Differential Expression of Connexins During Stratification of Human Keratinocytes

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To assess whether gap junctions and connexins change during keratinocyte differentiation, we have studied epidermal equivalents obtained in organotypic cultures of keratinocytes from the outer root sheath of human hair follicles. These reconstituted tissues exhibit a number of differentiation and proliferation markers of human epidermis, including gap junctions, connexins, and K6 and Ki67 proteins. Immunostaining and northern blots showed that gap junctions of the epidermal equivalents were made of Cx26 and Cx43. Cx26 was expressed in all keratinocyte layers, throughout the development of the epidermal equivalents. In contrast, Cx43 was initially observed only in the basal layer of keratinocytes and became detectable in the stratum spinosum and granulosum only after the epidermal equivalents had thickened. The levels of Cx26 and its transcript markedly increased as a function of stratification of the epidermal equivalents, whereas those of Cx43 remained almost constant. Microinjection of Lucifer Yellow into individual keratinocytes showed that gap junctions were similarly permeable at all stages of development of the epidermal equivalents. The data show that epidermal equivalents (i) feature a pattern of connexins typical of an actively renewing human interfollicular epidermis, and (ii) provide a model that reproduces the tridimensional organization of intact epidermis and that is amenable for experimentally testing the function of junctional communication between human keratinocytes. ***Key words: coupling/differentiation/epidermis/gap junctions. J Invest Dermatol 115:278–285, 2000***

In complex organisms, several mechanisms of communication assemble cells into functionally coordinated multicellular units, which are thought to play an important role in maintaining tissue homeostasis. One mechanism of cell-to-cell communication operates via direct exchanges of ions and molecules through highly permeable membrane channels located at gap junctions (Kumar and Gilula, 1996; Werner, 1998). These channels are made of a family of nonglycosylated integral membrane proteins, referred to as connexins (Cx) (Kumar and Gilula, 1996; Werner, 1998).

The gap junctions of human keratinocytes comprise mostly Cx43, which is abundantly expressed within interfollicular epidermis (Guo *et al*, 1992; Salomon *et al*, 1993, 1994), and Cx26, which is codistributed with Cx43 in skin adnexae (Salomon *et al*, 1993, 1994). Even though the role of gap junctions in epidermal homeostasis remains to be demonstrated, it is assumed that gap-junction-mediated communication is involved in the regulation of keratinocyte growth and differentiation (Salomon *et al*, 1993, 1994; Lucke *et al*, 1999). In this perspective, the differential expression of Cx43 and Cx26 within interfollicular epidermis and epidermal adnexae may be related to the specific differentiation program that keratinocytes undergo in these two different skin regions (Salomon *et al*, 1994). Consistent with this hypothesis are the observations that events leading to *in vivo* changes in the proliferation and/or state of differentiation of epidermis, e.g., chronic treatment with topical retinoic acid (Guo *et al*, 1992; Masgrau-Peya *et al*, 1997), psoriatic lesions (Rivas *et al*, 1997; Labarthe *et al*, 1998; Lucke *et al*, 1999), or skin carcinomas (Wilgenbus *et al*, 1992), are associated with marked changes in the expression of connexins, particularly of Cx26. Upregulation of Cx26 was also observed in normal, non-hyperproliferative tissues, such as vaginal and buccal epithelia, suggesting that if a Cx26 increase accompanied conditions of keratinocyte hyperproliferation it was also required for keratinocyte differentiation (Lucke *et al*, 1999).

Direct experimental testing of why and how Cx26 is upregulated in an activated epidermis requires a model that can reproduce *in vitro* the *in situ* organization of this tissue. We have previously shown that, when cocultured with fibroblasts in a tridimensional system, keratinocytes derived from the outer root sheath (ORS) of human hair follicles develop epidermal equivalents, which display histologic and biochemical characteristics close to those of an activated human epidermis (Limat *et al*, 1996, 1999). Here, we have studied these tridimensional epidermal equivalents to assess the presence of connexins, gap junctions, and cell coupling, and to follow the changes of these parameters as a function of keratinocyte stratification.

MATERIALS AND METHODS

Cells Human ORS keratinocytes were cultured out of anagen hair follicles explanted from four healthy volunteers, in a Dulbecco's modified Eagle's medium (DMEM)/F_{12} (3:1) medium, supplemented with 10% fetal bovine serum, epidermal growth factor, hydrocortisone, choleratoxin, adenine, and triiodothyronine (all from Sigma, St. Louis, MO) and 1.5 mM Ca^{2+} (Limat *et al*, 1996, 1999). Human dermal fibroblasts were obtained from skin explants of healthy individuals and cultured in DMEM supplemented with 10% fetal bovine serum (Limat *et al*, 1996, 1999).

Epidermal equivalents ORS keratinocytes (first subculture) were plated at a density of 5×10^5 cells per cm^2 in cell culture inserts

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(Transwell 3413, Corning Costar, Cambridge, MA) that were coated with 5×10^4 human dermal fibroblasts on the undersurface of their microporous membrane (Limat *et al*, 1996). Culture medium was the same as for primary cultures. After 48 h (this time is thereafter referred to as day 0), the medium inside the inserts was aspirated to expose the cells to air. Seven days later, the cells were switched to KGM (Clonetics, San Diego, CA) containing 0.5 μg per ml hydrocortisone, 5 μg per ml insulin, 5 ng per ml epidermal growth factor, 50 μg per ml gentamycin sulfate, and 1.5 mM Ca^{2+}, a switch that we have found in repeated experiments to favor the differentiation of epidermal equivalents. Thereafter, the medium was changed three times per week, exactly as reported by Limat *et al* (1999). For this study, four independent experiments were performed, each using keratinocytes from a different donor. In each experiment, keratinocytes were studied by the entire set of approaches described below, at each of the following time points: 2, 4, 7, and 14 d after exposure of the cells to the air–liquid interface.

