Roles of Aquaporin-3 in the Epidermis

Mariko Hara-Chikuma[1,2] and A.S. Verkman[1]

Aquaporin-3 (AQP3) is a membrane transporter of water and glycerol expressed in plasma membranes in the basal layer keratinocytes of epidermis in normal skin. AQP3 expression in human skin is increased in response to skin stress in diseases such as atopic eczema, to various agents such as retinoic acid, and in skin carcinomas. AQP3-knockout mice have reduced stratum corneum water content and elasticity compared with wild-type mice, as well as impaired wound healing and epidermal biosynthesis. Reduced AQP3-dependent glycerol transport in AQP3-deficient epidermis appears to be responsible for these phenotype findings, as evidenced by reduced glycerol content in epidermis and stratum corneum in AQP3-knockout mice, and correction of the phenotype abnormalities by glycerol replacement. Recent data implicate AQP3 as an important determinant in epidermal proliferation and skin tumorigenesis, in which AQP3-knockout mice are resistant to tumor formation by a mechanism that may involve reduced cell glycerol content and ATP energy for biosynthesis. AQP3 is thus a key player in epidermal biology and a potential target for drug development.

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INTRODUCTION

The aquaporins (AQPs) are a family of small, hydrophobic, integral membrane proteins that act primarily as water-selective pores, facilitating osmotically driven water transport across cell plasma membranes. There are at least 13 mammalian AQPs (AQP0–AQP12), which have been divided into two groups on the basis of their permeability. AQPs 1, 2, 4, 5, and 8 function primarily as water-selective transporters; AQPs 3, 7, 9, and 10, termed ''aquaglyceroporins'', transport water as well as glycerol and possibly other small solutes. The AQPs are expressed in plasma membranes in a variety of cell types, where they function as pore-like passive transporters responding to transmembrane osmotic gradients (for water transport) or glycerol gradients (for glycerol transport).

Phenotype analysis of transgenic mice lacking individual AQPs has revealed multiple physiological roles of AQPs (reviewed by Verkman, 2005). AQP-facilitated water transport is involved in the urinary concentration mechanism, epithelial fluid secretion, brain edema, neural signal transduction, and cell migration. AQPs in epithelia facilitate transepithelial water transport in response to osmotic gradients, such as fluid absorption by kidney proximal tubule (Schnermann *et al.*, 1998) and fluid secretion in salivary gland (Ma *et al.*, 1999). AQPs in microvascular endothelia and other cell types facilitate cell migration by enhancing water transport into lamellipodia at the leading edge of migrating cells (Saadoun *et al.*, 2005a). In contrast, the aquaglyceroporins are involved in metabolic pathways, such as adipose AQP7 in obesity (Hara-Chikuma *et al.*, 2005; Hibuse *et al.*, 2005), and AQP9 in glycerol metabolism (Liu *et al.*, 2007; Rojek *et al.*, 2007).

This review focuses on the experimental evidence for the involvement of AQP3-facilitated water and glycerol transport in skin physiology. AQP expression in skin is reviewed, followed by evidence, largely from phenotype analysis of AQP3-knockout mice, for involvement of AQP3 in skin hydration, wound healing, and tumorigenesis.

AQP EXPRESSION IN SKIN

Several AQPs have been reported to be expressed in various cell types in mammalian skin, providing indirect evidence for their involvement in some of the functions mentioned above. AQP3, the most studied and well-validated AQP in skin, was first reported in keratinocytes of rat epidermis (Frigeri *et al.*, 1995) after its cloning from rat kidney in 1994 by several laboratories (Echevarria *et al.*, 1994; Ishibashi *et al.*, 1994; Ma *et al.*, 1994). AQP3 expression in plasma membranes in the basal layer of keratinocytes was also detected in human and mouse skin (Sugiyama *et al.*, 2001; Ma *et al.*, 2002; Sougrat *et al.*, 2002). Figure 1a shows AQP3 immunofluorescence in the basal layer of keratinocytes in mouse skin. Expression of AQP9 was also reported in cultures of human differentiated keratinocytes (Sugiyama *et al.*, 2001) and in the stratum granulosum layer of mouse epidermis (Rojek *et al.*, 2007), and expression of AQP10 was reported by reverse transcriptase-PCR in human keratinocyte cultures (Boury-Jamot *et al.*, 2006).

