To understand the role of connexin43 (Cx43) in epidermal differentiation, we reduced Cx43 levels by RNA-mediated interference knockdown and impaired its functional status by overexpressing loss-of-function Cx43 mutants associated with the human disease oculodentodigital dysplasia (ODDD) in rat epidermal keratinocytes. When Cx43 expression was knocked down by 50–75%, there was a coordinate 55–65% reduction in Cx26 level, gap junction-based dye coupling was reduced by 60%, and transepithelial resistance decreased. Importantly, the overall growth and differentiation of Cx43 knockdown organotypic epidermis was severely impaired as revealed by alterations in the levels of the differentiation markers loricrin and involucrin and by reductions in vital and cornified layer thicknesses. Conversely, although the expression of Cx43 mutants reduced the coupling status of rat epidermal keratinocytes by ~80% without altering the levels of endogenous Cx43 or Cx26, their ability to differentiate was not altered. In addition, we used a mouse model of ODDD and found that newborn mice harboring the loss-of-function Cx43G60S mutant had slightly reduced Cx43 levels, whereas Cx26 levels, epidermis differentiation, and barrier function remained unaltered. This properly differentiated epidermis was maintained even when Cx43 and Cx26 levels decreased by more than 70% in 3-week-old mutant mice. Our studies indicate that Cx43 and Cx26 collectively co-regulate epidermal differentiation from basal keratinocytes but play a more minimal role in the maintenance of established epidermis. Altogether, these studies provide an explanation as to why the vast majority of ODDD patients, where Cx43 function is highly compromised, do not suffer from skin disease.

Gap junctions are intercellular channels that form from the pairing of two adjacent connexons from apposing cells. Connexons consist of six connexin subunits selected from the 21-member human connexin family. Gap junctions mediate gap junctional intercellular communication (GJIC), which allows ions, small molecules, and secondary messengers to pass from one cell to another (1). The epidermis is primarily composed of keratinocytes that temporally and spatially express as many as 10 different connexins depending on their degree of differentiation (2). Importantly, properly regulated GJIC is essential for efficient wound healing (3, 4) and for protection against diseases of the skin (5–7).

To elucidate the role of Cx43 in epidermal differentiation, we acquired an immortalized rat epidermal keratinocyte (REK) cell line that has the unique advantage of being suitable for two-dimensional monolayer cultures and can be induced to differentiate into three-dimensional organotypic skin (8). REKs express mRNA for at least nine connexin family members and differentially regulate the expression and spatial localization of Cx43 and Cx26 (9). We have previously shown that the expression profile and differentiation characteristics of organotypic epidermis provide an excellent cellular environment to examine the role of connexins in epidermal differentiation (9). In the current study we examine the specific role of Cx43 in regulating epidermal differentiation.

Cx43 is one of the more abundant members of the connexin family expressed in the epidermis, and it is primarily found in the stratum basale and stratum spinosum where keratinocytes undergo proliferation and commence differentiation (2, 10–12). Cx43 expression tends to diminish in the upper part of the stratum spinosum as the cells continue to differentiate (13). It is notable that Cx43 is down-regulated during wound healing (14) and during carcinogenesis of the epidermis (15). Interestingly, in only a few patients suffering from the Cx43-linked human disease oculodentodigital dysplasia (ODDD) (16) symptoms of palmoplantar keratodermas and hyperkeratosis have been identified (17–19). Recently, an N-ethyl-N-nitrosourea mutagenesis screen generated a mouse model of ODDD (20) (Gja1^rt-rt^rt- mutant mouse), which harbors an autosomal dominant mutation resulting in a G60S mutation in Cx43. Interestingly, the Gja1^rt-rt^rt- mouse exhibited no obvious gross skin abnormality. Although a Cx43 knock-out line exists (21), Cx43-null mice die at birth, restricting any assessment of skin disorders in mice to the early stages of development. The role of Cx43 in skin development was nevertheless established when mice harboring a truncated variant of Cx43 died within the first week after birth as a result of a major defect in skin barrier function (22). Collectively, these studies suggest that Cx43 plays a vital role in skin and epidermal development, yet it is unclear what role Cx43 plays in the differentiation, stratification, and maintenance of the epidermis during continual renewal.
Role of Cx43 in Epidermal Differentiation