Histology For conventional histology, epidermal equivalents were fixed in a Dubosq/Brazil solution and further processed according to standard procedures. For transmission electron microscopy, epidermal equivalents were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.4), embedded in Araldite (Serva, Heidelberg, Germany), cut, and contrasted with uranyl acetate and lead citrate, according to standard procedures. For freeze-fracture electron microscopy, the glutaraldehyde-fixed specimens were cryoprotected in 30% glycerol and frozen in liquid nitrogen. Photographs were taken with a Philips CM 10 or EM 300 electron microscope.

Connexins For indirect immunofluorescence, epidermal equivalents were excised from the insert dish with a scalpel blade, embedded in Tissue-Tech OCT Compound (Miles, Elkhart, IN), frozen, and kept at –80°C until further processing. Sections 5 μm thick were cut with a cryomicrotome (CM 3050 Leica, Heidelberg, Germany), collected on silane-treated slides, and exposed for 3 min to –20°C acetone. All slides were first rinsed in cold (4°C) phosphate-buffered saline (PBS), blocked for 30 min with PBS supplemented with 2% bovine serum albumin, and incubated for 2 h at room temperature with one of the following antibodies: (i) affinity-purified rabbit polyclonal antibodies against residues 108–122 of liver Cx26, diluted 1:200 (Goliger and Paul, 1994); (ii) mouse monoclonal antibodies against a segment of the cytoplasmic loop of Cx26 (Zymed Laboratory, San Francisco, CA), diluted 1:100 (Masgrau-Peya *et al*, 1997); (iii) affinity-purified rabbit polyclonal antibodies against residues 314–322 of heart Cx43, diluted 1:400 (Fishman *et al*, 1990); (iv) mouse monoclonal antibodies against 19 amino acids of the carboxy terminal portion of Cx43, diluted 1:1000 (Cat. No. 03–6900, Zymed Laboratories, South San Francisco, CA) [this is the original monoclonal antibody to Cx43 commercialized by Zymed, which recognizes both phosphorylated and nonphosphorylated forms of Cx43 (Kasper *et al*, 1996; Vozzi, Dupond, and Meda, unpublished)]; (v) mouse monoclonal antibodies against involucrin (Sigma), diluted 1:50; (vi) mouse monoclonal antibodies against cytokeratin K10 (Sigma), diluted 1:800; (vii) mouse monoclonal antibodies against human profilaggrin/filaggrin (BTI, Stoughton, MA), diluted 1:100; (viii) mouse monoclonal antibodies against human cytokeratin 6 (Novocastra Laboratories, Newcastle upon Tyne, U.K.), diluted 1:20. Sections were then rinsed in PBS and incubated for 60 min at room temperature with fluorescein-conjugated antirat, antirabbit, or antimouse antibodies, whichever applicable, diluted 1:400. After further rinsing, sections were stained with a 0.03% Evans' blue solution, covered with 0.02% paraphenylenediamine in PBS-glycerol (1:2 vol:vol), and photographed with an Axioplan microscope (Zeiss, Oberkochen, Germany) fitted with filters for fluorescein detection.

As positive controls, we used cryosections of normal human skin obtained from healthy volunteers who had given informed consent. In accordance with the guidelines of our institutional committee for clinical investigation, keratome samples were obtained from either neck, under intradermal anesthesia by 1% xylocaine plus epinephrine, or breast and abdomen during reduction surgery (Salomon *et al*, 1994; Masgrau-Peya *et al*, 1997; Labarthe *et al*, 1998). In all cases, samples were rapidly frozen by immersion in 2-methylbutane (Merck, Basel, Switzerland), which was cooled in liquid nitrogen, and stored at –80°C until cryostat sectioning. In these control tissues, the distribution of Cx26, which was restricted to hair follicles and ducts of eccrine sweat glands, Cx43, involucrin, K10, fillagrin, and K6 were as previously reported in normal human samples (Tyner and Fuchs, 1986; Ebling and Eayd, 1992; Guo *et al*, 1992; Salomon *et al*, 1994; Limat *et al*, 1996; Masgrau-Peya *et al*, 1997; Labarthe *et al*, 1998).

Fragments of the very same samples of normal skin and of the epidermal equivalents prepared in these experiments were also fixed in formol and embedded in paraffin. Sections 7 μm thick were deparaffinized, rehydrated, boiled three times for 5 min in a 0.01 M citrate buffer (pH 6) within a microwawe, cooled at room temperature, rinsed in PBS, and incubated 2 h at room temperature with a rabbit serum against human Ki67 antigen, diluted 1:50 (Dako, Glostrup, Denmark). Sections were then reacted 60 min with a biotinylated goat antirabbit IgG, diluted 1:100 (Jackson Immunoresearch Laboratories, West Grove, PA), and incubated 60 min with a fluorescein-conjugated streptavidin, diluted 1:100 (Jackson Immunoresearch Laboratories). The distribution of Ki67 in normal skin was as previously reported (Ralfkiaer *et al*, 1986; Lucke *et al*, 1999).