AQP1 is expressed in endothelial cells throughout microvessels outside of the central nervous system, such as in the kidney, lung, secretory glands, skeletal muscle, pleura, and peritoneum (Nielsen *et al.*, 1993; Hasegawa *et al.*, 1994). In

[1]*Departments of Medicine and Physiology, University of California, San Francisco, California, USA and* [2]*Department of Dermatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan*

Correspondence: Dr A.S. Verkman, 1246 Health Sciences East Tower, University of California, San Francisco, California 94143-0521, USA. E-mail: Alan.Verkman@ucsf.edu or http://www.ucsf.edu/verklab

Abbreviations: AQP3, aquaporin-3; SC, stratum corneum

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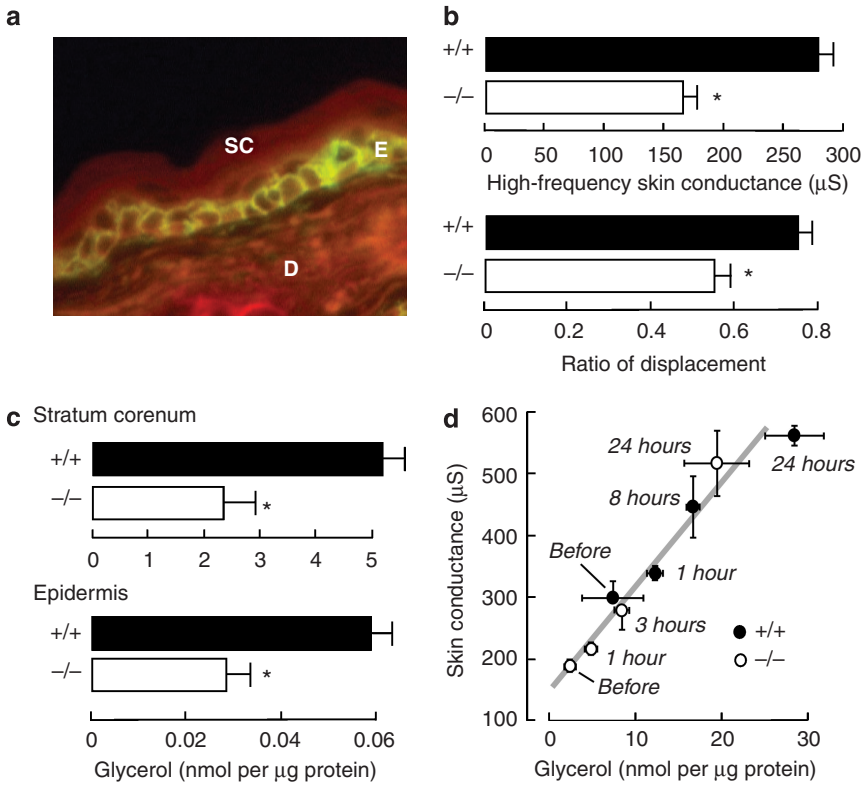

