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Differentiation of Organotypic Epidermis in the Presence of Skin Disease-Linked Dominant-Negative Cx26 Mutants and Knockdown Cx26

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Abstract In this study, we chose a differentiation-competent rat epidermal keratinocyte (REK) cell line to examine the role of Cx26 and disease-linked Cx26 mutants in organotypic epidermal differentiation. First, we generated stable REK cell lines expressing three skin disease-linked mutants (G59A, D66H and R75W). Second, we used an RNAi approach to knock down the expression of Cx26 in REKs. Interestingly, the three-dimensional (3D) architecture of the organotypic epidermis altered the intracellular spatial distribution of the mutants in comparison to 2D cultured REKs, highlighting the importance of using organotypic cultures. Unexpectedly, the presence of disease-linked mutants or the overexpression of wild-type Cx26 had little effect on the differentiation of the organotypic epidermis as determined by the architecture of the epidermis, expression of molecular markers indicative of epidermis differentiation (keratin 10, keratin 14, involucrin, loricrin) and stratification/cornification of the epidermis. Likewise, organotypic epidermis continued to differentiate normally upon Cx26 knockdown. While Cx26 has been reported to be upregulated during wound healing, no reduction in wound closure was observed in 2D REK cultures that expressed loss-of-function, dominant Cx26 mutants. In conclusion, we demonstrate that gain or loss of Cx26 function does not disrupt organotypic epidermal differentiation and offer insights into why patients harboring Cx26 mutations do not frequently present with more severe disease that encompasses thin skin.

Keywords Cx26 · Epidermis · Mutant · Differentiation · Disease

Introduction

Epidermal differentiation is a complex process involving the temporal and spatial regulation of a large number of key molecules (Fuchs & Raghavan, 2002). Several members of the connexin (Cx) family of gap junction proteins are exquisitely expressed and localized during epidermal proliferation and differentiation, and the fact that mutation of any one of six connexin members is associated with hyperproliferative skin disease highlights their importance in regulated skin development and maintenance (Richard, 2005). However, the mechanism(s) by which connexins contribute to healthy epidermal homeostasis is currently unclear.

In human epidermis, 10 connexins have been detected at the mRNA level, including Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx40, Cx43 and Cx45 (Di, Common & Kelsell, 2001a). Similar results have been reported for rodent epidermis (Budunova, Carbajal & Slaga, 1995; Butterweck et al., 1994; Choudhry, Pitts & Hodgins, 1997; Coutinho et al., 2003; Kamibayashi et al., 1993; Kibschull et al., 2005; King & Lampe, 2005; Kretz et al., 2003). In rat interfollicular epidermis, four of these have been confirmed at the protein level–Cx26, Cx31.1, Cx37 and Cx43–with Cx31 expressed in sebaceous glands (Goliger & Paul, 1994, 1995; Risek, Klier & Gilula, 1992). Spatially, low levels of Cx43 are expressed in the strata basale and granulosum, with much higher amounts expressed within

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the stratum spinosum (Guo et al., 1992; Masgrau-Peya et al., 1997; Risek et al., 1992). Conversely, Cx26 is restricted to the upper suprabasal layers in rats (Risek et al., 1992) and is not normally expressed at high levels in interfollicular adult human epidermis except in some patches of thick skin of the palms and foot soles (Di et al., 2001a; Labarthe et al., 1998; Masgrau-Peya et al., 1997). Cx26 is upregulated and present throughout all layers in cases of psoriasis in humans and during wound healing in humans and rats (Brandner et al., 2004; Goliger & Paul, 1995; Labarthe et al., 1998; Lucke et al., 1999). As a result, it has been proposed that Cx26 plays an essential role in the proliferation and differentiation that occurs in response to certain pathological conditions as well as during skin regeneration.

Mutations in Cx26, Cx30, Cx30.3, Cx31, Cx31.1 and Cx43 are linked to sensorineural deafness and/or hyperproliferative skin diseases (Richard, 2005). In the case of skin disease, the type and severity of clinical symptoms vary depending on the mutant connexin involved as well as the molecular location of the mutation. Analysis of some of the mutants has demonstrated defects in trafficking to the cell surface, loss of channel function and, in some cases, gain of channel function (Bakirtzis et al., 2003a; Laird, 2006; Marziano et al., 2003; Richard, 2001, 2005; Thomas et al., 2003; Thomas, Telford & Laird, 2004). As a result, in addition to providing information about the etiology of the skin disease, studies on the different mutants may add important information regarding the function of specific domains within the connexin molecules (Oshima et al., 2003; Thomas et al., 2004). One of the most intriguing features is that most Cx26 mutations only cause deafness while others cause syndromes involving the skin. We and others have demonstrated the potential for some of these syndromic Cx26 mutants (used in our current study) to have *trans*-dominant effects on other members of the connexin family *in vitro* (Marziano et al., 2003; Rouan et al., 2001, 2003; Thomas et al., 2004). These studies support a mechanism whereby autosomal dominant Cx26 mutants leading to deafness and skin disease may inhibit the function of Cx26 as well as other connexins coexpressed in the epidermis.

We chose a newborn rat epidermal keratinocyte (REK) cell line as a model to specifically examine the role of Cx26 in the differentiation of organotypic epidermis. When grown on a collagen gel support at a liquid-air interface, these cells develop into a stratified organotypic epidermis with three to five vital cell layers and a thick cornified layer that closely resembles rat skin (Baden & Kubilus, 1983; Maher et al., 2005; Pasonen-Seppanen et al., 2001b; Tammi et al., 2000). Since Cx26 is upregulated in certain pathological conditions throughout the layers and during wound healing (Brandner et al., 2004; Coutinho et al., 2003), we expressed wild-type Cx26 and disease-linked Cx26 mutants and used an RNAi strategy to knock down Cx26 to examine its role in organotypic epidermal differentiation, cell proliferation and wound healing.

The studies presented here highlight the fact that expression of loss-of-function, dominant Cx26 mutants does not significantly impair normal REK growth and differentiation into organotypic skin, although REKs often exhibited a distinct localization pattern from that found in two-dimensional (2D) cultures. Furthermore, expression of disease-linked Cx26 had no impact on 2D wound healing of REKs. Finally, a full complement of endogenous Cx26 was deemed not necessary for proper REK differentiation into organotypic epidermis. Collectively, these studies suggest that either compensatory connexins coexpressed in REKs may be sufficient to maintain normal cell growth, proliferation and differentiation or endogenous Cx26 is expressed in great excess of the minimal need for the basic properties of epidermal differentiation to be achieved and maintained. These findings could explain why patients harboring Cx26 mutants do not exhibit even more severe disease in all areas of thick and thin skin.