In the present study we used two different approaches to assess the role of Cx43 in epidermal differentiation. First, Cx43 expression levels were reduced by a RNA-mediated interference strategy, and differentiation was assessed in organotypic epidermis from REKs. The organotypic epidermis model was chosen because of the ease with which connexin expression can be manipulated in cells undergoing differentiation in a three-dimensional configuration, mimicking epidermal development in vivo. Second, the functional status of Cx43 was inhibited by the overexpression of dominant-negative ODDD-linked Cx43 mutants. The effect of ODDD-linked Cx43 mutants in epidermis differentiation was assessed in an organotypic model in vitro and in mouse skin in vivo. Knockdown of Cx43 expression in keratinocytes significantly altered their ability to properly stratify and assemble into an epidermis, thus resulting in a loss of architectural organization of the organotypic epidermis. The reduction of Cx43 expression also resulted in a decrease in Cx26 and an up-regulation in the expression of differentiation markers involucrin and loricrin. As opposed to the Cx43 knockdown studies, the inhibition of GJIC function by overexpressing dominant-negative Cx43 mutants did not alter the ability of keratinocyte to reach terminal differentiation in vitro. In vivo, newborn Gja1<sup>G60S</sup> mice harboring the loss-of-function Cx43<sup>G60S</sup> mutant exhibited only a slight reduction of total Cx43 expression compared with control animals without any change in Cx26 levels, suggesting that the amounts of Cx43 and Cx26 present during embryonic epidermal differentiation were similar in mutants and control mice. Accordingly, newborn G60S mice did not show any difference in the levels of keratinocyte differentiation markers or in barrier function integrity of the epidermis. As these mice age, although there was a significant reduction in Cx43 and Cx26, the integrity of the properly and fully stratified epidermis observed in newborn skin and its continual renewal were maintained.

EXPERIMENTAL PROCEDURES

Cell and Organotypic Epidermal Culture—REKs were cultured as previously described (9). Briefly, cells were cultured in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All cell culture reagents were purchased from Invitrogen.

For organotypic cultures REKs were subcultured on collagen I-coated transwells as previously described (9). Once cells reached confluence, the medium from the upper chamber was removed to expose the cells to a liquid/air interface for 14 days.

shRNA Constructs and Retroviral Infections—Two Cx43-targeted shRNA constructs designated S1 (GAAGTTCAAGTACGGGATT) and S2 (GGTGTGCTGTCAGTACTT) together with a control vector containing a nonsense sequence were designed and used to make infectious viral supernatants as we described previously (23). REKs were infected and cultured in the same manner as we described (23). Infected REKs were cultured in selection medium containing 50 μg/ml hygromycin, and antibiotic-resistant cells were passed at least 3 times before further experimentation.

Generation of constructs containing cDNA for human Cx43-GFP, G21R-GFP, and G138R-GFP within the AP2 replication-defective retroviral vector (24) were previously described (25). Stable REK cell lines expressing Cx43-GFP, G21R-GFP, and G138R-GFP cDNAs were generated by retroviral infection as previously described (26, 27).

Cx43 Mutant Mice—Mice were maintained in accordance with the University of Western Ontario Guide for the Care and Use of Laboratory Animals. Gja1<sup>G60S</sup> mutant mice were obtained from Dr. Janet Rossant, Centre for Modeling Human Disease (Toronto, ON). These mice exhibit similar characteristics to the Cx43-linked human disease, oculodentodigital dysplasia, as previously described (20). Gja1<sup>G60S</sup> mutant mice and their wild-type littermates were collected at the day of birth and at 3 weeks of age.

Transepithelial Resistance Assay—REKs were plated on 3.0-μm pore transwell inserts (VWR) at 15,000 cells/insert. After 24 h a Millicel-ERS voltmeter (Millipore) was used to measure resistance in ohms. Measurements were taken for three consecutive days. Three independent experiments were performed where three measurements were taken per well, and three wells were used per cell type.

Preparation of Cryosections, Histological Staining, and Immunofluorescence—Organotypic epidermis and thick skin from hind paws of euthanized 21-day-old wild-type and Gja1<sup>G60S</sup> mutant mice was embedded in Optimal Cutting Temperature compound (Tissue-Tek). To observe histological epidermal structure, hematoxylin and eosin staining was performed as previously described (9). Immunofluorescence and confocal microscopy were used to examine endogenous and GFP-tagged wild-type and Cx43 mutants in REKs as modified from our previous study (28). Briefly, cultured cells were plated on 12-mm glass coverslips and fixed with an ice-cold solution of 80% methanol and 20% acetone at 4 °C for 15 min. Cryosections and organotypic cultures were fixed with 3.7% formaldehyde overnight at room temperature or with methanol/acetic acid. The localization of endogenous Cx43 was analyzed using rabbit anti-Cx43 (1:500, Sigma-Aldrich). Cell nuclei were stained for 15 min with Hoechst 33342 (10 μg/ml) in phosphate-buffered saline. The samples were mounted in Airvol or Vectashield (Vector Laboratories, Burlington, ON). Immunolabeled monolayer cultures of REKs and organotypic epidermis were imaged using a Zeiss LSM 510 META system as previously described (28).