Figure 1. Reduced SC hydration and glycerol content in AQP3-null mice. (**a**) Immunofluorescence showing AQP3 staining in mouse epidermal cells. E, epidermis; D, dermis; sc, stratum corneum. (**b**) (Top) High-frequency superficial skin surface conductance in dorsal skin of hairless wild-type (+/+) and AQP3-null (−/−) mice (SEM, $n=20$, $^*P<0.001$). (Bottom) Skin elasticity measured in dorsal skin using a 2-mm diameter suction probe and 50 mbar pressure transient (SEM, $n=7$, $^*P<0.01$). (**c**) Glycerol content in the SC and epidermis (SEM, $n=5$–6, $^*P<0.01$). (**d**) Correlation between SC glycerol content and skin conductance for wild-type (filled circles) and AQP3-null (open circles) mice in a 90% atmosphere. Glycerol was administered orally. Adapted with permission from Ma *et al.* (2002), Hara *et al.* (2002), and Hara and Verkman (2003).

skin, AQP1 expression was found by immunocytochemistry in rat dermal capillaries (Agren *et al.*, 2003) and human neonatal dermis (Marchini *et al.*, 2003). AQP1 is also expressed in microvessels in tumors implanted in skin, where as mentioned above it is involved in tumor angiogenesis (Saadoun *et al.*, 2005a). Whether AQP1 is functionally important in normal skin functions is unknown.

AQP5 is expressed in sweat glands in humans, rat, and mice (Nejsum *et al.*, 2002; Song *et al.*, 2002). AQP5 deletion in mice did not, by multiple measurement methods, affect sweat secretion (Song *et al.*, 2002). However, whether AQP5 is important in sweat secretion in humans is not known, as there are notable differences in sweat gland physiology between rodents and humans. As mentioned above, AQP7 is expressed in the adipocytes in subcutaneous tissue. AQP7-knockout mice manifest progressive adipocyte hypertrophy as a consequence of reduced AQP7-facilitated plasma membrane glycerol exit in adipocytes. AQP7 is thus likely involved in hypertrophy of adipocytes in hypodermis.

Regarding other cell types in skin, AQP3 was found to be expressed in sebaceous glands (Frigeri *et al.*, 1995). Recent reverse transcriptase-PCR analysis of human primary skin cultures showed expression of AQP1 in melanocytes and fibroblasts, and of AQP3 and AQP9 in Langerhans cells using monocyte-derived dendritic cells (Boury-Jamot *et al.*, 2006). However, significance of these observations is unknown.

FUNCTIONS OF AQP3 IN KERATINOCYTES

Skin hydration

The most superficial layer of skin is the stratum corneum (SC), which consists of terminally differentiated keratinocytes that originate from actively proliferating keratinocytes in the lower epidermis and contain a lipid extracellular matrix secreted from lamellar bodies (Elias, 2005). Hydration of the SC is an important determinant of skin appearance and physical properties, and depends on a number of factors, including external humidity; and its structure, lipid/protein composition, barrier properties, and concentration of water-retaining osmolytes; or ''natural moisturizing factors'' such as free amino acids, ions, and other small solutes (Rawlings and Matts, 2005). Reduced SC hydration is found in aged skin and in skin diseases including atopic dermatitis, eczema, psoriasis, senile xerosis, and hereditary ichthyosis (Tagami *et al.*, 2001; Chuong *et al.*, 2002).

Compared with wild-type mice, the skin of AQP3-null mice, as examined in a hairless, SKH1 genetic background, is relatively dry, rough, and aged. SC hydration, as measured by high-frequency skin conductance (Figure 1b, top) and ^3H_2O

partitioning (not shown), was significantly reduced in AQP3-null mice, as was skin elasticity as measured by displacement ratio following pulsed suction (Figure 1b, bottom) (Ma *et al.*, 2002). A series of experiments were performed to investigate the mechanism by which AQP3 deficiency reduces SC hydration. Remarkably, exposure of mice to high humidity or skin occlusion increased SC hydration in the wild type, but not in AQP3-null mice, suggesting an intrinsic defect in water-holding capacity, as verified by sorption–desorption measurements (Ma *et al.*, 2002). The inability of high humidity or occlusion to correct reduced SC hydration also provides evidence against involvement of AQP3-facilitated water permeability in SC hydration, which is not unexpected, as water movement into the SC to offset evaporative water losses is many orders of magnitudes slower than that where AQPs are involved.