Materials and Methods

Cell Culture

REK cell lines were cultured in Dulbecco's modified essential medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, Burlington, ON, Canada). Cells were passed twice weekly by treatment with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and replated in complete culture medium. In some cases, REKs were grown on glass coverslips coated with 10% type I collagen (phosphate-buffered saline [PBS]) or on glass-bottomed collagen-coated dishes (MatTek, Ashland, MA).

cDNA Constructs and Retroviral Infections

Generation of constructs containing cDNA for human Cx26-green fluorescent protein (GFP), G59A-GFP, D66H-GFP, D66K-GFP and R75W-GFP within the AP2 replication-defective retroviral vector (Galipeau et al., 1999) was as described (Thomas et al., 2003, 2004). shRNA-1/-2 Cx26 targeted sequences (TGGTGAAGTGTAACGCC TGGC 165-172aa and TGGCTGTAAGAATGTGTGCTA, 57-64aa) were generated and cloned into the pH1.1-QCXIH retroviral vector. Stable REK cell lines expressing these cDNAs and shRNA were generated by retroviral infection as previously described (Mao et al., 2000; Qin et al., 2002; Shao et al., 2005; Thomas et al., 2003, 2004). Cells expressing GFP-tagged constructs were maintained in

culture and checked periodically by fluorescence microscopy to ensure continued expression. In general, >80% of the cells stably expressed the appropriate fusion protein for a minimum of 10 cell passages. Stable cell lines exhibiting RNAi-induced Cx26 knockdown were selected with 50 μm/ml hygromycin medium for at least five passages.

Growth of Organotypic REK Epidermis

Rat tail type I collagen was purchased from BD Biosciences (San Diego, CA). Collagen gels were poured on top of 24-mm transwell filter inserts containing 3-μm pores (BD Biosciences, Mississauga, Canada) exactly as described (Pasonen-Seppänen et al., 2001a). Cells (5×10^5) were plated on top of the collagen gel with growth medium in the upper and lower chambers. Upon reaching confluence 3 days later, the medium from the upper chamber was removed to expose the cells to air and allow them to differentiate at the liquid-air interface. For 14 days, 1.5 ml of medium in the lower chamber was replaced daily.

Preparation of Cryosections

Transwell filters with collagen gels and 2-week-old organotypic epidermis were removed from the transwell and cut in half; each half was rolled up and embedded in optimal cutting temperature compound (Tissue-Tek, Hatfield, PA) by freezing in melting isopentane. Embedded samples were stored at –80°C until sectioning. Sections (12 μm) were cut with a cryostat and mounted on Superfrost PLUS slides (VWR International, Mississauga, ON). Slides were stored at –20°C until further processing.

Measurement of Organotypic Epidermis Thickness

For morphometric analysis, cryosections were labeled with hematoxylin and eosin. Cryosections were fixed in neutral buffered 3.7% formaldehyde for 20 min, rinsed under tap water and stained with 1% hematoxylin for 5 min. After a rinse in tap water, excess hematoxylin was removed by a brief rinse in acid-alcohol (1% hydrochloric acid in 70% ethanol) and stained with 1% eosin for another 5 min. Finally, samples were rinsed once in 90% ethanol and twice in 100% ethanol, followed by two rinses in xylene prior to mounting in low-viscosity Cytoseal (Canadawide Scientific, Ottawa, ON, Canada). The thicknesses of the total, vital and cornified layers were measured by imaging five random areas with a x40 oil objective lens mounted on a Zeiss (Thornwood, NY) Axioscope microscope workstation equipped with a Sony (Tokyo, Japan) PowerHAD camera and Northern Eclipse imaging software (Empix, Mississauga, ON, Canada). The epidermal thicknesses of three regions within each image were measured using Adobe (Mountain View, CA) Photoshop so that 15 measurements were obtained per sample. Measurements were obtained from three separate preparations of organotypic cultures. One-way analysis of variance (ANOVA) was performed using GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA).

Immunolabeling

Cells grown in monolayer on glass coverslips (coated with type I collagen) or cryosections were fixed in a solution of 80% methanol and 20% acetone at –20°C for 15 min. Nonspecific labeling was blocked by incubating samples in a solution of 2% bovine serum albumin (BSA, Sigma) in PBS for 30 min. Samples were incubated in primary antibody (anti-Cx26 antibody, 10 μg/ml, from Fred Hutchinson Cancer Research Center Antibody Development Group [Seattle, WA]; Sigma [St. Louis, MO] anti-Cx43 antibody at a dilution of 1/500; or anti-bromodeoxyuridine (BrdU) antibody at a dilution of 1/100 from bank (Iowa City, IA) for 1 h. Samples were washed 3×5 min in PBS, incubated in secondary antibody for 1 h, washed 3×5 min in PBS and when necessary incubated for 5 min with Hoechst 33342 at 10 μg/ml (PBS). Finally, samples were mounted on glass slides in airvol.

Immunolabeling and Imaging of Intact Organotypic Epidermis

Two-week cultures of organotypic REKs were peeled off the collagen gel as an intact stratified layer of cells (epidermis). The epidermis was fixed in 3.7% formaldehyde overnight at room temperature or for 1 h in a –20°C solution of 80% methanol/20% acetone, followed by rinses in PBS. Immunolabeling was carried out as described earlier except that 0.1% Triton X-100 was added to the blocking, primary antibody and secondary antibody solutions to increase antibody penetration into the tissue and incubations were 2 h each for primary and secondary antibodies. The samples were mounted in Vectashield (Vector Laboratories Inc., Burlington, ON, Canada).

For imaging the tissue, a x40 water objective lens mounted on a Zeiss LSM 510 Meta system was used to generate z-stacks using the following laser and filter settings: Texas red was excited with a 543 nm helium-neon laser line and collected on a spectral analysis detector configured to accept 600–650 nm wavelengths, GFP was excited with the 488 nm argon laser line and captured after passage through a band pass 500–550 filter and Hoechst 33342 was detected using a Chameleon multiphoton laser set to 730 nm with fluorescence emissions collected after passage through a 400–450 band pass filter. Motor steps of

1 μm were used for optical sectioning of epidermis with the first image of a z-stack set just below the detection of nuclei stained with Hoechst 33342.