Western Blot Analysis—Confluent monolayer cultures and organotypic cultures were solubilized in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM sodium vanadate, 1 mM sodium fluoride, and proteases inhibitors), sonicated, and centrifuged to remove the cell debris. In addition, dorsal mouse skin was excised from euthanized newborn and 3-week-old mice and rinsed in phosphate-buffered saline. The skin was homogenized in lysis buffer and treated as described for REK organotypic cultures (9).

Western blots were developed with rabbit anti-Cx43 (1:1000, Sigma-Aldrich), rabbit anti-Cx26 (1:500, Zymed Laboratories Inc.), mouse anti-keratin 14 (dilution 1:5000, Lab Vision Co. Fremont, CA), rabbit anti-involucrin (dilu-
Role of Cx43 in Epidermal Differentiation

Cx43 Knockdown Alters the Coupling Status, the Transepithelial Resistance, and the Differentiation Characteristics of Keratinocytes—Cx43 is abundantly localized to gap junction plaques at the cell surface between adjoining REKs (Fig. 1A, arrow) and within intracellular compartments (Fig. 1A, arrowhead). To begin to assess the role of Cx43 in keratinocyte differentiation, we reduced Cx43 protein expression levels using shRNA in REKs. Immunofluorescence localization of Cx43 in two different shRNA-treated cell lines revealed that whereas evidence of intracellular localized Cx43 remained (Fig. 1B and C, arrowheads), there was little evidence for gap junction plaques. Western blot and densitometric analysis (Fig. 1D and E) confirmed that the two different shRNA sequences reduced Cx43 expression by ∼50–70% compared with the nonsense vector control. The reduction in Cx43 levels also resulted in a ∼50% decrease in the incidence of dye coupling (Fig. 1F). Transepithelial resistance measurements of Cx43-reduced REKs grown on cell culture inserts revealed no change in resistance in comparison to controls until day 3 when a small but significant decrease in transepithelial resistance was observed (Fig. 1G).

To determine the effects of Cx43 knockdown on terminal differentiation of keratinocytes, we generated organotypic epidermis from nonsense vector control and Cx43-reduced REKs. The histological staining and the localization patterns in control organotypic epidermis revealed a tightly organized basal layer (Fig. 2A and C, first layer above the dotted line) and a two- or three-cell thick suprabasal layer (Fig. 2A and C, dotted line to first dashed line) with abundant evidence for Cx43 at cell-cell interfaces (Fig. 2C, arrows). Control organotypic epidermis also gave rise to a thick cornified layer (Fig. 2A and C, between dashed lines) that contained sparse staining that we believe is nonspecific anti-Cx43 immunostaining or remnants of Cx43 trapped in the cornified layer (Fig. 2C). When Cx43 was partially reduced, evidence for sparse amounts of Cx43 could still be detected by immunofluorescence (Fig. 2D, arrows). Interestingly, whereas there was some basal cell layer organization, the overall thicknesses of the vital layer was reduced to one or two cell layers (between the dotted line and first dashed line), and the cornified layer (between dashed lines) was typically thinner compared with the wild-type (Fig. 2B and D). In addition, the organotypic cultures generated from Cx43-reduced REKs were fragile and difficult to peel compared with wild-type and control cultures. Reduction of Cx43 in REKs did not significantly affect cell proliferation as assessed by the incorporation of bromodeoxyuridine or the overall rate of cell growth in two-dimensional cell cultures (data not shown).

To assess whether Cx43 knockdown altered epidermal differentiation with respect to known molecular markers of keratinocyte differentiation, organotypic epidermis from vector control and Cx43-reduced REKs were immunoblotted for Cx26, keratin 14, involucrin, and loricrin. Quantification studies from six matched control and Cx43-S1 and -S2 knockdown organotypic cultures revealed that when Cx43 was reduced by ∼70–75%, Cx26 expression was diminished by ∼55–65%. Loricrin and involucrin were up-regulated in Cx43-reduced organotypic epidermis compared with control, whereas keratin 14 was not affected (Fig. 2, E and F). The extent of up-regulation of loricrin and involucrin was variable (∼1.2–4-fold compared with control organotypic epidermis) but consistently observed in all sets of epidermal differentiation assays.