A systematic analysis of epidermis and SC in AQP3-deficient mice revealed reduced glycerol content in SC and epidermis compared with wild-type mice (Figure 1c), with normal glycerol content in dermis and serum. No significant differences were found in wild-type *versus* AQP3-deficient mice regarding SC structure, lipid profile, protein content, and the concentrations of amino acids, ions, and other small solutes (Hara *et al.*, 2002). As expected, water and glycerol permeabilities were reduced in AQP3-null keratinocytes. Reduced glycerol transport from blood into the epidermis and SC through the relatively glycerol-impermeable basal keratinocyte layer was found, which accounted for the reduced steady-state epidermal and SC glycerol content (Hara and Verkman, 2003). SC glycerol is also derived from sebaceous glands where AQP3 is expressed (Fluhr *et al.*, 2003). Further studies are needed to determine whether AQP3 facilitates secretion of glycerol by sebaceous glands, as well as the relative contributions of epidermis *versus* sebaceous glands to SC glycerol.

As further evidence that reduced epidermal and SC glycerol content was responsible for the decreased hydration and elasticity in AQP3-null mice, SC glycerol content and hydration were correlated in wild-type and AQP3-null mice in which SC glycerol content was varied by systemic glycerol administration (Hara and Verkman, 2003). Figure 1d shows strong correlation between SC water content, as assessed by skin conductance, and SC glycerol content in mice placed in a 90% humidity atmosphere and given oral glycerol. Oral glycerol administration also corrected the reduced skin elasticity in AQP3 null mice.

These results implicated an important role for AQP3-facilitated glycerol transport in regulating SC and epidermal glycerol content, and for glycerol as a key determinant of skin hydration. These findings also provided a rational scientific basis for the longstanding practice of including glycerol in cosmetic and skin medicinal preparations.

Wound healing

Cutaneous wound healing is a multi-step process that involves several cell types, including epidermal keratinocytes, fibroblasts, endothelial cells, and peripheral nerve cells. Re-epithelialization is a crucial step during wound healing, which involves migration and proliferation of keratinocytes from the surrounding epidermis and appendages such as hair follicles and sweat glands (Martin, 1997; Hunt *et al.*, 2000). Our initial study found delayed healing of cutaneous wounds in AQP3-null mice (Figure 2a; Hara *et al.*, 2002). More recent studies have established the likely mechanisms for this phenotype: reduced AQP3-facilitated water transport in keratinocytes from AQP3-null mice, resulting in impaired keratinocyte migration, and reduced AQP3-facilitated glycerol transport, resulting in impaired AQP3-facilitated proliferation.

The motivation for studying AQP3-facilitated migration of keratinocytes was our earlier observation of defective angiogenesis and endothelial cell migration in AQP1 deficiency (Saadoun *et al.*, 2005a), and follow-up findings of defective astroglial cell migration in AQP4 deficiency (Saadoun *et al.*, 2005b; Auguste *et al.*, 2007) and defective migration of kidney proximal tubules cells in AQP1 deficiency (Hara-Chikuma and Verkman, 2006). Migration measurements using transwell assay, in which cell migration through a porous filter is measured in response to a chemotactic stimulus, showed impaired migration in AQP3-knockdown (small interfering RNA) human keratinocyte cultures and AQP3-knockout mouse keratinocyte cultures (Hara-Chikuma and Verkman, 2008a; Figure 2b). Both AQP3 and AQP1 (a water-only AQP) adenoviral infection corrected the migration defect and restored cell membrane water permeability. In an *in vitro* scratch assay of wound healing, wound closure was delayed in AQP3-deficient keratinocytes, with reduced cell protrusions seen at the wound edge. These results suggested the involvement of AQP3-facilitated water transport in keratinocyte migration, perhaps, as found for AQP1 in endothelial cells, by increasing water influx into protruding lamellipodia at the leading edge of migrating cells.