Cell Proliferation Analysis

To estimate the number of cells in S phase, REKs in monolayer or REKs expressing wild-type or mutant Cx26-GFP were incubated in 10 μM BrdU for 1 h. After fixation in 80% methanol/20% acetone, samples were incubated in 2N HCl for 15 min at room temperature and immunolabeled for BrdU using the G_3G_4 monoclonal antibody. A proliferation index was calculated as the percentage of the number of BrdU-positive nuclei divided by the total number of nuclei (stained with Hoechst 33342) counted in 10 fields imaged on a Leica (Deerfield, IL) microscope equipped with a x40 objective. This experiment was repeated three times, and at least 1,000 nuclei were counted per cell line.

Wound Healing

A 2D wound-healing assay was used to assess the migratory potential of REKs expressing wild-type or Cx26 mutants. Cells (3×10^6) were plated in 35-mm glass-bottomed dishes (MatTek). Wounds were made in REK cell cultures that had reached confluence and were starting to show evidence of stratification. A scalpel was used to remove approximately a 1-cm^2 area from the plastic portion (surrounding the glass coverslip center) of the plate. Samples were rinsed and the cells allowed to grow for 48 h in Optimem I reduced serum medium (Invitrogen). Finally, the cells were fixed in 80% methanol/20% acetone and 10 areas of wound edges from three separate experiments were imaged with the x20 objective mounted on a Leica microscope. The distance from the wound to the migrating front was measured using Adobe Photoshop. One-way ANOVA was performed using GraphPad Prism version 4.02.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Confluent monolayers or 2-week organotypic REK epidermis were lysed in ice-cold buffer as described (Pasonen-Seppanen et al., 2001a) except the lysis buffer was supplemented with 0.1% sodium dodecyl sulfate (SDS), 1% NP-40 and 1% deoxycholic acid and protease inhibitor (Sigma). After 20 min, the lysate was sonicated and a Bradford assay (Bio-Rad, Richmond, CA) was used to estimate protein concentrations. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and immunoblotted with antibodies from Zymed (San Francisco, CA) to Cx26 (250 ng/ml); from Sigma to Cx43 (1/20,000); from Chemicon (Temecula, CA) to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1/10,000); from Stressgen (Vancouver, Canada) to Hsc70 (1/10,000); from Lab Vision (Fremont, CA) to keratins 10 and 14 (1/10,000); and from Covance Research Products (Princeton, NJ) to involucrin and loricrin (1/1,000 and 1/5000, respectively). Nonspecific binding was blocked by incubation for 30 min in 5% milk in PBST (PBS plus 0.05% Tween-20), followed by 1-h incubation with primary antibody, 3 x 5 min rinses in PBST, 1-h incubation with secondary antibody conjugated to horseradish peroxidase and 3 x 5 min rinses in PBST. Membranes were then incubated in Supersignal horseradish peroxidase substrate (Pierce, Rockford, IL) and exposed to film. The membranes were stripped using Restore (Pierce) per the manufacturer's instructions, and GAPDH or Hsc70 detection was used as internal control for protein loading. Unsaturated blots were scanned, and densitometry was performed using Scion Image Beta 4.02 for Windows (Scion, Frederick, MD).

Results

Cx26 and Cx43 Expression during REK Differentiation in Organotypic Epidermis

Recently, we reported that REKs grown in a collagen-coated chamber and exposed at a liquid-air interface can differentiate into organotypic epidermis complete with three to five vital cell layers and a cornified layer (Maher et al., 2005). To further characterize organotypic epidermis, we compared Cx26 and Cx43 expression levels and localization patterns in the context of the expression of protein markers of epidermal differentiation and tissue architecture (Fig. 1). Cx26 was only detected in overgrown monolayer cultures in which differentiation had begun (Fig. 1a, arrows) and in the upper differentiated layer of organotypic epidermis (Fig. 1b). The efficacy and specificity of the anti-Cx26 antibody were determined by Western blot using a Cx26-expressing rat mammary tumor cell line (BICR) and Cx26-negative L6 myoblasts (Fig. 1e). Cx43 (Fig. 1c, d) was detected in all cells grown in monolayer cultures (Fig. 1c, arrows) and in basal and suprabasal cells of organotypic epidermis (Fig. 1d). Differentiation of REKs in organotypic culture was assessed by immunoblotting for keratin 14 (K14), K10, involucrin and loricrin. As expected, the relative expression of K14 decreased during differentiation, while the molecular indicators of differentiation–K10, involucrin and loricrin–all increased in the organotypic culture (Fig. 1e). The characteristics of the differentiated epidermis were revealed in cryosections stained with hematoxylin and eosin by the presence of three or four cell layers and a thick stratum corneum (Fig. 1f).