Overexpression of ODDD-linked Cx43 Mutants Alters the Coupling Status of Keratinocytes but Not Their Ability to Reach Terminal Differentiation—We examined the effects of two dominant-negative Cx43 mutants, G21R and G138R (16, 30), on REK differentiation. These overexpressed mutants have been shown to traffic to the cell membrane but fail to form functional gap junction channels (25). Similar to wild-type REKs, Cx43 was found in gap junction plaques at the cell surface (Fig. 3A and B, arrows) and in intracellular compartments (Fig. 3A and B, arrowheads) in REKs overexpressing Cx43-GFP. Consistently, confocal microscopy revealed that both G21R and G138R mutants were found at the cell surface when overexpressed in REKs (Fig. 3C and D, arrowheads) as well as within intracellular compartments (Fig. 3C and D, arrowheads).

Toluidine Blue Penetration Assay—To test for intact epidermal barrier, euthanized newborn Gja1+/− mice and wild-type littermates were incubated for 5 min in 100% methanol and rinsed 3 × 5 min with phosphate-buffered saline followed by a 30-min incubation in 0.1% toluidine blue (29). After 3 × 5-min washes in phosphate-buffered saline, pups were examined for blue staining of the epidermis and imaged using a digital camera.

Dye Transfer—Confluent REKs infected with Cx43-targeted shRNA constructs or transiently transfected with cDNAs encoding Cx43-GFP, G21R-GFP, or G138R-GFP were used for microinjection. One cell within a cluster of REKs expressing the GFP-tagged connexin or random cells selected within Cx43-knockdown cell cultures were pressure-microinjected with 1% Lucifer yellow in double-distilled H2O (Molecular Probes) until the cell was brightly fluorescent (<5 s) using an Eppendorf FemtoJet automated pressure microinjector. After 1 min, the percent of microinjected cells that transferred Lucifer yellow to at least one contacting cell was determined using a Leica DM IRE2 inverted epifluorescence microscope. Digital images were collected with a charged coupled device camera (Hamamatsu Photonics, Japan) using OpenLab software (distributed by Quorum Technologies, Inc., Guelph, ON). At least 25 microinjections were performed for each experimental condition.

Statistics—Statistical data were analyzed using an analysis of variance followed by a Tukey test for significance. A two-tailed unpaired t test was used for the data presented in Fig. 6. Error bars reflect S.D.

RESULTS
Western blots indicated that the expression levels of wild-type and Cx43 GFP-tagged mutants were similar in these cells and that the overexpression of these constructs does not affect the level of endogenous Cx43 (Fig. 3E). The expression of the Cx43 mutants inhibited more than 50% of the incidence of dye coupling (Fig. 3F), suggesting that they had dominant-negative effects on co-expressed connexins in REKs.

