Figure 4b, cell proliferation, assessed by BrdU incorporation, was significantly increased in CCL17-treated cells as compared with control cells. Finally, cell growth was assayed in controls and AQP3 knockdown keratinocytes to determine the involvement of AQP3 in CCL17-induced cell proliferation. Transfection of small-interfering RNA-AQP3 into HaCaT cells consistently reduced AQP3 mRNA expression by ~90% (9.1 ± 1.4% of controls). Figure 4c shows reduced CCL17-induced cell proliferation in AQP3 knockdown keratinocytes.

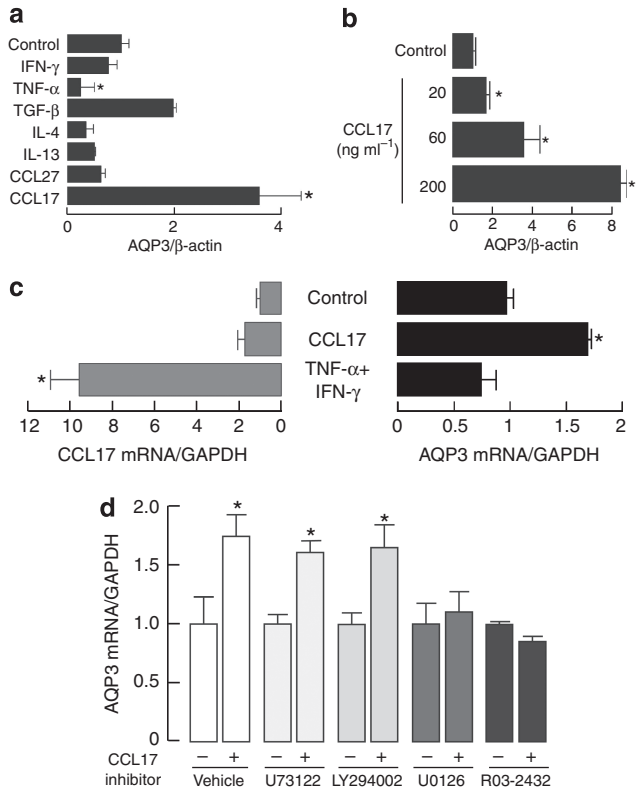


Figure 3. Effects of cytokines/chemokines on AQP3 expression in human keratinocytes. (**a**) HaCaT cells were treated with Th1/Th2 cytokines or chemokines for 2 days, and AQP3 expression was analyzed by immunoblot. Data are expressed as AQP3/β-actin ratio (mean ± SE) ($n = 3$). *$P < 0.05$. (**b**) Cells were treated with CCL17 (20, 60, 200 ng ml^{-1}) for 2 days. AQP3 expression was analyzed by immunoblot. AQP3/β-actin ratios are expressed as mean ± SE ($n = 2$–6). *$P < 0.05$. (**c**) Cells were treated with CCL17 (20 ng ml^{-1}) or a mixture of TNF-α and IFN-γ (10 ng ml^{-1} each) for 24 hours. The mRNA levels of CCL17 and AQP3 were analyzed by quantitative RT-PCR. CCL17 or AQP3/GAPDH ratios are expressed as mean ± SE ($n = 6$). *$P < 0.01$. (**d**) Cells were incubated for 1 hour with U73122 (PLC inhibitor), LY294002 (PI3 kinase inhibitor), U0126 (MAP kinase inhibitor), or R03-2432 (PKC inhibitor), which was followed by treatment with CCL17 (20 ng ml^{-1}) for 24 hours. AQP3/GAPDH ratios are expressed as mean ± SE ($n = 5$). *$P < 0.01$ versus without CCL17. AQP, aquaporin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP kinase, mitogen-activated protein kinase; PI3, phosphoinositide-3; PKC; protein kinase C; PLC, phospholipase C; RT-PCR, reverse transcription-PCR; TNF-α; tumor necrosis factor-α.