The motivation for investigating the involvement of AQP3 in keratinocyte proliferation were the observations of defective proliferation of corneal epithelial cells in AQP3 deficiency resulting in delayed corneal wound healing (Levin and Verkman, 2006), and defective proliferation in AQP3-deficient colonocytes resulting in severe colitis in experimental models of colitis (Thiagarajah *et al.*, 2007). Experiments using AQP3-null mice and human keratinocyte cultures indicated impaired wound-induced cell proliferation in AQP3 deficiency during wound repair (Figure 2c). Epidermal growth factor-induced cell proliferation was reduced in AQP3-deficient keratinocytes, with impaired p38 mitogen-activated protein kinase cell signaling. Glycerol supplementation restored the impairment in cell proliferation during wound healing, supporting the involvement of AQP3-facilitated glycerol transport in keratinocyte proliferation. These data suggest the possibility of pharmacological modulation of AQP3 to accelerate wound healing in traumatic, burn, and other forms of injury.

Our initial study also found delayed barrier recovery after tape stripping in AQP3-null mice (Hara *et al.*, 2002). Oral glycerol administration corrected the impairment in barrier recovery, which was suggested to involve improved biosynthetic function (Hara and Verkman, 2003). The more recent

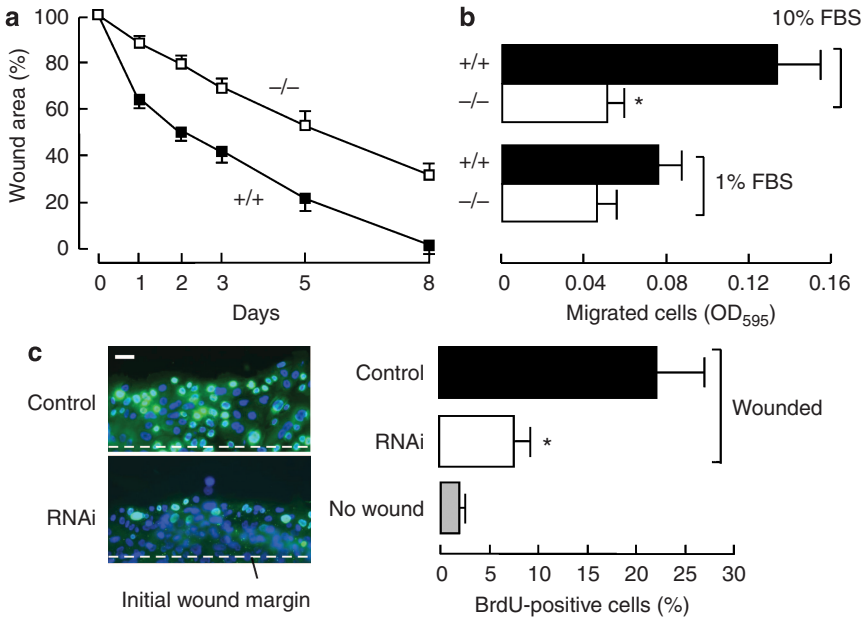

Figure 2. Delayed wound healing with decreased keratinocyte proliferation and migration in AQP3-null mice. (**a**) *In vivo* wound-healing assay. Two full-thickness punch biopsies (diameter 5 mm) were created on the backs of wild-type (+/+) and AQP3-null (−/−) mice. The wound area was measured daily. The percentage of initial wound area (SEM, $n=5$, $P<0.01$ at 1–8 days). (**b**) Transwell cell migration assay (SEM, $n=6$, $^*P<0.01$). The bottom chamber contained 10% or 1% fetal bovine serum. (**c**) (Left) Bromodeoxyuridine staining during wound closure in control and RNA interference-knockdown human keratinocyte cultures. Bar = 50 μm. (Right) The percentage of bromodeoxyuridine-positive cells within 250 μm of the wound margin (SEM, $n=4$, $^*P<0.01$ for control *vs* RNA interference). Adapted from Hara-Chikuma and Verkman (2008a) with kind permission of Springer Science & Business Media.

studies suggest the possible involvement of AQP3-facilitated cell proliferation in epidermal barrier function.