Connexin transcripts cDNAs containing the entire coding region of Cx43 (Fishman *et al*, 1990) and Cx26 (Lee *et al*, 1992) were subcloned in plasmid Bluescript II KS and linearized with Xho I and EcoR I (for Cx43) and Hind III and EcoR I (for Cx26) restriction site enzymes (Boehringer Mannheim, Germany), as previously described (Salomon *et al*, 1994). Connexin antisense probes were synthesized by *in vitro* transcription (Meda *et al*, 1993). Skin samples and epidermal equivalents were homogenized in 2.5 ml 0.1 M Tris-HCl, pH 7.4, containing 2 M β-mercaptoethanol and 4 M guanidium thiocyanate. After addition of solid CsCl (0.4 g per ml), the homogenate was layered on a 2 ml cushion of 5.7 M CsCl and 0.1 M ethylenediamine tetraacetic acid (EDTA) (pH 7.4) and centrifuged for 20 h at 35,000 rpm and 20°C. Pelleted RNA was resuspended in 10 mM Tris-HCl, pH 8.1, supplemented with 5 mM EDTA and 0.1% sodium dodecyl sulfate (SDS), extracted twice with phenol-chloroform, precipitated in ethanol, and resuspended in water. For northern blots, total cellular RNA was denatured with 1 M glyoxal in 0.01 M phosphate buffer containing

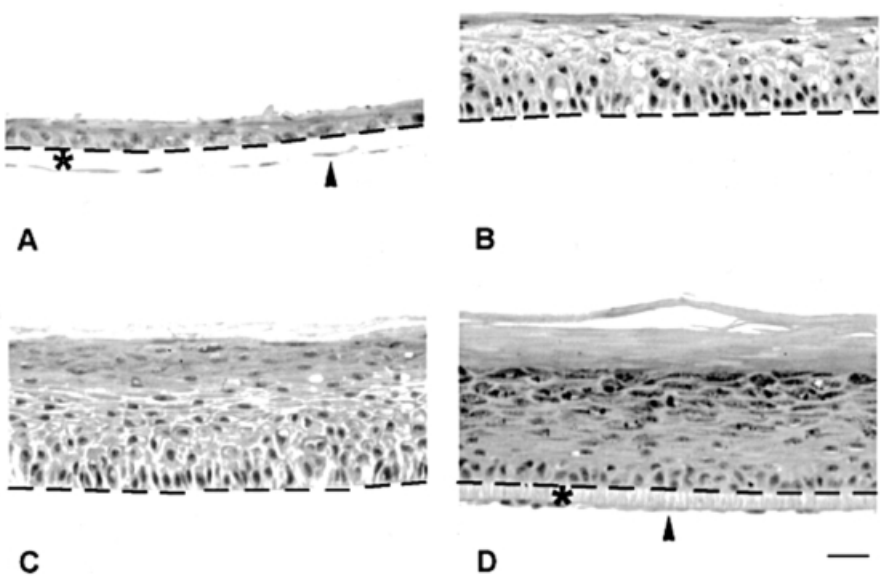

Figure 1. ORS keratinocytes reconstitute a phenotypically normal epidermis *in vitro*. Paraffin sections (hematoxylin and eosin staining) through epidermal equivalents at days 2 (*A*), 4 (*B*), 7 (*C*) and 14 (*D*) after exposure of the cells to the air–liquid interface. The dotted line indicates the upper surface of the insert membrane in contact with basal keratinocytes. In (*A*) and (*D*), the insert membrane is still present (asterisk) and the arrows point to fibroblasts attached to its undersurface. *Scale bar*: 50 μm.

50% dimethylsulfoxide, separated by electrophoresis in a 1.2% agarose gel (5 μg total cellular RNA per lane), and transferred overnight onto nylon membranes (Hybond N; Amersham International). Filters were exposed for 30 s to 302 nm light, stained with methylene blue, and prehybridized for 2 h at 65°C in a 50% formamide solution buffered with 0.05 M Pipes (pH 6.8) and supplemented with 2 mM EDTA, 0.1% SDS, 0.1 mg per ml salmon sperm DNA, 0.8 M NaCl, and 2.5 × Denhardt's solution. Filters were hybridized for 18 h at 65°C with 1.5×10^6 cpm per ml of one of the ^{32}P-labeled probes mentioned above, washed twice at 65°C in 3 × sodium citrate/chloride buffer (SSC) and 2 × Denhardt's solution, then three times at 70°C in 0.2 × SSC, 0.1% SDS, and 0.1% sodium pyrophosphate, and eventually exposed to XAR-5 film (Eastman Kodak, Rochester, NY), between intensifying screens, at –80°C for 1–6 d.

Junctional coupling Epidermal equivalents were split from the insert membrane by a 30 min exposure to 1.2 U per ml dispase II (Boehringer Mannheim) and rinsed three times with PBS. They were then placed upside down on 4 μl Tissucol Duo (Immuno, Vienna, Austria), which was freshly prepared by mixing equal volumes of fibrinogen and a 1:100 dilution of thrombin. After 5 min, the insert membrane was removed with fine tweezers and the epidermal equivalents were covered with culture medium.