Taken together, these findings suggest that exogenous CCL17 increases AQP3 expression and enhances keratinocyte proliferation.

Reduced epidermal hyperplasia in AQP3-null mice in an AD model

To investigate the requirement of AQP3 for the development of AD, we applied an established murine model of AD in wild-type (WT) and AQP3-null mice. Dermatitis was induced by repeated epicutaneous application of ovalbumin (OVA) in a patch to tape-stripped skin, as described previously (Spergel *et al.*, 1998).

Hematoxylin and eosin staining showed that the OVA-treated epidermis in WT mice was thicker than that in AQP3-null mice (Figure 5a, left), with the representative data summarized in Figure 5a (right). Immunostaining showed strong expression of the AQP3 protein on the plasma membranes in the OVA-applied epidermis of WT mice (Figure 5b). Immunoblot analysis confirmed that repeated OVA sensitization significantly increased AQP3 expression (Figure 5c), supporting the utility of the OVA-AD model in investigating the role of AQP3 in AD pathogenesis. To quantify keratinocyte proliferation, immunostaining with anti-proliferating cell nuclear antigen (PCNA) was performed (Supplementary Figure S1 online). Figure 5d shows that OVA sensitization resulted in an ~10-fold increase in PCNA-positive cells in the WT epidermis, while the number of PCNA-positive cells was much lower in the AQP3-null than in the WT-OVA-treated epidermis. To determine epidermal permeability, transepidermal water loss (TEWL) was measured on the OVA- and saline-treated skin. After five treatments with OVA, TEWL was significantly elevated in the WT-OVA treated skin compared with the control- and saline-treated skin (Figure 5e).

Total IgE and OVA-specific IgE were significantly higher in OVA sensitization than saline application in both WT and AQP3-null mice, indicating that repeated OVA application induced comparable allergic sensitization in WT and AQP3-null mice (Supplementary Figure S2 online). These findings suggest that AQP3 deficiency suppresses OVA-induced keratinocyte hyperproliferation, which may be responsible for epidermal hyperplasia and barrier disruption during AD development.

AQP3 deficiency prevents hapten-induced AD development

To confirm the involvement of AQP3 expression in epidermal hyperplasia and barrier disruption during AD development, we investigated a different, hapten-induced mouse model of AD (Man *et al.*, 2008). WT and AQP3-null mice were challenged 10 times with an application of oxazolone (Ox) after 1 week of sensitization. Figure 6a shows that Ox-treated WT mice developed mild erythema and a rough-textured skin surface, whereas there were only minor changes in AQP3-null mice, suggesting that WT mice are more susceptible to atopic disorders than AQP3-null mice. TEWL values were much greater in Ox-applied WT than in the AQP3-null skin, indicating that AQP3 deficiency prevented the barrier disruption induced by Ox applications (Figure 6b). Hematoxylin and eosin staining showed that AQP3 deficiency sustained hapten-induced epidermal hyperplasia compared with the WT epidermis (Figure 6c). Immunostaining with AQP3 showed strong expression in the thickened epidermis of Ox-treated WT mice (Figure 6d). Immunoblotting verified an ~10-fold increase in AQP3 expression in the WT AD epidermis (Figure 6e). CCL17 was comparably elevated in Ox-applied WT and the AQP3-null epidermis as assessed by ELISA assay (Figure 6f). Figure 6g showed that total IgE was significantly elevated in both WT and AQP3-null mice, indicating that allergic sensitization occurred in both WT and AQP3-null mice. These data from the Ox model are in agreement with those from the OVA-AD model: AQP3 expression is required for epidermal hyperplasia, which might contribute to barrier disruption during AD development.