Skin tumorigenesis

At least 13 different tumor cell types have been found to express various AQPs, with AQP expression correlating with tumor aggressiveness in some tumor types where studied, such as AQP4 and malignant astroglioma (reviewed by Verkman *et al.*, 2008). AQP-facilitated water transport is involved in tumor angiogenesis (Saadoun *et al.*, 2005a), as mentioned above, as well as in the migration, invasiveness, and metastatic potential of tumor cells (Hu and Verkman, 2006).

Our motivation for studying the possible involvement of AQP3 in skin tumorigenesis was the strong overexpression of AQP3 in basal cells in human skin squamous cell carcinomas (Figure 3a), and the role of AQP3 in proliferation of keratinocytes during wound healing described above. In experiments to address the role of AQP3 on skin tumorigenesis, we discovered that AQP3-null mice do not develop skin tumors following exposure to a tumor initiator and phorbol ester promoter, a well-established multistage carcinogenesis model (Yuspa *et al.*, 1996), whereas under the same conditions wild-type mice developed multiple tumors (Figure 3b) (Hara-Chikuma and Verkman, 2008b). Strong expression of AQP3 was found in papilloma cells, which colocalized with the proliferation marker keratin-14, but not with differentiation markers, similar to human squamous cell carcinoma.

Studies to establish the cellular mechanisms responsible for AQP3-dependent tumorigenesis, using live mice and keratinocyte cell cultures, showed greatly impaired promoter-induced cell proliferation in AQP3-deficient and knockdown keratinocytes (Figure 3c). Comparable initiator-induced apoptotic response and promoter penetration into the epidermis were found in the wild-type and AQP3-null epidermis, excluding differences in the initiation step or promoter exposure as accounting for the differences in tumorigenesis and cell proliferation. Epidermal differentiation was similar in the wild-type and AQP3-null epidermis under basal conditions and after promoter application. These results suggest that impaired 12-*O*-tetradecanoylphorbol-13-acetate-induced cell proliferation in AQP3 deficiency is responsible for the absence of papillomas. Our data did not verify the proposed involvement of AQP3 in keratinocyte differentiation based on prior indirect evidence (Zheng and Bollinger Bollag, 2003).

Analysis of mechanism showed reduced epidermal cell glycerol, its metabolite glycerol-3-phosphate, and ATP in AQP3 deficiency, without impairment of mitochondrial function (Hara-Chikuma and Verkman, 2008b). Bollag *et al.* (2007) recently reported evidence that AQP3 participates with phospholipase-D in a glycerol–phosphatidylglycerol-signalling module, although we found little difference in lipid composition between wild-type and AQP3-null epidermis. Further, the functional relevance of an AQP3–phospholipid-D module is unclear because glycerol is freely diffusible. In primary keratinocyte cultures, glycerol supplementation corrected the defects in keratinocyte proliferation and reduced ATP generation, providing evidence for the involvement