Permeability of junctional channels was evaluated by impaling individual cells with a glass microelectrode (150–200 MΩ) filled with HEPES-buffered (pH 7.2) 150 mM LiCl that contained 4% Lucifer Yellow CH (Sigma) (Salomon *et al*, 1988). The microelectrode was connected to a pulse generator for passing current and recording membrane potentials. After successful cell impalement, 0.1 nA negative square pulses of 900 ms and 0.5 Hz frequency were applied for 2–15 min. At the end of the injections, the epidermal equivalents were fixed in 4% paraformaldehyde in PBS and photographed under fluorescence illumination. Injected areas (about six per culture time) were further scanned using a coulour chilled 3CCD video camera (Hamamatsu C5810) and scored for the number of cells containing Lucifer Yellow, including the injected one, by two independent investigators. Results were expressed as both mean + SEM and median values.

RESULTS

Epidermal equivalents comprised differentiated and proliferating keratinocytes When monolayers of ORS keratinocytes were exposed to the air–liquid interface (day 0), cells began to form a stratified epithelium. After 2 d, this epithelium was two to four layers thick and already comprised flattened cells at the air surface (**Fig 1*A***). Two days later, the epithelium showed a basal layer of columnar cells and eight to ten layers of polygonal cells that became flattened and parakeratotic close to the air surface (**Fig 1*B***). Between days 4 and 7, the number of cell layers almost doubled. Thus, the epithelium comprised a prominent stratum spinosum, a faint stratum granulosum, and an orthokeratotic layer of moderate thickness (**Fig 1*C***). In 14-d-old epidermal equivalents, the keratinocyte organization was comparable to that observed in human epidermis, i.e., comprised a layer of small, basal cells, several layers of progressively flattening spinous cells, two to three layers of granular keratinocytes, and several layers of orthokeratotic cells that detached at the tissue surface (**Fig 1*D***).

At this stage, several markers of keratinocyte differentiation could be immunolocalized in the epithelium. Thus, involucrin was seen in several layers of suprabasal cells, starting from the mid-stratum spinosum (**Fig 2*B***), and fillagrin decorated upper spinous and granular cells (**Fig 2*C***). Cytokeratin K10 was found expressed in almost all suprabasal cells (**Fig 2*D***). At the ultrastructural level, keratinocytes of 14-d-old epidermal equivalents contained abundant bundles of intermediate filaments and were connected by numerous desmosomes and gap junctions (**Fig 3**).

Immunolabeling of the same cultures showed that keratinocytes also expressed K6 and Ki67, two protein markers of active proliferation, throughout the development of the epidermal equivalents. Thus, antibodies to K6, which stained the epidermis of psoriatic plaques but not of control human skin (not shown), revealed abundant levels of this cytokeratin in the cytoplasm of all basal and suprabasal keratinocytes, from day 2 onwards (**Fig 4**). Similarly, antibodies to Ki67, which revealed numerous proliferating keratinocytes in the epidermis of psoriatic plaques but rare dividing cells in control human skin (not shown), showed clusters of basal keratinocytes featuring an immunostained nucleus in the basal layer of all the epidermal equivalents studied (**Fig 4**).

Cx26 and Cx43 are expressed in epidermal equivalents Immunolabeling with two different antibodies that recognized both the phosphorylated and nonphosphorylated forms of Cx43 failed to detect this protein in the first days of development of the epidermal equivalents (**Fig 5*A***). From day 4 onward, however, expression of Cx43 was clearly detectable in all layers of living keratinocytes (**Fig 5*C***, ***E***). At day 14, the expression of this protein appeared to be low in the basal layer of keratinocytes and in the lower half of the stratum spinosum, strong in the upper stratum spinosum and stratum granulosum, and null in the superficial layers of keratinized cells (**Fig 5*G***). The pattern of Cx26 distribution was markedly different, as judged using two different antibodies. Thus, this protein was already detectable in 2-d-old epidermal equivalents, in which it showed a patchy distribution in the basal layer of keratinocytes (**Fig 5*B***). Thereafter, the expression of Cx26 markedly increased in all layers during the stratification of the epidermal equivalents, except in the lower half of the stratum

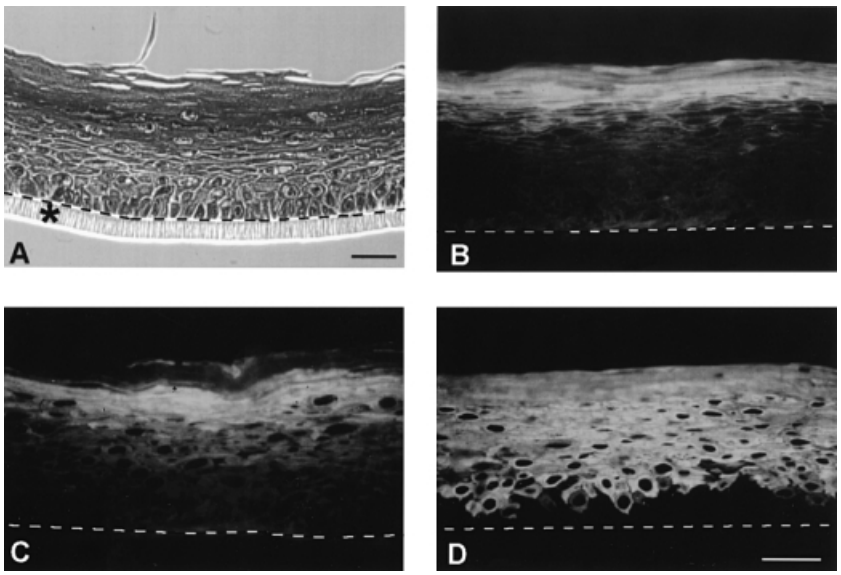

Figure 2. Epidermal equivalents display markers of differentiated human keratinocytes. (*A*) Phase-contrast view of an epidermal equivalent at day 14, lying on the insert membrane (asterisk). (*B*) Involucrin was detected from the mid-stratum spinosum up to the stratum corneum. (*C*) Filaggrin was expressed in the granular layer and in some of the uppermost spinous cell layers. (*D*) Cytokeratin K10 was distributed in most suprabasal keratinocytes. The dotted line indicates the upper surface of the insert membrane in contact with basal keratinocytes. *Scale bar*: 50 μm.