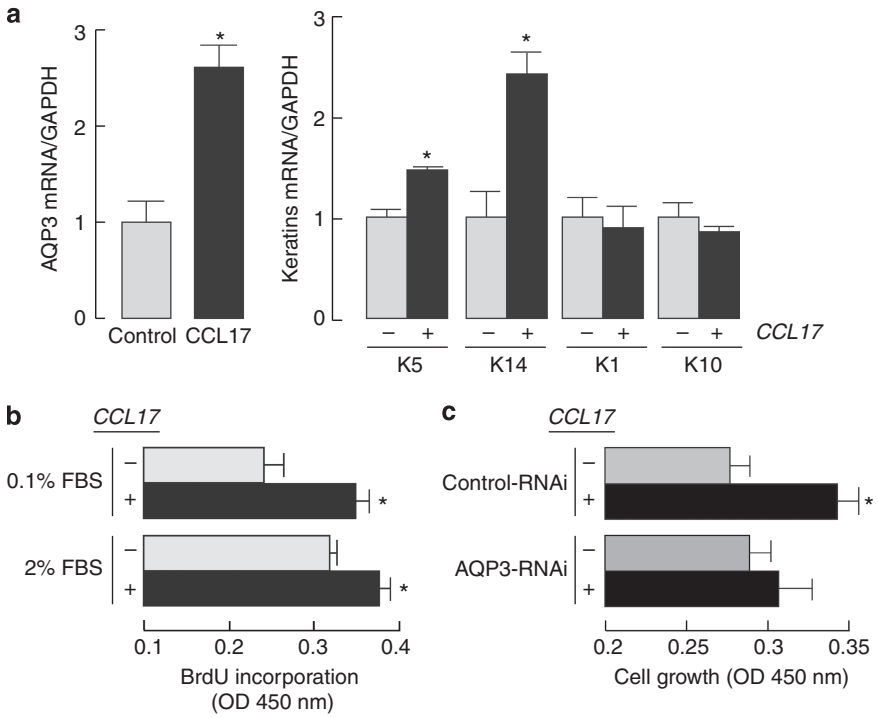
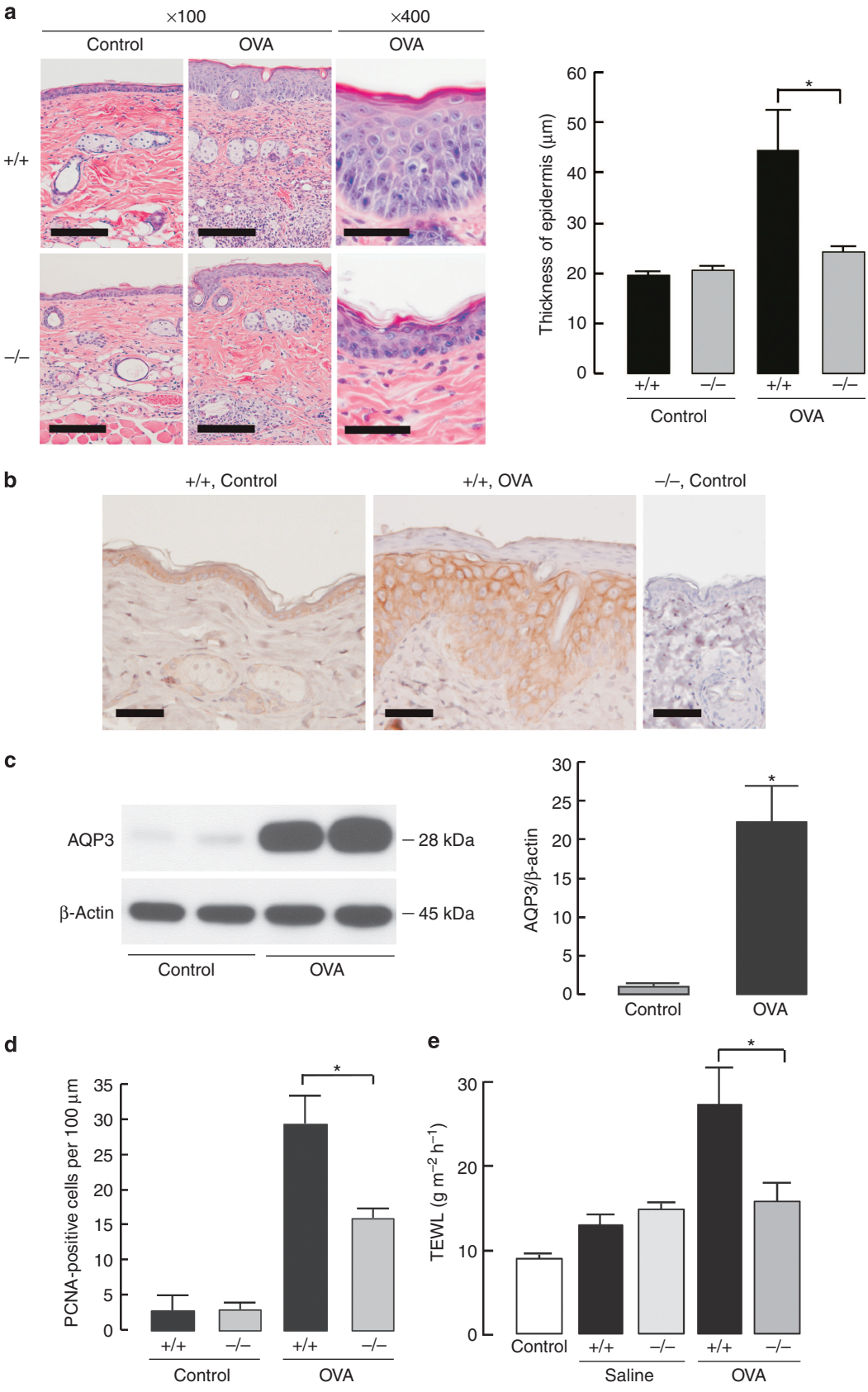