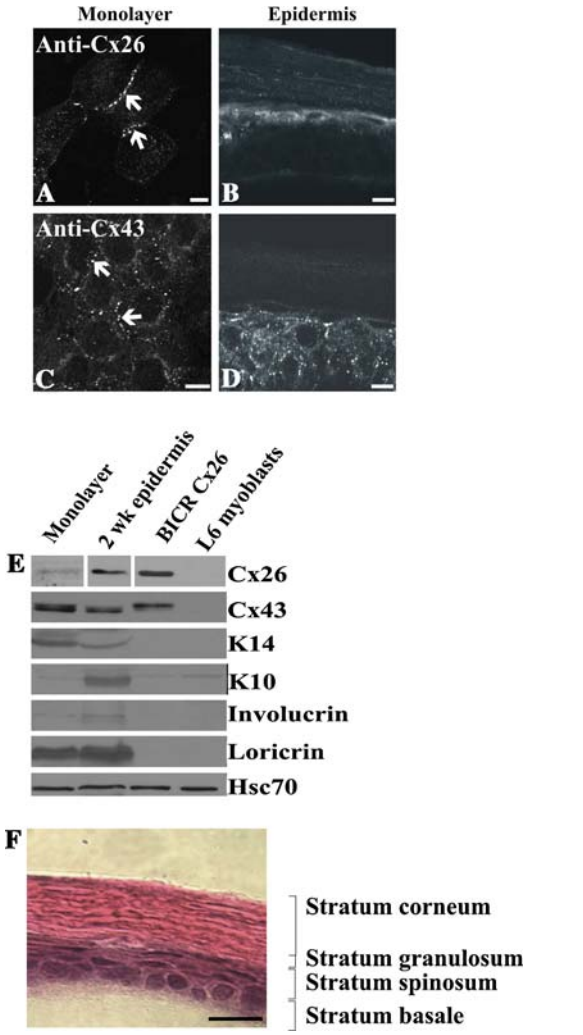

Fig. 1 Organotyic rat epidermis as a reference model for examining the role of Cx26 in keratinocyte differentiation. Monolayers of REKs and cryosections prepared from organotypic epidermis were immunolabeled for Cx26 (**a, b**) or Cx43 (**c, d**). Cx26 expression was only detected in sporadic differentiated areas of monolayer cultures (**a**, *arrows*) and in the upper suprabasal layers of epidermis (**b**). Cx43 was detected in all cells grown in monolayers (**c**, *arrows*) as well as in the basal and suprabasal layers of epidermis (**d**). Western blot analysis of monolayer and epidermal cultures confirmed that Cx26 was upregulated upon differentiation of REKs into epidermis (**e**). Western blot analysis also revealed that K14 decreased while epidermal differentiation markers K10, involucrin and loricrin increased during differentiation (**e**). As a loading control, blots were immunolabeled for Hsc70 (**e**). A representative hematoxylin and eosin-stained cryosection of organotypic epidermis demonstrated the presence of both vital and cornified epidermal layers (**f**). Bars = 10 μm (**a-d**), 25 μm (**f**)

Cx26 Mutants Relocalize in Differentiated Organotypic Epidermis

To assess the localization patterns of disease-linked mutants in 2D and 3D cultures and to determine whether disease-linked mutants can inhibit REK differentiation into epidermis, we stably expressed GFP-tagged Cx26, G59A, D66H and R75W in REKs and grew organotypic epidermis. We also included in this study a nonhuman disease-linked D66K variant of the D66H mutant as this mutant was found previously to exhibit characteristics similar to the D66H mutant in being localized to the Golgi apparatus in gap junction-deficient HeLa cells (Thomas et al., 2004). Cx26-GFP, G59A-GFP, D66H-GFP and R75W-GFP (Fig. 2a, c, e, I; arrows) were assembled into gap junction-like structures at the cell surface, although G59A-GFP and D66H-GFP also exhibited a significant population localized to the Golgi apparatus (Fig. 2c, e; double arrows). These findings suggest that the Golgi apparatus-localized D66H mutant is partially rescued to the cell surface by the presence of endogenous Cx26 or another connexin family member. The D66K-GFP mutant was retained almost exclusively within the Golgi apparatus (Fig. 2g, double arrow) in these 2D cultures consistent with the localization pattern of D66H and D66K in a number of other cell lines (Thomas et al., 2004).

In cryosections of organotypic epidermis formed from REKs overexpressing the variants of Cx26-GFP, the presence of a stratified and cornified epidermis was evident (Fig. 2b, d, f, h, j). In all cases, three to five vital layers and a distinct cornified layer were noted (Fig. 2, layer above the red line). The fluorescent structures observed in the cornified layer represent incompletely degraded GFP-tagged Cx26 or mutants that were trapped in the stratum corneum.

To more closely analyze the localization characteristics of Cx26-GFP, G59A-GFP, D66H-GFP, D66K-GFP and R75W-GFP and to determine if the 3D architecture of the organotypic epidermis would alter the localization patterns of any of the mutants, high-resolution optical sectioning was performed on intact organotypic epidermis (Fig. 3). An optical slice from the basal layer and one from the suprabasal layer are shown with the approximate position within the epidermis as denoted by the schematic diagram (Fig. 3). Gap junction-like plaques were observed in the narrow, columnar basal cells when wild-type Cx26-GFP and G59A-GFP were expressed. In the case of the G59A mutant, a cell surface rim pattern of GFP fluorescence was readily detectable along with punctate plaques in basal cells; but as the cells differentiated and presumably began to keratinized, this pattern was replaced by intracellular GFP fluorescence (Fig. 3, Thomas-G59A Movie 1). Importantly, there were increased incidences of intracellular structures in suprabasal cells immediately below the cornified layer for all Cx26 variants. These structures likely represent internalized connexins en route to degradation as the cells in this region undergo keratinization. In the cases of D66H-GFP and D66K-GFP, although there was