To assess the effect of overexpressing loss-of-function and dominant-negative Cx43 mutants (25) on keratinocyte differentiation, we generated organotypic epidermis from REKs overexpressing GFP-tagged wild-type or mutants (G21R and G138R) of Cx43. Similar to the organotypic epidermis from REKs-expressing Cx43-GFP, the organotypic epidermis from cells overexpressing Cx43 mutants showed a tightly organized...
basal layer (Fig. 4, A–F, above the dotted line) and a two- or three-cell-thick suprabasal layer (Fig. 4, A–F, dotted line to first dashed line) with abundant evidence for Cx43 at cell-cell inter-
faces (Fig. 4, D–F, arrows) as well as at intracellular locations. Similar to Cx43-GFP, organotypic epidermis generated from REKs expressing the Cx43 mutants gave rise to a thick cornified layer (Fig. 4, A–F, between dashed lines) with considerable evi-
FIGURE 2. Cx43 knockdown alters the architecture and expression of differentiation markers in organotypic epidermis. Histological staining and confocal images revealed that in wild-type epidermis, a thick cornified layer (A and C, between dashed lines) and vital cell layers (A and C, dotted line to first dashed line) are evident, whereas these layers are both reduced when Cx43 expression is knocked down (B and D). Cx43 immunofluorescence (C and D, green) of cryosectioned organotypic epidermis revealed abundant Cx43 throughout the vital layer (C, arrows) of the vector control, a situation not evident in Cx43 knockdown epidermis (D). Western blot (E) and densitometric analysis (F, n = 6) of Cx43-reduced organotypic cultures revealed a notable decrease in Cx26 expression and an increase in loricrin and involucrin compared with control cultures. Dotted lines represent the bottom of the basal layer, and the space between the dashed lines denotes the cornified layer. The green staining between the dashed lines in C represents nonspecific staining or remnants of Cx43 trapped in the cornified layer. Protein expression was normalized to β-actin (F). Blue, nuclei; B, bar = 50 μm; D, bar = 20 μm.
FIGURE 3. Subcellular localization of overexpressed Cx43 mutants in REKs and their effect on gap junctional coupling. Confocal imaging of monolayer cultures of REKs expressing endogenous Cx43 (A, green), Cx43-GFP (B, green), G21R-GFP (C, green), or G138R-GFP (D, green) revealed gap junction plaques at the cell surface (A–D, arrows). A subpopulation of both mutant connexins was also found within intracellular compartments (C and D, arrowheads). A Western blot (E) revealed similar expression levels of Cx43-GFP, G21R-GFP, and G138R-GFP. β-Actin was used as a loading control. Anti-Cx43 antibodies were used to label endogenous Cx43 in A. The expression of either Cx43 mutant reduced the incidence of Lucifer yellow dye coupling by more than 50% (F). The number of injections per treatment is denoted by n (asterisks = p < 0.05). No variance is indicated on the Cx43-GFP control cells, as 100% of the cells were coupled. Bar = 20 μm. WT, wild type.
density of GFP trapped in the cornified layer during keratinization. Although the thicknesses of the vital and cornified were variable, overall no difference in the architecture of the organotypic epidermis from REKs overexpressing the G21R and G138R GFP-tagged Cx43 mutants was observed when compared with organotypic epidermis derived from REKs expressing Cx43-GFP. Organotypic epidermis from REKs overexpressing Cx43-GFP and mutants Cx43 were immunoblotted for Cx43, Cx26, involucrin, keratin 14, and loricrin (Fig. 4G). There was an ~60% decrease in keratin 14, a basal cell marker of keratinocytes, when either the G21R or G138R mutants were expressed (Fig. 4, G and H), but there was no change in the levels of endogenous Cx43, Cx26, involucrin, or loricrin. However, the reduction of keratin 14 in the organotypic epidermis from REKs overexpressing Cx43 mutants did not affect the ability of these cells to reach terminal differentiation as assessed by the levels of involucrin and loricrin and their overall architecture.

Keratinocytes in Gja1Jrt/Jrt Mice Differentiate Normally—Western blot (Fig. 5A) and densitometric analysis (Fig. 5B) of skin obtained from newborn wild-type and Gja1Jrt/Jrt mice revealed that the total Cx43 expression level was slightly reduced by ~15–25%. There was no change in the expression level of Cx26 and of the differentiation markers keratin 14, involucrin, and loricrin (Fig. 5, A and B). Toluidine blue staining of 1-day-old pups revealed that there was no obvious change in epidermal barrier function in Gja1Jrt/Jrt mice when compared with controls (Fig. 5C).

Because connexin-linked skin diseases have been usually observed during aging and in the presence of mechanical stress (31), the epidermis from 3-week-old wild-type and Gja1Jrt/Jrt mice was also examined. The reduction of Cx43 and Cx26 levels in these Gja1Jrt/Jrt mice compared with their control littermates was more pronounced than in the newborn animals, as Western blotting revealed at least a 75% reduction in both Cx43 and Cx26 (Fig. 6, A and B). However, there was no change in the expression levels of keratin 14, involucrin, and loricrin (Fig. 6, A and B). Confocal images of skin from wild-type animals revealed punctate Cx43 staining in the basal and suprabasal cell layers (Fig. 6E, arrows). In mutant littermates, Cx43 was evident, particularly in the more basal layers, but punctate staining typical of gap junctions was not very apparent