Figure 4. Effects of CCL17 on keratinocyte proliferation in human keratinocytes. (**a**) HaCaT cells were starved for 1 day, and treated with CCL17 (100 ng ml^{-1}) in DMEM containing 10% FBS for 6 hours. AQP3 (left) and keratins 5, 14, 1, and 10 (right) mRNA expressions were analyzed by quantitative RT-PCR. Data are expressed as the ratio to GAPDH ($n=5$, SE, *$P<0.01$). (**b**) Cells were cultured with or without CCL17 (20 ng ml^{-1}) for 2 days in starved medium, after which the medium was switched to 0.1 or 2.0% FBS for 2 days. Cell proliferation was assessed by BrdU incorporation ($n=5$, SE, *$P<0.01$). (**c**) Cells were transfected with AQP3-siRNA or non-targeting-siRNA (control-RNAi), and cultured with or without CCL17 (20 ng ml^{-1}) for 2 days in the starved medium. Cell proliferation was assessed by the modified MTT assay ($n=5$, SE, *$P<0.01$). AQP, aquaporin; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; siRNA, small-interfering RNA.

DISCUSSION

We found that AQP3 upregulation enhanced keratinocyte proliferation, which may be responsible for epidermal hyperplasia found in a number of skin disorders. We previously suggested the involvement of AQP3 in keratinocyte proliferation, in view of the observation that AQP3 deficiency impaired keratinocyte proliferation and reduced cellular glycerol and ATP content (Hara-Chikuma and Verkman, 2008c). We proposed that AQP3-mediated glycerol transport is an important determinant of keratinocyte proliferation, in which glycerol works as a key regulator of cellular ATP energy. In this study, we showed that increased AQP3 expression by plasmid-DNA transfection or the AQP3 inducer CCL17 enhanced keratinocyte proliferation with increased proliferation markers, and increased cellular glycerol and ATP content. Although further studies are required to elucidate the exact mechanisms by which AQP3 expression increases cell proliferation, AQP3 upregulation might be one of the determinants of keratinocyte hyperproliferation in several skin diseases, such as AD.