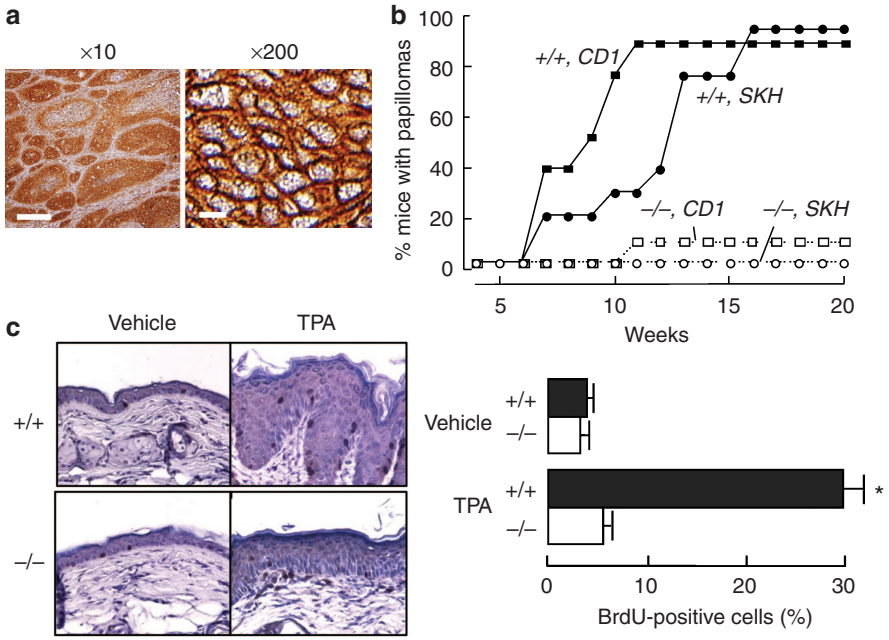

Figure 3. AQP3 expression in human squamous cell carcinoma and impaired skin tumorigenesis in AQP3-null mice. (**a**) Representative AQP3 immunostaining in human skin squamous cell carcinoma (male, age 58 years). Bars = 200 μm. (**b**) Percentage of mice with papillomas. The dorsal skin of wild-type and AQP3-null mice (SKH and CD1 genetic backgrounds) was treated with a single dose of the tumor initiator, 7,12-dimethylbenz[*a*]anthracene (DMBA), followed by multiple applications of the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate, for 20 weeks (9–12 mice per group). (**c**) Impaired epidermal cell proliferation in AQP3-deficient epidermis. The percentage of bromodeoxyuridine-positive cells in epidermal basal layer following vehicle or four 12-*O*-tetradecanoylphorbol-13-acetate applications (TPA) (SEM, $n = 4$, $^*P < 0.01$). Adapted from Hara-Chikuma and Verkman (2008b).

of AQP3-facilitated glycerol transport in ATP generation and keratinocyte proliferation. Further studies revealed correlations between cell proliferation, and ATP and glycerol concentrations. Oral glycerol administration also corrected the impaired epidermal proliferative response in AQP3-null mice epidermis.

Although ATP is thought to be the major energy source in the epidermis as well as in tumor formation (Moreno-Sánchez *et al.*, 2007), the detailed metabolic pathways of ATP production/synthesis in the epidermis have not been established, nor has the role of glycerol metabolism, which is quite tissue-specific (Brisson *et al.*, 2001), been explained. The expression of glycerol kinase, which catalyzes the phosphorylation of glycerol to yield glycerol-3-phosphate, has not been determined in epidermis. Studies are much needed to elucidate the precise metabolic pathways in epidermal cells linking glycerol and ATP generation.

From these findings, we proposed that AQP3-facilitated glycerol transport is an important determinant of epidermal cell proliferation and tumorigenesis by a novel mechanism in which glycerol is a key regulator of cellular ATP energy. Tumor-committed cells generally have an aggressive energy metabolic profile, allowing them to compete with surrounding cells, proliferate, and form characteristic structure. The remarkable resistance to skin tumorigenesis in AQP3 deficiency provides a rational basis for AQP3 inhibition/suppression in the therapy of skin and possibly other cancers associated with overexpression of aquaglyceroporins.