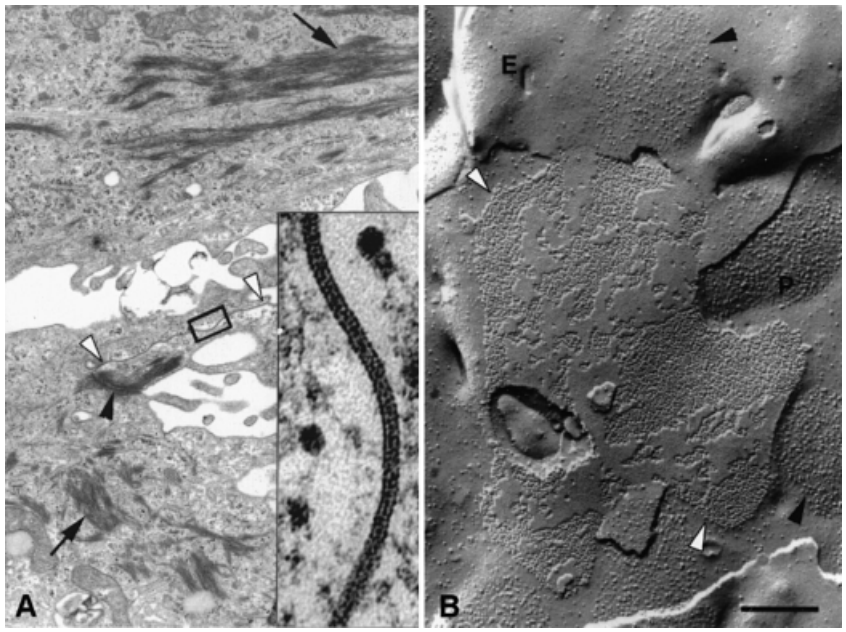

Figure 3. Gap junction plaques form in epidermal equivalents. (*A*) At day 14, suprabasal cells of epidermal equivalents showed several ultrastructural characteristics of differentiated keratinocytes, including bundles of intermediate keratin filaments (*arrows*), desmosomes (*black arrowhead*), and areas of close membrane apposition (*white arrowheads*). The higher magnification view of one such area (*rectangle*) illustrates the pentalaminar, slightly striated appearance of two adjacent cell membranes at a gap junction (*inset*). (*B*) Freeze-fracture showed that the plasma membrane of keratinocytes contained aggregates of uniformly large particles (on one fracture face, p) and pits (on the other fracture face, E), typical of gap junction plaques (*white arrowheads*). Smaller aggregates of heterogeneous particles are characteristic of desmosomes (*black arrowheads*). *Scale bar*: (*A*) 15 μm; (*inset*) 160 nm; (*B*) 270 nm.