In this study, we showed in two different murine models that AQP3-null mice exhibit defective epidermal hyperplasia with suppressed barrier disruption during AD development. Repeated application of OVA or Ox induced AD-like skin lesions with irregularly acanthotic epidermis, high TEWL, and increased AQP3 expression in WT mice. These data provide evidence for the involvement of AQP3 in excessive keratinocyte proliferation and disturbed barrier function during the development of AD. Enhanced keratinocyte proliferation might induce disturbed differentiation and barrier function, as there is no sufficient time for normal differentiation or development of a functional epidermal barrier during accelerated cell renewal. AD is a common chronic inflammatory skin disease, which is classified into extrinsic and intrinsic types according to the presence or absence of sensitization toward environmental allergens (Tokura, 2010). The extrinsic and allergic AD lesions display impaired epidermal barrier function by inherited and acquired factors, which not only enhances allergen sensitization but also leads to systemic allergic responses (Spergel *et al.*, 1998; Novak *et al.*, 2003; Elias and Steinhoff, 2008; O'Regan *et al.*, 2008). Recent human genetic studies have shown that loss-of-function mutation in filaggrin was associated with impaired skin barrier function in AD patients (Palmer *et al.*, 2006; Morar *et al.*, 2007). Coincidentally, the flaky-tail mouse, which exhibited low filaggrin gene expression, showed barrier abnormality with epidermal hyperplasia like a severe AD (Scharschmidt *et al.*, 2009; Moniaga *et al.*, 2010). Although further studies are necessary, it is expected that AQP3 expression might be increased in the other murine AD model, including filaggrin deficiency. The application of the AQP3 inhibitor will provide a, to our knowledge, previously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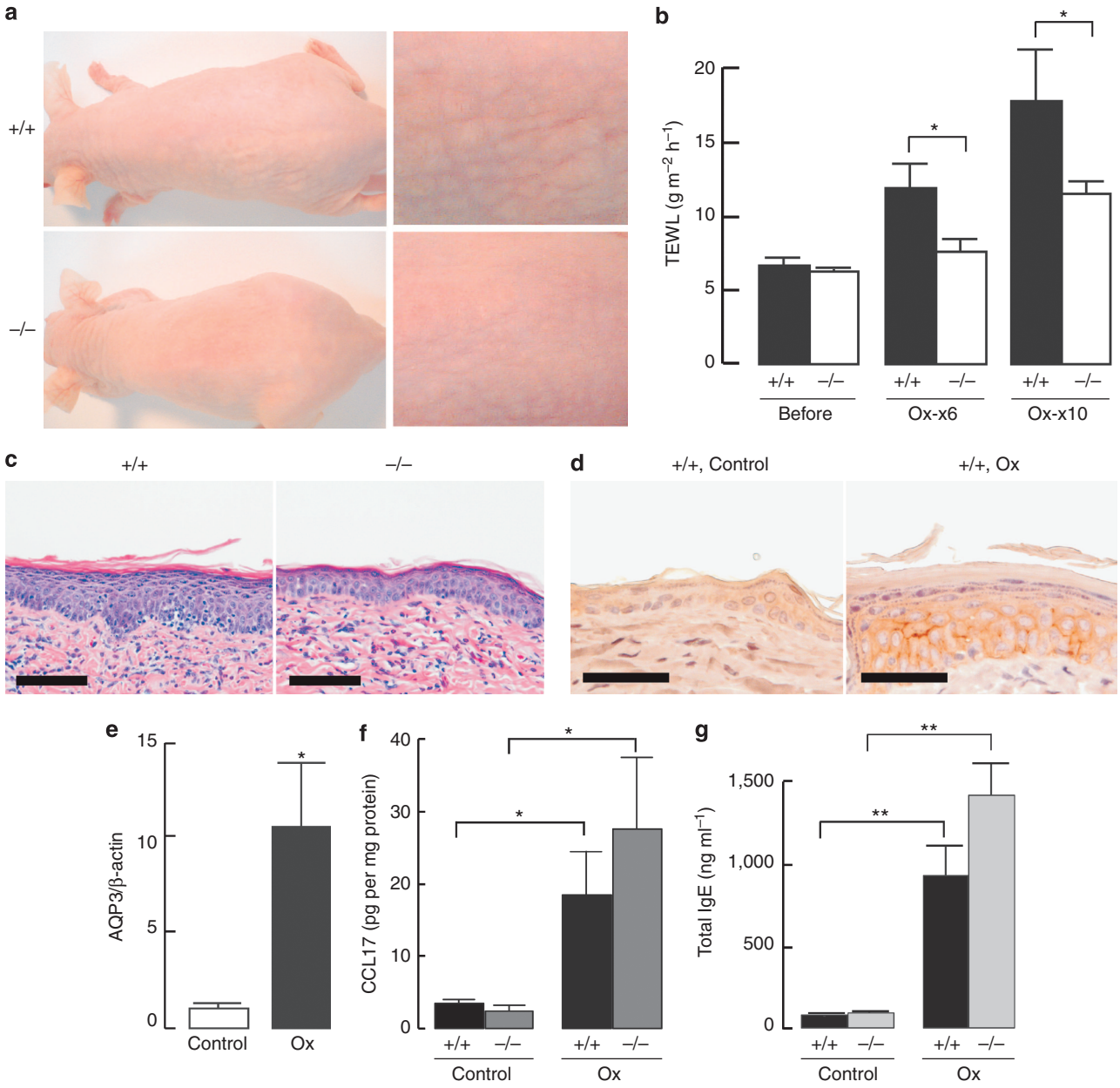