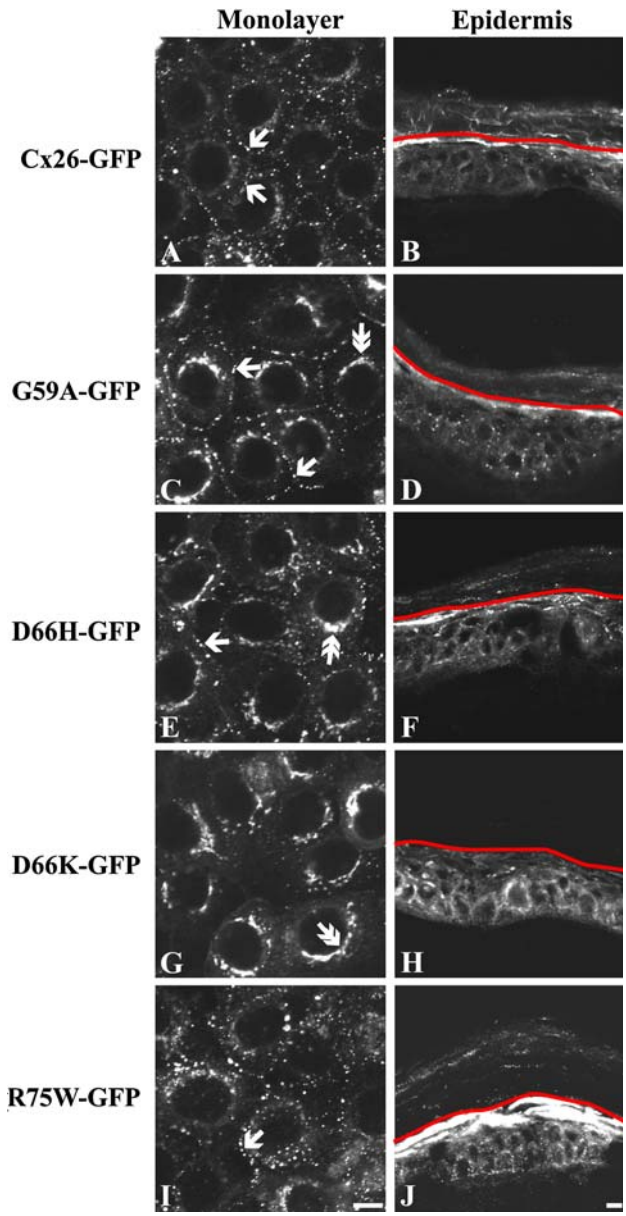

Fig. 2 REKs expressing wild-type or mutant Cx26 formed a stratified and cornified epidermis. Wild-type or mutant Cx26 variants were stably expressed in REKs and grown in monolayer or organotypic cultures. In monolayer, Cx26 (**a**), G59A (**c**), D66H (**e**) and R75W (**i**) were detected at the cell surface in punctate structures reminiscent of gap junction plaques (*arrows*). G59A and D66H (**c** and **e**, *double arrows*) exhibit partial intracellular localization reminiscent of the Golgi apparatus, while D66K (**g**, *double arrow*) was almost exclusively localized to the Golgi apparatus. R75W (**i**, *arrow*) was more commonly observed within intracellular structures compared to wild-type Cx26. Cryosections prepared from organotypic cultures revealed a stratified and cornified epidermis regardless of the overexpression of Cx26 or Cx26 mutants (**b, d, f, h, j**). *Red line* indicates the boundary between the vital cellular layers and the cornified layers. Bars = 10 μm

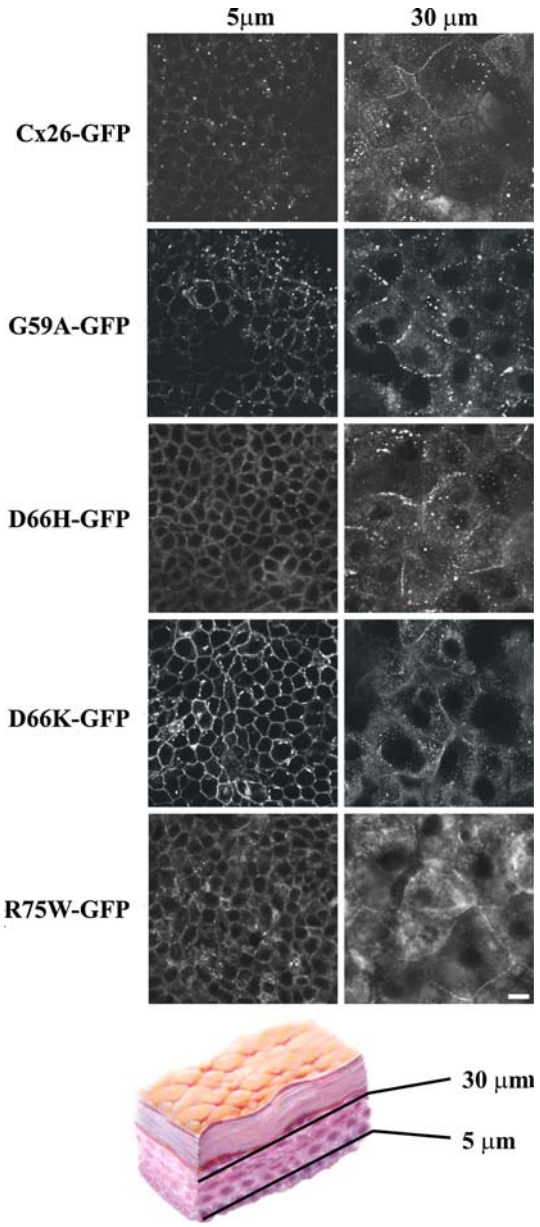

Fig. 3 Differential intracellular spatial localization of Cx26 mutants in 2D and 3D cultures as revealed by optical sectioning of organotypic epidermis. Optical sections of organotypic epidermis expressing Cx26 variants were acquired by confocal microscopy. Optical slices from positions 5 and 30 μm are noted by the schematic diagram of the epidermis. Bars = 10 μm

evidence of evenly distributed cell surface fluorescence and an intracellular Golgi population of the mutant, there was little evidence of gap junction plaques (Fig. 3, Thomas-D66H Movie 2). This distribution pattern was distinctly different from that found in 2D REK cultures as perinuclear localization of these mutants was rarely evident. In addition, unlike the punctate localization pattern seen in 2D REKs, the R75W-GFP mutant exhibited a diffuse localization pattern within the cytoplasm of the suprabasal cells and gap junction plaques were not easily distinguished even in basal cells. Collectively, these studies suggest that the 3D architecture of organotypic epidermis alters the