FIGURE 4. REKs expressing dominant, loss-of-function Cx43 mutants differentiate into organotypic epidermis. Histological staining and confocal images revealed that REKs overexpressing either Cx43-GFP or Cx43 mutants (G21R-GFP or G138R-GFP) differentiated into organotypic epidermis exhibiting a thick cornified layer (A–F, between dashed lines) and vital cell layers (A–F, dotted line to first dashed line; arrows denote Cx43 or mutant). Western blot (G) and densitometry (H) of multiple organotypic cultures generated from REKs expressing the Cx43 mutants revealed a decrease in keratin 14, whereas endogenous Cx43, Cx26, involucrin, and loricrin remained unchanged (n = 4). Dotted lines represent the bottom of the basal layer, and the space between the dashed lines represents the cornified layer. The green staining between the dashed lines in D, E, and F represents GFP trapped within the cornified layer. Protein expression was normalized to β-actin (E). Blue, nuclei; bar, 20 μm.
In keeping with our Western blot data on the expression levels of epidermal differentiation markers, histological staining revealed no differences in the thickness of the vital or cornified layers in the thick skin of wild-type (Fig. 6C) and mutant mice (Fig. 6D).

DISCUSSION

In this study we set out to test the importance of Cx43 in epidermal differentiation and maintenance equipped with the knowledge that mice harboring a truncated variant of Cx43 died shortly after birth due to the loss of barrier function (22). Conversely, mice harboring a loss-of-function autosomal dominant mutation of Cx43 were viable with apparently normal skin (20). We thus hypothesized that the expression levels of full-length Cx43 may be of critical importance as opposed to its functional capacity in passing transjunctional molecules within the layers of the epidermis. To test this hypothesis, we first employed an organotypic model where REKs could be genetically manipulated to express Cx43 mutants or engineered to reduce their Cx43 levels while maintaining the capacity to grow into a stratified epidermis when cultured at a liquid/air interface.

Role of Cx43 in Organotypic Epidermis—REKs can be grown in two-dimensional or differentiated into a three-dimensional organotypic epidermis that has two-three vital layers with a thick cornified layer not unlike typical rat skin (8, 32). We previously showed that REKs have a connexin mRNA profile that encompasses all of the connexins identified in rodent epidermis (9). We first used an RNA-mediated interference approach that targets different locations of the Cx43 sequence...
and generated two variant cell lines that exhibited an $\sim 50$–75% reduction in Cx43 expression and an $\sim$50% reduction in GJIC. Intriguingly, Cx26 was also reduced to a similar level, suggesting some cross-talk relationship between Cx26 and Cx43. Epithelial barrier function was altered in Cx43-reduced REKs, as indicated by the reduction in transepithelial resistance. In addition, organotypic epidermis generated from Cx43 knockdown REKs failed to properly stratify, cornify, or express normal levels of involucrin and loricrin. Collectively, these indexes suggest that the expression of Cx43 is required for proper and complete organotypic epidermal differentiation. The reduction of Cx43 observed in Cx43 knockdown REKs might also contribute to their failure to properly differentiate and stratify. It has been previously reported that loss of Cx43 in keratinocytes, obtained from patients carrying a stop mutation, resulted in a reduced stratification of in vitro reconstructed tissue, although dye coupling was maintained (33). These data would support a model where keratinocyte differentiation is dysregulated when Cx43 and Cx26 expressions are reduced, resulting in premature elevation of the late keratinocyte differentiation markers involucrin and loricrin in the absence of coordinated proliferation and differentiation of the keratinocytes. Interestingly, it has been shown that mutations in loricrin result in Vohwinkel syndrome not unlike disease linked to autosomal dominant mutations in the gene encoding Cx26 (34). In addition, the increase in involucrin found in Cx43-reduced organotypic epidermis might reflect an immature and fragile cornified envelope. In fact, it has been reported that involucrin-positive, fragile cornified envelopes are immature and less hydrophobic and that their occurrence is closely related to impairment of the barrier function of the skin (35). Interestingly, involucrin is also highly expressed in the thinner skin of patients suffering from psoriasis (36, 37).

In a second stream of experimental studies designed to mechanistically assess the role of a full complement of functional Cx43 in organotypic epidermis differentiation, we established REKs where endogenous Cx43 function was inhibited by the expression of dominant mutants of Cx43. Two ODDD-linked mutants, G21R and G138R (25, 38), were overexpressed in REKs to dominantly inhibit the function of Cx43 in epidermal differentiation in vitro. Importantly, our laboratory recently demonstrated using Lucifer yellow microinjection and patch clamp electrophysiology assays that the G21R mutant was considerably more potent than the G138R mutant at inhibiting the function of wild-type Cx43. Thus, even though both mutants caused a similar reduction in dye transfer when expressed in REKs, one cannot conclude that the Cx43 channels are equally inhibited by the two Cx43 mutants if the cells were subjected to more sensitive analysis. Unlike Cx43 knockdown approaches, normal levels of endogenous Cx43 and Cx26 were maintained in the REKs overexpressing the G21R and G138R mutants, allowing for continued interactions between these connexins and their binding partners (39–41), but the coupling status of Cx43 is known to be greatly impaired (25, 38). When Cx43-based GJIC was inhibited by the co-expression of these ODDD-linked Cx43 mutants, keratin 14 expression was reduced in organotypic epidermis. However, the reduction in keratin 14 did not affect the ability of these cells to differentiate and stratify into both basal and suprabasal cells as well as to form a cornified layer in vitro. This is not particularly surprising as it has been previously reported that keratin 14-deficient keratinocytes can still undergo morphological and biochemical differentiation (42). Thus, the effect of the Cx43 mutants on keratin 14 level alone is unlikely to cause a decrease in organotypic epidermis differentiation.