Figure 6. AQP3 deficiency prevents the development of hapten-induced AD. (**a**) Clinical photographs of Ox-treated mice (upper, WT (+/+) mice; lower, AQP3−/− mice). (**b**) TEWL on the dorsal skin was measured before and after 6 or 10 episodes of Ox treatment ($n=5$, SE, *$P<0.05$; WT versus AQP3-null mice). (**c**) H&E staining of Ox-treated WT (+/+) and AQP3-null (−/−) mice. Bar = 100 μm. (**d**) Immunostaining of AQP3 in the epidermis of control and Ox-treated WT (+/+) mice. Bar = 50 μm. (**e**) Immunoblot analysis of epidermal homogenate was performed with anti-AQP3 and β-actin. AQP3/β-actin ratios are expressed as mean ± SE ($n=4$, *$P<0.05$). (**f**) CCL17 protein levels in control and Ox-treated mice. ($n=4$, SE, *$P<0.05$). (**g**) Total serum IgE levels were measured in control and Ox-treated mice ($n=5$, SE, **$P<0.01$). AD, atopic dermatitis; AQP, aquaporin; H&E, hematoxylin and eosin; Ox, oxazolone; TEWL, transepidermal water loss; WT, wild type.

Figure 5. Suppressed epidermal hyperplasia in AQP3-null OVA-AD mice. (**a**) (Left) H&E staining of WT (+/+) and AQP3-null mice (−/−). Bar = 100 μm (× 100) and bar = 50 μm (× 400). (Right) Epidermal thickness in control and OVA-treated mice ($n=3$, three locations per mouse, *$P<0.05$). (**b**) Immunostaining of AQP3 in the epidermis of control and OVA-treated WT (+/+) mice. −/−, AQP3-null control mice. Bar = 50 μm. (**c**) (Left) Immunoblot analysis of epidermal homogenate with anti-AQP3 and β-actin. (Right) The evaluation of AQP3 protein levels in control and OVA-treated WT (+/+) mice ($n=4$, SE, *$P<0.05$). (**d**) PCNA staining was performed in control and OVA-treated mice. The number of PCNA-positive cells in the epidermal basal layer was counted ($n=3$, three locations per mouse, SE, *$P<0.05$). (**e**) TEWL on the dorsal skin was measured at the end of the fifth cycle of treatment ($n=5$–8, SE, *$P<0.05$). AD, atopic dermatitis; AQP, aquaporin; H&E, hematoxylin and eosin; OVA, ovalbumin; PCNA, proliferating cell nuclear antigen; TEWL, transepidermal water loss; WT, wild type.

unreported therapeutic strategy for controlling unwanted increased keratinocyte proliferation in the AD epidermis.