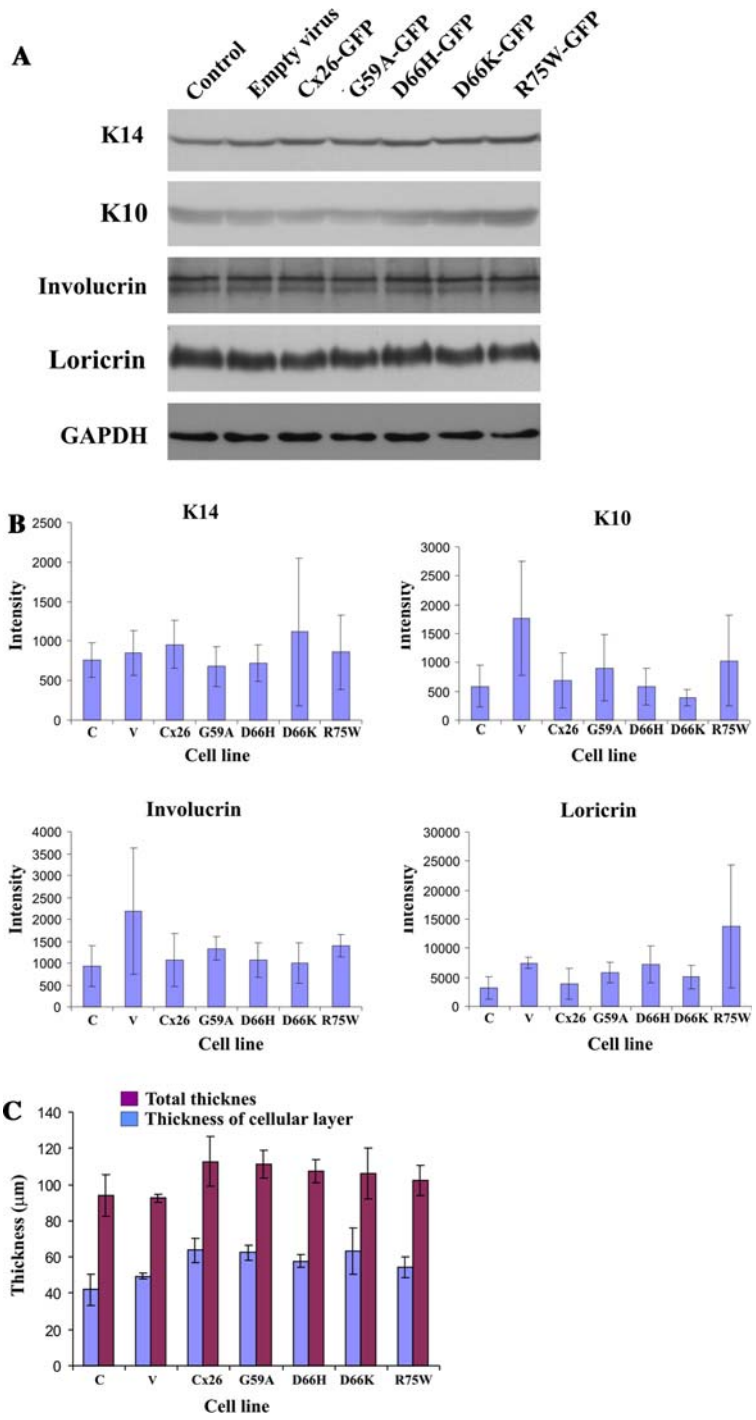

Fig. 4 Expression of Cx26 and mutants does not impair epidermal differentiation. Western blot analysis was performed from epidermal lysates using antibodies against K14, K10, involucrin and loricrin (**a**). Densitometric analysis of four Western blots did not reveal any significant difference in the expression levels of any of the reporter proteins indicative of epidermal differentiation (**b**). Western blot quantification was normalized to GAPDH or hsc70 expression levels (the GAPDH blot presented here corresponds to the involucrin blot). Data are presented as mean intensity ± standard deviation. Cryosection analysis of epidermis grown from REKs expressing the various Cx26 mutants revealed no significant differences in either the total thickness or the vital cell layer thickness of the epidermal cultures (**c**). Data are presented as mean thickness ± standard error. In charts, *C*, control cells; *V*, empty vector-treated cells.

cellular distribution profile of three out of the four mutants studied.

Differentiation Status of Organotypic Epidermis Generated from Cx26 Mutant-Expressing REKs

We next tested whether the constitutive expression of wild-type or mutant Cx26 had an effect on epidermal differentiation markers that included K14, K10, involucrin and loricrin (Fig. 4a, b). Surprisingly, there was no significant difference in the levels of any of these differentiation markers in organotypic cultures upon overexpression of Cx26 or dominant-negative Cx26 mutants. Further organotypic culture analysis of cryostat sections of the total, cellular and cornified layer thicknesses revealed no significant differences between any of the REK cell lines

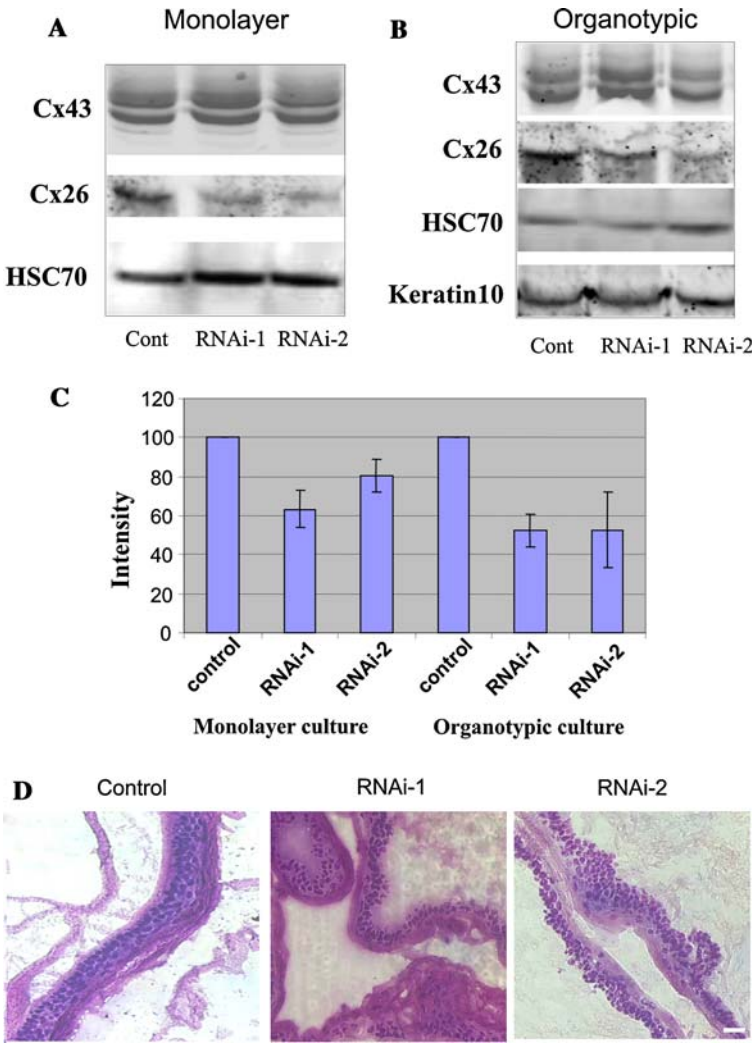

Fig. 5 Downregulated Cx26 did not affect the differentiation of REKs into organotypic epidermis. Protein lysates were collected from monolayer cultures of REKs (**a**) and organotypic epidermis (**b**) that were treated with untargeted shRNA (*Cont*) or shRNA targeting Cx26 (*RNAi-1, RNAi-2*). Western blots were preformed to detect Cx26, Cx43, HSC70 and K10. Densitometric analysis from four Western blots revealed the downregulation of Cx26 (**c**). Data are presented as mean intensity ± standard deviation and are the averages of three separate experiments. (**d**) Hematoxylin and eosin staining of cryosections prepared from organotypic cultures of control or Cx26 knockdown REKs. Bar = 50 μm

expressing wild-type or mutant Cx26 (Fig. 4c). Consequently, we conclude that the overexpression of loss-of-function Cx26 mutants exhibited no notable effect on either the molecular indices of epidermal differentiation or the overall architecture of the epidermis.