AQP3 IN SKIN DISEASES

Altered AQP3 expression has been found in a variety of skin diseases. Immunostaining showed AQP3 expression in Langerhans cell, dendritic cells, macrophages, neutrophils, and eosinophils in erythema toxicum neonatorum, as well as in epidermal keratinocytes, suggesting involvement of AQP3 in the skin immune system at birth (Marchini *et al.*, 2003). Increased AQP3 transcript and protein expression was found in atopic eczema (Olsson *et al.*, 2006), with the authors suggesting that increased AQP3 contributed to water loss and dry skin. Perhaps increased AQP3 promotes hyperproliferation of immature keratinocytes, resulting in abnormal barrier function. AQP3 expression was absent in epidermal spongiosis associated with eczema (Boury-Jamot *et al.*, 2006), with the authors suggesting a possible relationship between absence of AQP3 and intercellular edema. Whether AQP3 expression is altered in uninvolved skin in diseased subjects was not measured, but is unlikely. As shown above, AQP3 is expressed strongly in skin squamous cell carcinomas, suggesting involvement of AQP3-facilitated glycerol transport in cell proliferation during tumorigenesis (Hara-Chikuma and Verkman, 2008b). A human keratocarcinoma cell line has also been found to express AQP3 (Nakakoshi *et al.*, 2006). Whether altered AQP3 expression is involved in a significant way in the pathophysiology of skin diseases remains to be established. Changes in AQP3 expression may represent only secondary responses to various disease processes.

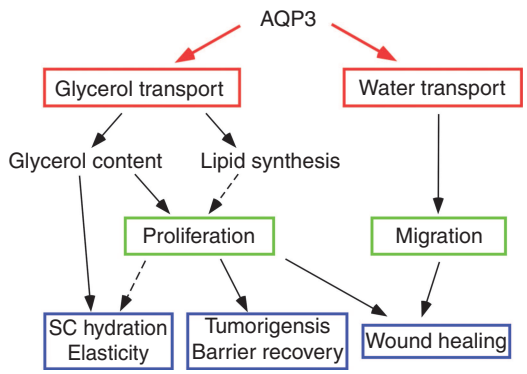

Figure 4. Proposed mechanism for AQP3-dependent skin hydration, wound healing, and tumorigenesis.

Several studies have shown regulated AQP3 expression in keratinocytes. Hyperosmolarity increased AQP3 expression by more than twofold in human keratinocyte cultures (Sugiyama *et al.*, 2001). Retinoic acid, a known regulator of keratinocyte proliferation and differentiation, increased AQP3 gene expression by ~2-fold after 2 hours in the same culture system (Cao *et al.*, 2008; Bellemère *et al.*, 2008). Phorbol ester application to mouse epidermis *in vivo* increased AQP3 protein expression by 10-fold by 4 hours, which was followed by a strong hyperproliferative response (Hara-Chikuma and Verkman, 2008b). Although relevance of these observations to skin physiology and disease pathogenesis is not known at this time, the ability to regulate keratinocyte AQP3 expression suggests the possibility of developing pharmacological agents to alter AQP3 expression (or function) for treatment of skin diseases associated with abnormal proliferation or water homeostasis.

SUMMARY AND PERSPECTIVE

Phenotype analysis of AQP3-null mice supports several distinct roles of AQP3 in keratinocyte biology (Figure 4). AQP3-facilitated water transport is involved in cell migration, accelerating healing of cutaneous wounds. AQP3-facilitated glycerol transport is involved not only in skin hydration and elasticity, based on the humectant properties of glycerol, but also in cell proliferation. We propose AQP3-facilitated glycerol transport to be of central importance in generating ATP, which facilitates the cell growth and tumorigenesis. AQP3 may serve other functions in keratinocytes as well, based perhaps on AQP3 protein–protein interactions.

Although there remain many basic mechanistic questions about the involvement of AQP3 in cell proliferation and the relevance of AQP3 to human skin diseases, the available data suggest interesting new possibilities for clinical therapies. AQP3 modulation by topical drugs may be of benefit in a variety of common skin disorders associated with epidermal hyperproliferation, such as skin carcinogenesis, psoriasis and atopic dermatitis, and repair of burn and other wounds.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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