The acute-phase AD skin displayed an allergen-derived Th2 cell-dominant infiltrate of T lymphocytes and increased Th2-type cytokine secretion, which induced an elevation in serum IgE and inflammation. In the chronic phase, AD lesions displayed infiltration with both Th1 and Th2 cells. The screening assay was performed based on the hypothesis that some Th1 and/or Th2 cell-derived chemokines/cytokines might increase AQP3 expression, which enhances keratinocyte proliferation during AD development. We found that the CCL17 Th2 chemotactic chemokine increased AQP3 expression in keratinocytes through mitogen-activated protein kinase and/or protein kinase C cell signaling. Indeed, we verified that CCL17 activated extracellular signal-regulated kinase of mitogen-activated protein kinase (not shown). It has been reported that CCL17 is produced by dendritic cells, T cells, and keratinocytes, and induces Th2-type T-cell migration (Reiss *et al.*, 2001). CCL17 was found to be increased to a greater extent in the AD serum and epidermis than in healthy controls (Kakinuma *et al.*, 2001; Saeki and Tamaki, 2006). Consistent with previous observations, we found significant elevation of CCL17 in both the WT and the AQP3-null AD epidermis as assessed by ELISA assay. Our findings implicate the involvement of CCL17 in the increased AQP3 expression in the AD epidermis, although further studies are required to establish the mechanisms of AQP3 upregulation by CCL17. CCL17-induced AQP3 upregulation might accelerate keratinocyte proliferation during AD development.

In conclusion, our data provide several lines of evidence that AQP3 is involved in keratinocyte proliferation and epidermal hyperplasia. We propose that upregulated AQP3 expression enhances keratinocyte proliferation, which is involved in hyperplasia and barrier disruption in AD. Our findings suggest that AQP3 suppression by topical drugs may be useful for treatment of skin diseases associated with excessive epidermal proliferation.

MATERIALS AND METHODS

Human subjects

A total of 7 patients with AD (lesion, $n=7$; non-lesion, $n=4$) and 6 healthy non-AD volunteers were enrolled in this study. AD was diagnosed according to the consensus criteria as described previously (Williams *et al.*, 1994). Informed consent was obtained from all subjects involved in this study. The study was approved by the Ethics Committee of the Kyoto University and was conducted according to the Declaration of Helsinki Principles. None of the patients had received local or systemic treatment with glucocorticoids or immunosuppressants within 1 week before the study. Skin biopsies were analyzed with real-time PCR and immunohistochemistry. For real-time PCR, the skin was first separated into the epidermis and the dermis by incubation in 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA) at 37 °C for 1 hour, and total RNA was extracted from the epidermis as described below.

Murine AD model

The AQP3-null mice (hairless genetic background) were generated by targeted gene disruption as described previously (Ma *et al.*, 2002). All animal experiments were approved by the Committee on Animal Research of the Kyoto University. Mice aged 6–8 weeks were treated with OVA (Sigma-Aldrich, St Louis, MO) or Ox (Sigma-Aldrich), as described previously (Spergel *et al.*, 1998; Man *et al.*, 2008). In brief, for the OVA model, the dorsal skin was tape stripped six times, and OVA (100 μg in 100 μl saline) or saline alone (100 μl) was placed on a round patch (16 mm in diameter) (Torii Pharmaceutical, Tokyo, Japan), which was secured to the skin with an elastic tape (Alcare, Tokyo, Japan). Each mouse was treated with five 4-day periods of epicutaneous application of OVA or saline under occlusion at 3-day intervals. For the Ox model, each mouse was sensitized by one topical treatment on the dorsal skin with 60 μl of 2.5% Ox (in ethanol). One week later, the mouse was treated topically with 120 μl of 0.1% Ox on the dorsal area once every other day for an additional 3 weeks. TEWL was measured with a Tewameter Vapo Scan (Asahi Biomed, Tokyo, Japan). CCL17 level was assayed in the epidermal homogenate by ELISA (R&D Systems, Minneapolis, MN).