REKs with Downregulated Cx26 Differentiated into Organotypic Epidermis

To further assess the role of Cx26 in epidermal differentiation, we stably downregulated Cx26 in REKs using an RNAi approach and two targeted sequences designated RNAi-1 and RNAi-2. Cell lysates from retrovirus-infected REKs grown in monolayer or as organotypic cultures were collected and Western blots were assessed for the levels of Cx43, Cx26, HSC70 and K10 (organotypic cultures only) (Fig. 5). Both RNAi vectors targeting Cx26 were successful in knocking down Cx26 levels by approximately 50% in REKs grown as organotypic epidermis. The Cx26 knockdown did not affect the levels of Cx43, HSC70 or K10. Interestingly, cryosections of organotypic epidermis generated from REKs with reduced levels of Cx26 revealed architecturally similar epidermis consisting of three to five vital layers and a distinct cornified layer (Fig. 5).

REKs Expressing Cx26 Mutants Have Similar Cell Proliferation and Wound-Healing Properties

To determine whether the expression of wild-type or Cx26 mutants caused a change in proliferation, REKs expressing wild-type or various Cx26 mutants were exposed to BrdU to estimate a proliferation index. Based on this analysis, there were no significant changes in the proliferation index (Fig. 6a).

Since clinical lesions associated with Cx26-linked skin disorders are often in locations subject to wounds and mechanical stress, we tested whether Cx26 mutant-expressing REKs exhibited alterations in 2D wound healing.

A

	Control	Empty virus	Cx26	G59A	D66H	D66K	R75W
BrdU-positive nuclei	39.7%	54.1%	49.9%	53.1%	54.3%	55.2%	47.1%

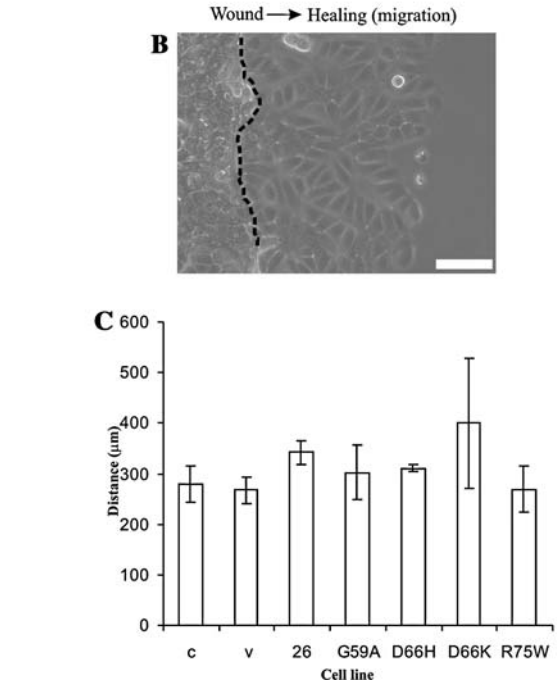

Fig. 6 REK proliferation and wound healing are not significantly affected by the expression of Cx26 or mutants. Monolayer cultures of REKs were incubated with BrdU and assessed for BrdU-positive cells (**a**). Monolayer cultures of REKs were scraped, wounded and allowed to grow for 48 h (**b**). The distance traveled was measured as the distance between the wound edge (**b**, *dotted line*) and the migrating front. Data are presented as mean distance ± standard deviation (**c**). *C*, control cells; *V*, empty vector-treated cells; *26*, Cx26. Bar = 50 μm

Results from the wounding assay did not reveal any significant difference in any REK cell lines expressing the Cx26 mutants (Fig. 6b, c).

Discussion

Organotypic Culture as a Reference Model for Examining the Functional Role of Cx26 in Keratinocyte Proliferation, Differentiation and Function

In humans, Cx26 has been established as being associated with the maintenance of epidermal function, particularly in the thick skin of the hands and feet. This fact was established when various palmoplantar keratodermas were identified in patients harboring Cx26 mutations (Kelsell et al., 2000). However, the mechanism by which Cx26 is linked to clinically detectable skin disease is poorly understood. The vast majority of Cx26-linked skin disease is linked to autosomal dominant Cx26 mutations and not to the more frequently found autosomal recessive mutations associated with hearing loss (Kelsell et al., 2000). We have previously established that the Cx26 mutations G59A, D66H and D66K are loss-of-function mutations and that G59A and D66H can dominantly inhibit the function of coexpressed Cx26 and other connexins (Thomas et al., 2004). These prior findings raised the possibility that these mutants may in fact act in the skin by inhibiting the function of coexpressed endogenous Cx26 or even other members of the connexin family. Consequently, we hypothesized that if these and other mutants were expressed in keratinocytes, they would directly impact the differentiation of keratinotyes and the formation of a complete epidermis.

In order to test this hypothesis, we first fully characterized an *in vitro* 3D organotypic culture model where REKs were differentiated into epidermis. In organotypic epidermis, Cx43 was detected in basal and suprabasal cells while Cx26 was present only in upper suprabasal cells. This epidermal layer-specific connexin distribution pattern was previously reported for rat epidermis *in vivo* as well as for human organotypic cultures (Wiszniewski et al., 2000; Wiszniewski, Salomon & Meda, 2001a) and *in vivo* (Arita et al., 2002; Di et al., 2001b; Salomon et al., 1994). In our organotypic cultures, keratinocytes underwent terminal differentiation as assessed by increased levels of K10, involucrin and loricrin as well as by the architectural parameters of exhibiting three to five vital cell layers and a thick cornified layer mimicking rat epidermis *in vivo*. Consequently, we established an excellent epidermal differentiation reference model in

which to investigate the consequence of keratinocytes expressing Cx26 mutants.

Differential Localization of Cx26 Mutants in 2D and 3D Environments

We generated stable REK cell lines expressing three skin disease-linked and GFP-tagged mutants (G59A, D66H and R75W) and one additional mutant that exhibited a Golgi-restricted localization within the cells (D66K). In monolayer, all except D66K could be found to some extent in punctate structures at the cell surface, although R75W appeared to make few gap junction-like structures. G59A, D66H and D66K were also localized to prominent intracellular compartments, as we and others have previously reported in other cell lines (Bakirtzis et al., 2003a,b; Di et al., 2001a; Thomas et al., 2004). Collectively, these somewhat diverse distribution patterns would suggest that Cx26 mutants variably intermix with wild-type connexins coexpressed in REKs. Of the three disease-linked Cx26 mutants, D66H exhibited the greatest trafficking defect in our previous studies as it was retained within the Golgi apparatus in gap junctional intercellular communication (GJIC)-deficient cell lines (Thomas et al., 2004), whereas G59A and R75W could be found in punctate structures at the cell surface (Thomas et al., 2003, 2004).