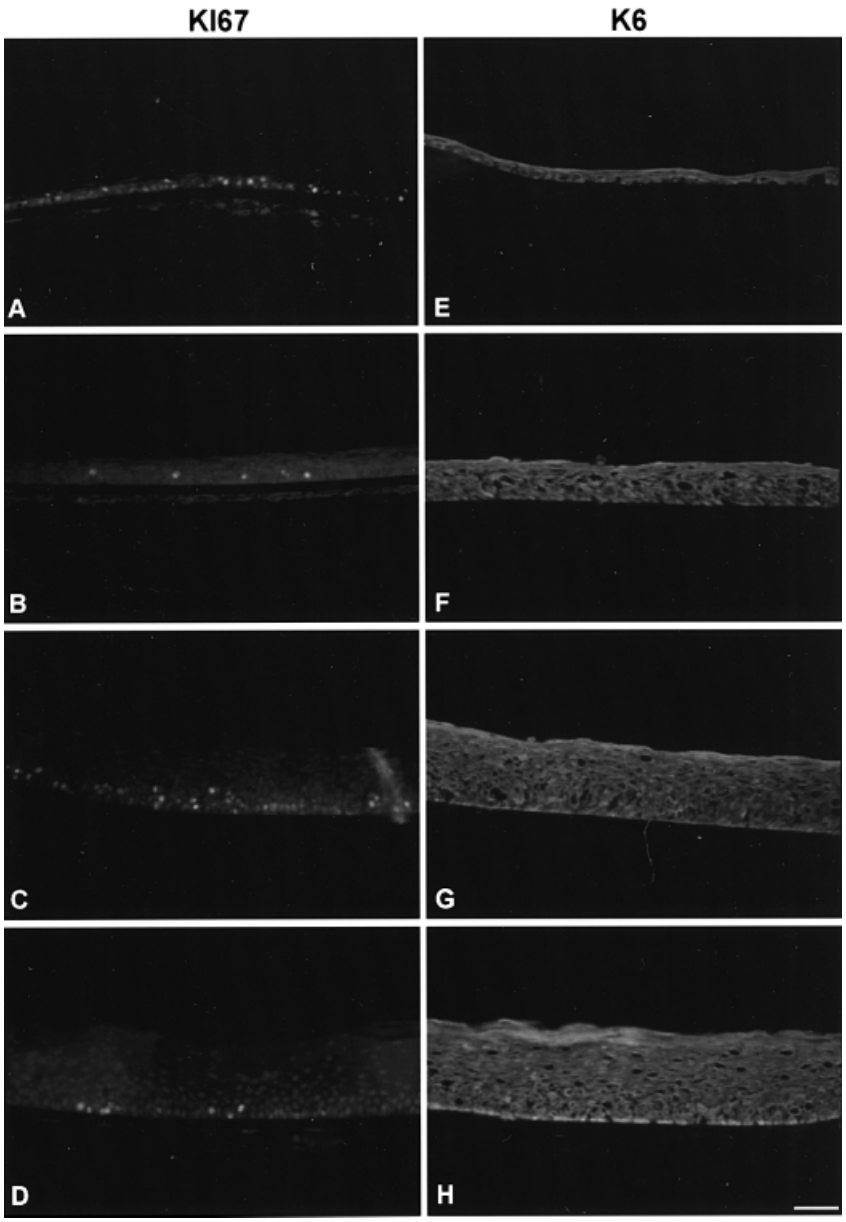

Figure 4. Keratinocytes of epidermal equivalents express proliferation markers. *Left panel*: Antibodies to protein Ki67 immunolabeled the nuclei of numerous keratinocytes, often distributed in clusters, along the basal and parabasal layers of 2-d-old (*A*), 4-d-old (*B*), 7-d-old (*C*), and 14-d-old (*D*) epidermal equivalents. *Right panel*: Antibodies to protein K6 immunolocated this cytokeratin in the cytoplasm of virtually all keratinocytes, throughout the entire thickness of the epidermal equivalents at days 2 (*E*), 4 (*F*), 7 (*G*), and 14 (*H*). *Scale bar*: 50 μm.

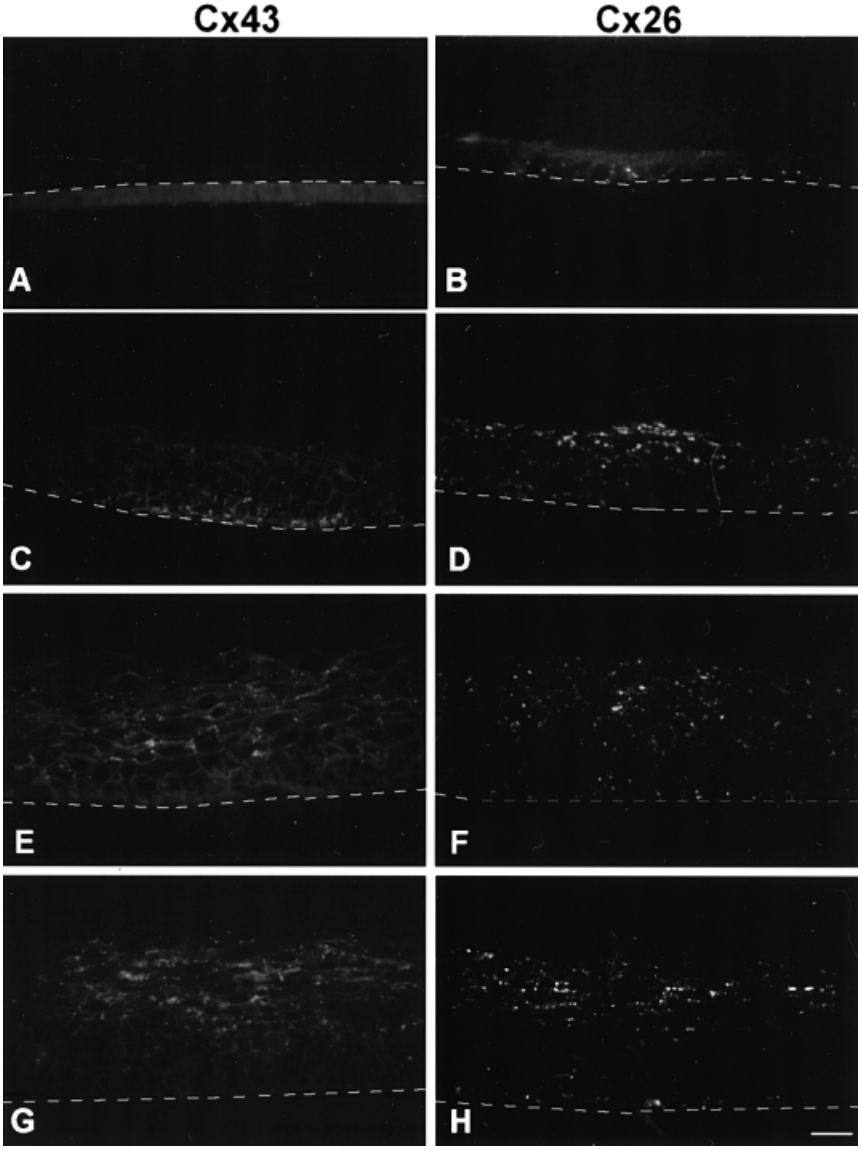

Figure 5. Cx43 and Cx26 are differentially expressed during stratification of epidermal equivalents. *Left panel*: Cx43 was not detectable at day 2 (*A*) but was seen in the basal layer of keratinocytes at day 4 (*C*) and throughout the suprabasal layers at day 7 (*E*). At day 14, the protein predominated in the upper stratum spinosum and in the stratum granulosum (*G*). *Right panel*: Cx26 had a patchy distribution in basal keratinocytes at day 2 (*B*) and was markedly increased at days 4 (*D*) and 7 (*F*). At day 14, Cx26 was detected in all the layers of the epidermal equivalents, except the lower half of the stratum spinosum (*H*). *Scale bar*: 50 μm.

spinosum (**Fig 5*D***, ***F***, ***H***). Cx26 and Cx43 were still observed in 21-d-old epidermal equivalents (not shown).