Human keratinocyte cell cultures

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were grown in Humedia-KG2 medium (Kurabo). HaCaT cells (a kind gift of Dr Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in low-glucose DMEM (Invitrogen) with 10% fetal bovine serum (Funakoshi, Tokyo, Japan). After the cells grew to 80–90% confluence, they were treated with 10 ng ml^{-1} IFN-γ (R&D Systems), 10 ng ml^{-1} TNF-α (Miltenyi Biotec, Bergisch Gladbach, Germany), 1 ng ml^{-1} transforming growth factor-β1 (PeproTeck, Rocky Hill, NJ), 50 ng ml^{-1} IL-4 (PeproTeck), 50 ng ml^{-1} IL-13 (PeproTeck), 20 ng ml^{-1} CCL27 (PeproTeck), and 20–200 ng ml^{-1} CCL17 (Miltenyi Biotec), respectively. For treatment with cell signaling inhibitors, cells were incubated for 1 hour with 10 μM U73122 (Cayman Chemical, Ann Arbor, MI), 50 μM LY294002 (Jena Bioscience, Jena, Germany), 10 μM U0126 (Cell Signaling Technology, Danvers, MA), or 10 μM R03-2432 (Enzo Life Sciences, Plymouth Meeting, PA), followed by treatment with CCL17 (20 ng ml^{-1}). Experiments were performed 6–48 hours after incubation for quantitative reverse transcription-PCR, immunoblot analysis, and cell growth assay.

The constructs yielding human AQP3 (NM_004925) and control vector pCMV6-XL4 were obtained from Origene TrueClone (Rockville, MD). NHKs were transfected with purified plasmid DNA using Lipofectamine 2000 (5–25 ng per 8×10^3 cells; Invitrogen). HaCaT cells were transfected with AQP3 small-interfering RNA or non-targeting small-interfering RNAs (Dharmacon, Lafayette, CO) at 40–50% confluence using Lipofectamine 2000. Cell proliferation was analyzed using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan) or the BrdU Cell Proliferation Assay kit (Calbiochem, San Diego, CA). Cell homogenates (3,500 g, 10 minutes, 4 °C) were assayed for glycerol and ATP using commercial kits (glycerol, Sigma-Aldrich; ATP, Roche, Basel, Switzerland).

Histology

Paraffin-embedded sections were stained with hematoxylin and eosin or immunostained with anti-AQP3 (Millipore, Billerica, MA) or anti-PCNA (Dako, Glostrup, Denmark) with biotinylated IgG and horseradish peroxidase-conjugated ABC reagent (Vector Laboratories, Burlingame, CA). Epidermal thickness and PCNA-positive cells per 100 μm were measured at three locations per mouse.

Immunoblot analysis

The epidermis of each mouse was separated from the dermis by incubation in phosphate-buffered saline solution at 60 °C for 20 seconds. The epidermis and cultured HaCaT cells were lysed with extraction buffer containing 250 mM sucrose, 1 mM EDTA, and 1% protein inhibitor cocktail (Sigma-Aldrich). For immunoblot analysis, polyclonal AQP3 antibody (Millipore) and horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Cell Signaling Technology) were used for detection by ECL (GE Healthcare, Piscataway, NJ).

Quantitative reverse transcription-PCR

Total RNA was isolated using RNeasy kits and digested with DNase I (Qiagen, Hilden, Germany). The cDNA was reverse transcribed from total RNA samples using the Prime Script RT reagent kit (Takara Bio, Otsu, Japan). Quantitative reverse transcription-PCR was performed using SYBR Green I (Takara Bio) and primers listed in Supplementary Table S1 online using the Light Cycler real-time PCR apparatus (Roche).

Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test or analysis of variance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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