The D66H and D66K mutants in organotypic cultures revealed a much different pattern from that found in 2D culture as they were now readily found evenly distributed at the cell surface in addition to the Golgi apparatus. Relocation of the R75W mutant was also evident as the punctate distribution pattern seen in 2D was replaced by a more cytoplasmic and rim-like distribution pattern in 3D. These results strongly suggest that the 3D architecture of organotypic epidermis, possibly in conjunction with the onset of expression of other connexins or molecules, collectively caused a distinctly different localization pattern for the D66H, D66K and R75W mutants. Previously, we showed in GJIC-deficient HBL100 and HeLa cell lines that D66H was retained within the Golgi apparatus but transported to the cell surface upon coexpression with Cx26, Cx30 and Cx32 but not Cx43 (Thomas et al., 2004). Therefore, it is likely that the onset of endogenous Cx26 expression (and possibly another connexin[s]) in the upper suprabasal layers was able to rescue D66H to the cell surface in the REK epidermis. Interestingly, in the D66H-expressing mouse, Bakirtzis and colleagues (2003b) failed to detect any D66H at the cell surface of keratinocytes and furthermore found that wild-type Cx26 and Cx30 were retained within intracellular compartments in the presence of D66H. A plausible reason for this is that mouse epidermis expresses only low amounts of endogenous Cx26 (Kretz et al., 2003) compared to rat epidermis; thus, rescue of the D66H mutant to the cell surface would not be as likely. Additionally, in their study, it is difficult to resolve precise connexin localization in cryosections since the upper suprabasal cells become flattened.

Loss-of-Function, Gain-of-Function or Reduced Cx26 Levels Failed to Detectably Alter Organotypic Epidermal Differentiation

At present, only one study has reported that epidermis reconstructed from a patient encoding a Cx26 truncation mutant exhibits evidence of reduced stratification *in vitro*, yet preliminary evaluation of patients harboring this mutation revealed an apparent normal epidermal barrier (Wiszniewski et al., 2001b). To begin to understand the importance of Cx26 in the skin, it is necessary to evaluate Cx26 expression during development and differentiation. Cx26 is expressed earlier than other connexins in the embryonic development of the epidermis throughout all layers in humans and rats (Arita et al., 2002; Risek et al., 1992) and is upregulated in psoriasis (Labarthe et al., 1998) and during skin regeneration (Brandner et al., 2004). Consequently, we hypothesized that if the regulation of functional Cx26 is critical, then the overexpression of Cx26 (gain of function) throughout organotypic epidermis would result in an increase in the number of cellular epidermal layers and dominant-negative-acting Cx26 mutants (loss of function) would inhibit this effect.

The symptoms of Cx26 mutant-associated diseases begin shortly after birth or during childhood and are most often restricted to the palms and soles (Richard, 2003). Thus, the organotypic model presented here is more likely a reflection of the developing epidermis rather than a steady-state epidermis undergoing continuous mechanical stress. This difference may partially explain why wild-type Cx26 or mutant forms of Cx26 and knockdown Cx26 failed to affect the stratification, differentiation or proliferation of REK epidermis, even though G59A, R75W and D66H all have been shown to exert strong dominant negative effects on the function of Cx26 (Marziano et al., 2003; Rouan et al., 2001; Thomas et al., 2004). The lack of any Cx26 mutant-induced changes in the levels of key molecules known to be directly related to epidermal differentiation is consistent with a model in which Cx26 loss or mutation can be overcome by other connexins coexpressed in the epidermis during stratification. This hypothesis is also supported by the reverse transcriptase polymerase chain reaction results, which revealed the expression of at least nine connexin isoforms in REK cells (Maher et al., 2005). However, it is also possible that Cx26 is not an essential connexin of thin skin or that only low levels of Cx26 function are adequate to facilitate epidermal

differentiation. Alternatively, Cx26 may partially mediate its effects on keratinocyte differentiation by mechanisms independent of GJIC, as has been demonstrated in other systems with other connexins (Lin et al., 2003; McLachlan et al., 2006; Moorby & Patel, 2001; Qin et al., 2002, 2003; Zhang, Kaneda & Morita, 2003).

In the present study, we did not observe any differences in the migratory ability of any of the mutant Cx26 cell lines in a 2D wound-healing assay. It has been reported that during wound healing there is coordinated up- and down-regulation of several connexin isoforms (Brandner et al., 2004; Coutinho et al., 2003; Goliger & Paul, 1995). Prior studies where Cx43 was eliminated or silenced revealed enhanced wound healing (Kretz et al., 2003; Qiu et al., 2003), which may not have been surprising as Cx43 downregulation is a normal feature of wound healing (Brandner et al., 2004; Coutinho et al., 2003; Goliger & Paul, 1995). Cx26, on the other hand, is upregulated throughout the layers. Coutinho et al. (2003) hypothesized that this upregulation is necessary for the migration of keratinocytes into the wounded area, although the results presented here would suggest that functional Cx26 is not required. Kretz, Maass & Willecke (2004) further suggested that the upregulation of Cx26 (and other connexins) maintains GJIC throughout the epidermal layers during the regeneration of the wounded area while preventing the reestablishment of a calcium gradient. Normally, the calcium concentration is low in the basal layers and high in the differentiated suprabasal layers, and this is vital for proper epidermal differentiation (Elias et al., 2002). This hypothesis awaits evidence that the calcium gradient is indeed disrupted or at least decreased in epidermis constitutively expressing Cx26 or mutant variants of Cx26. It is conceivable that if the mutants disrupted the calcium gradient, a clear epidermis phenotype may not present itself for several weeks of organotypic culturing. Such subtle changes would likely have a more exaggerated effect and, hence, a visible phenotype in a tissue undergoing mechanical stress such as the palmar and plantar epidermal surfaces.

In conclusion, we demonstrated that gain- and loss-of-function Cx26 mutants did not alter epidermal differentiation. It is plausible that other connexins coexpressed within the REK epidermis compensate for the loss of Cx26 function or that a full complement of Cx26 is not necessary for epidermal differentiation and maintenance in thin skin. Future studies will be necessary to determine if Cx26 plays a more direct role in thick skin when placed under mechanical stress further mimicking the environment of palmoplantar epidermis.

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