Northern blot hybridization of total RNA using specific cRNA probes revealed that, relative to the level of 18S, that of the Cx43 transcript was essentially unchanged throughout the development of the epidermal equivalents. Thus, this transcript was also detected at the early time points, when Cx43 could not be visualized by immunofluorescence labeling. In contrast, the levels of the transcript for Cx26 markedly increased with time in the same samples (**Fig 6**).

Junctional channels are permeable in epidermal equivalents Microinjection of Lucifer Yellow into individual keratinocytes of the epidermal equivalents was consistently followed by the transfer of the tracer into a number of neighboring cells, within both basal and suprabasal layers (**Fig 7**). Within each communication territory, a gradient of fluorescence was seen that decreased as a function of the distance from the injected cell. Under the injection conditions we used, the Lucifer Yellow assay failed to demonstrate significant variations in coupling at any time point in parametric as well as nonparametric tests. Thus, mean ± SEM and median number of coupled keratinocytes per territory were, respectively, for 2-, 4-, 7-, and 14-d-old epidermal equivalents: 25.6 ± 8.0 and 19.2 (n = 6); 30.0 ± 4.0 and 31.0 (n = 6); 47.9 ± 7.6 and 47 (n = 7); 37 ± 3.2 and 38.5 (n = 5).

DISCUSSION

Communication via gap junctions has been implicated in the regulation of cell growth, differentiation (Wilgenbus *et al*, 1992; Kumar and Gilula, 1994), and migration (Pepper *et al*, 1989), three processes that play a central role in the development of epidermis and in the maintenance of this tissue. Accordingly, conditions that modify epidermis homeostasis, due to changes in the proliferation and desquamation rate of keratinocytes and/or in their differentiation program, are associated with alterations of connexin expression, in both animal models (Pitts *et al*, 1988; Brissette *et al*, 1994; Goliger and Paul, 1994, 1995; Salomon *et al*, 1994; Sawey *et al*, 1996; Risek *et al*, 1998) and humans (Guo *et al*, 1992; Wilgenbus *et al*, 1992; Masgrau-Peya *et al*, 1997; Rivas *et al*, 1997; Labarthe *et al*, 1998; Richard *et al*, 1998a). It is still uncertain, however, whether these alterations are causal to the epidermal changes and, if so, what the mechanism is whereby altered connexin expression leads to abnormal epidermal homeostasis. Investigation on these central questions implies the availability of a model that can faithfully reproduce *in vitro* the tridimensional organization and differentiation program of native epidermis. As an approach to the identification of such a model, we have used epidermal equivalents generated with keratinocytes of human follicular ORS, a cell population that contributes to epidermal regeneration *in vivo* (Limat *et al*, 1996, 1999).

In this model, we have found that keratinocytes reconstitute a tissue phenotypically like native human interfollicular epidermis (Ebling *et al*, 1992; Limat *et al*, 1996, 1999). Thus, in epidermal equivalents, the morphology of keratinocytes changed from the basal to the superficial layers leading to the formation of a surface horny layer of orthokeratotic cells, as observed *in situ*. Also, the distribution of cytokeratin K10, involucrin, filaggrin, hemidesmosomes, desmosomes, keratohyaline granules, and keratinosomes was similar to that of human epidermis (Ebling *et al*, 1992; Limat *et al*, 1996, 1999). The finding that epidermal equivalents were formed by keratinocytes expressing cytokeratin K6 and comprised sizable numbers of basal and parabasal cells positive for the nuclear antigen Ki67, however, clearly indicates that these reconstructed tissues feature characteristics of a hyperproliferative rather than resting epidermis (Ralfkiaer *et al*, 1986; Tyner *et al*, 1986; Lucke *et al*, 1999), throughout the entire stratification process. Keratinocytes of epidermal equivalents also expressed the two connexin isoforms, Cx43 and Cx26, that predominate in human epidermis (Guo *et al*, 1992; Salomon *et al*, 1993, 1994) and, at least under conditions of advanced stratification, assembled these proteins in typical gap junction plaques, much like those observed *in situ* (Salomon *et al*, 1993). Gap junctions of epidermal equivalents comprised functional cell-to-cell channels, as indicated by the intercellular diffusion of Lucifer Yellow, and the topographical relationships of the keratinocytes that were coupled by these channels were very like those observed within intact human epidermis (Salomon *et al*, 1988). Thus, in both epidermal equivalents and human interfollicular epidermis, keratinocytes exchanged Lucifer Yellow across basal and suprabasal layers, usually within a rather large territory surrounding the injected cell, irrespective of the site of microinjection. The coexpression of Cx43 and Cx26 within epidermal equivalents, which reproduce the histologic organization of an interfollicular epidermis, is intriguing, as the keratinocytes of this tissue do not express Cx26 in normal humans (Salomon *et al*, 1993, 1994). This finding might be accounted for by the origin of the keratinocytes we used to produce the epidermal equivalents. Indeed, cells of the ORS of human hair follicles are rich in both Cx26 and Cx43 (Salomon *et al*, 1993, 1994). The finding of Cx43 and Cx26 also in epidermal equivalents that had been generated using keratinocytes derived from the interfollicular epidermis, however (not shown), indicates that the origin of keratinocytes is not the only factor determining the pattern of connexins that these cells express *in vitro*.