Consequently, how does one reconcile the differences in these findings where Cx43 knockdown results in significantly altered organotypic differentiation while functional impairment of Cx43 had no detectable consequences on cultured epidermis? In both cases, total GJIC, as determined by dye coupling assays, was reduced by $\sim 50$–75%. Although similar reduction in Lucifer yellow dye permeability does not necessarily mean that the conductance properties of the channels and the influence on Cx43 are the same, these data suggest that diminution on GJIC alone is insufficient to explain the differences observed between the two models. In essence, the primary difference in these findings rest on the fact that in our Cx43 knockdown experiments, total Cx43 and Cx26 levels are reduced, suggesting that movement of transjunctional molecules that pass through these distinct channels is reduced and cellular properties governed by Cx43/Cx26 binding proteins are dysregulated. However, in REKs containing Cx43 mutants, endogenous Cx43 levels remained intact, and no notable change in the level of Cx26 was observed, suggesting that GJIC mediated by Cx26 remained intact as did the opportunity for the regulatory involvement of Cx43/Cx26 binding proteins. Based on our results using Cx43 knockdown and mutants organotypic epidermis, we predicted that the continued presence of Cx43 as well as Cx26, regardless of the functional status of Cx43, would establish and maintain barrier function. We tested this hypothesis in an autosomal dominant mouse model (Gja1<sup>+/−</sup>) of a Cx43-linked disease where the mutant variant of Cx43 was functionally dead and, importantly, where the level of Cx43-based coupling expected from the co-expressed wild-type counterpart was dominantly reduced (20). As hypothesized, epidermal differentiation and function were maintained in the Gja1<sup>+/−</sup> mice.

Role of Cx43 in Epidermal Differentiation in Vivo—Our Cx43 studies in organotypic epidermis argue for a pivotal role of sufficient Cx43 expression for the initial differentiation of basal cells from one layer to a complete and properly differentiated and stratified rat epidermis. Nevertheless, organotypic epidermis lacks potential regulatory interactions with the underlying dermis and the array of factors that accompany skin development and differentiation in vivo. In addition, it is not uncommon for a complex expression pattern of connexin family members in vivo to be reduced to primarily Cx43 expression in vitro. Thus, we felt it was important to assess the role of Cx43 in epidermal differentiation in mice where the functional status of Cx43 was greatly reduced. Previous studies by Maass et al. (22, 43) supported a central role for Cx43 in the skin since the expression of a C-terminal truncated variant of Cx43 caused a lethal epidermal barrier dysfunction in knock-in mice, although the truncated Cx43 protein could still form open gap junctional channels in transfected cells. Thus, the phenotypic abnormalities observed in these
mice were more likely due to defective regulation of Cx43 rather than Cx43 channel closure (43). To study the role of Cx43 in epidermal differentiation in vivo, we circumvented the limitations of using Cx43-null mice which die at birth by using a mouse model of human ODDD that harbors a dominant Gja1 (Cx43) gene missense mutation (20). Interestingly, the majority of human ODDD-linked Cx43 mutations do not cause skin disease (16), but two dinucleotide deletions in the C terminus caused frameshift and a premature truncation of Cx43 resulting in the associated skin diseases palmoplantar keratoderma and palmar hyperkeratosis (17, 18). We, thus, propose that the Gja1Jrt/H11001 mutant mouse is relevant to elucidating the role of Cx43 in epidermis differentiation and maintenance as this is a complete loss of function mutation and dominantly reduces the overall Cx43-linked GJIC to ~15% that of wild type (20). Although this Cx43 mutant caused a slight reduction of total Cx43 expression in the skin from newborn mice, barrier function and markers of epidermis differentiation were found to be similar to wild-type littermates. The distribution patterns of Cx43 and Cx26 in the epidermis during transition of prenatal to newborn rats have been reported to be constant (44), suggesting that the amounts of Cx43 and Cx26 found in the epidermis of neonatal Gja1/Jrt +/+ and control mice are likely indicative of what is found in fetal development, and a full complement of functional Cx43 is not essential to support initial epidermis differentiation. Similar to the effect of overexpressing the ODDD-linked Cx43 mutants (G21R and G138R) in vitro, these results suggest that inhibition of Cx43 channel function alone does not alter epidermal differentiation when the expression of endogenous Cx43 is maintained at similar levels without affecting the amount of Cx26.

Given the very young age of these mice and the lack of exposure to mechanical and environment stresses, we also compared the status of the epidermis of 3-week-old Gja1/Jrt +/+ mutant mice to their control littermates to assess changes that might develop with aging. Interestingly, the Cx43 mutant caused a drastic reduction of both total Cx43 and Cx26 expression in 3-week-old animals compared with control littermates, again supporting cross-talk between Cx43 and Cx26. As seen in Cx43 knockout studies. However, the expression levels of keratin 14, loricrin, and involucrin remained similar to those from control mice, and the integrity of the mutant epidermis was not altered. Taking into account our results from Cx43 knockout epidermis, these results might suggest that Cx43 and Cx26 expression levels are important for the initiation of the differentiation of basal cells during the development of the epidermis from one layer to a proper stratified differentiated epidermis but that the maintenance and the continuous differentiation of an established epidermis proceeds independently of a full complement of Cx43 and Cx26. Accordingly, although Cx43 and Cx26 are expressed in the newly formed rat epidermis, their mRNA and protein levels markedly decrease after birth, although their distribution pattern remained the same, suggesting that Cx43 and Cx26 are most important in epidermal differentiation during fetal development (44). Although the reasons for the notable reduction of total Cx43 and Cx26 expression in older Gja1/Jrt +/+ mutants compared with their control littermates are still unknown, our results indicate that reduction of Cx43-linked GJIC and of Cx43 and Cx26 levels did not appear to alter the maintenance of the previously established differentiated epidermis.

Because many connexins are known to be expressed in epidermis with overlapping expression patterns, it is possible that other members of the connexin family may also be regulated and contribute to the differentiation of the epidermis as seen for Cx26. At present, it is poorly understood how the specific down-regulation of Cx43 in REKs results in a reduction of Cx26 expression particularly since these connexin are not expected to co-oligomerize (45). Because both RNA-mediated interference sequences targeting Cx43 were carefully selected and behaved similarly, we do not believe that the down-regulation of Cx43 is due to off target effects. Furthermore, we have also observed this regulatory cross-talk in 3-week-old mutant mice. The nature of this cross-talk remains elusive, but it has been reported that mutants of Cx26 trans-dominantly inhibit Cx43 channel function (46), raising the possibility that mutant Cx43 may in fact interact with Cx26 even though their wild-type counterparts are thought to make distinct channels (45). Complex interplay among connexin family members has been suggested in other in vivo studies where Cx31 null mice have been reported to have reduced levels of Cx43 (47). Interestingly, Plum et al. (48) found that there was no change in epidermal differentiation in Cx31 null-mice, perhaps due to the up-regulation of other connexin family members, yet we know that mutations in Cx31 cause human skin disease (49), perhaps highlighting differences between rodent and human epidermis.

At present, limitations in the accessibility of quality antibodies to all epidermis connexins prevents an exhaustive analysis of possible compensatory connexins in Cx43-manipulated mice, and we cannot rule out compensatory mechanisms that extend outside the connexin family of proteins that may be contributed by the underlying dermis.

In summary, our organotypic and mouse model studies point to a mechanism whereby initial epidermis differentiation and barrier function proceed normally providing that Cx43 and Cx26 are present in sufficient abundance regardless of Cx43 being fully functional. Conversely, Cx43 knockdown together with a coordinate reduction in Cx26 results in impaired epidermal differentiation and barrier function, suggesting that the additional loss of Cx26-based transjunctional movement of small molecules or interactions with important regulatory proteins are key to the initial establishment of the epidermal architecture. Finally, the maintenance of an established epidermis is less dependent on the availability of a full complement of Cx43 and Cx26. Because ODDD patients are anticipated to have greatly reduced levels of functional Cx43, these findings may help explain why ODDD patients generally do not suffer from skin symptoms.

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