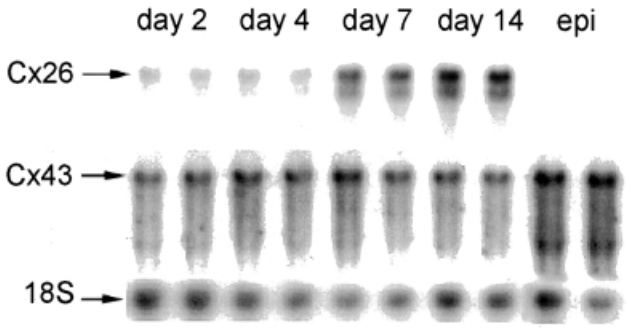

Figure 6. Differential changes of Cx43 and Cx26 transcripts during stratification of epidermal equivalents. *Upper panel*: A transcript for Cx26 was observed in the epidermal equivalents at days 2, 4, 7, and 14, but not in normal human epidermis (epi). With time, the level of this transcript increased markedly, relative to the 18S signal (*lower panel*). *Middle panel*: A transcript for Cx43 was seen in the epidermal equivalents at all time points studied, as well as in normal human epidermis. The levels of this transcript remained constant thoughout the culture time, relative to those of 18S RNA. *Lower panel*: A probe hybridizing to ribosomal 18S RNA was used to control for an equivalent loading of all lanes. Each sample (5 μg mRNA) was run in duplicate for each time point.

As stressed above, epidermal equivalents display features (K6 and Ki67 expression etc.) characterizing proliferating epidermis and thus mimic the situation that keratinocytes face during wound healing, in psoriasis, and in carcinomas (Grinnell, 1992; Wilgenbus *et al*, 1992; Rivas *et al*, 1997; Labarthe *et al*, 1998; Lucke *et al*, 1999), as well as after pharmacologic treatments of skin leading to increased turnover and altered differentiation (Guo *et al*, 1992; Masgrau-Peya *et al*, 1997). Under these conditions, a remarkable *de novo* expression of Cx26 is consistently observed, which is restricted to phenotypically altered regions of human interfollicular epidermis (Wilgenbus *et al*, 1992; Masgrau-Peya *et al*, 1997; Rivas *et al*, 1997; Labarthe *et al*, 1998; Lucke *et al*, 1999). The selective upregulation of Cx26 that we observed in epidermal equivalents, and that can be accounted for by a specific increase in the transcription rate of this connexin, is reminiscent of the situation observed *in vivo* under pathologic conditions. The coexpression of Cx43 and Cx26 in

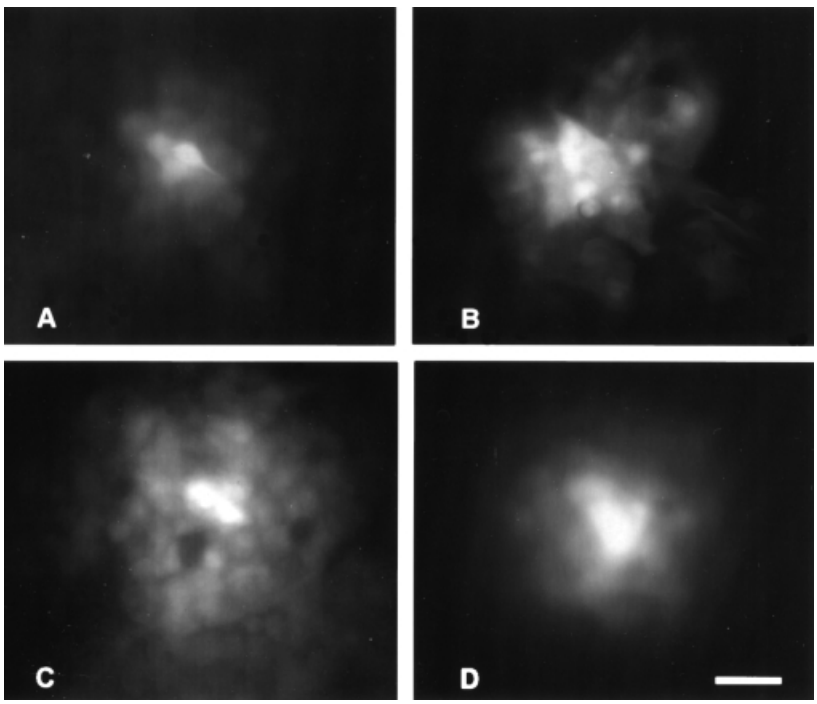

Figure 7. Junctional channels are functional within epidermal equivalents. Microinjection of Lucifer Yellow in one cell resulted in a diffusion of the tracer into numerous neighboring keratinocytes of epidermal equivalents at days 2 (*A*), 4 (*B*), 7 (*C*), and 14 (*D*). This coupling did not change markedly with culture time. *Scale bar*: 25 μm.

keratinocytes of epidermal equivalents, and the selective upregulation of the latter connexin with stratification, in the absence of major changes in functional cell coupling, are also findings that have been made in animal models. Thus, in both rat and mouse skin, Cx43 and Cx26 are coexpressed in the undifferentiated epidermis and are differentially regulated during the differentiation of the tissue, without obvious alterations in the intercellular transfer of Lucifer Yellow and neurobiotin (Risek *et al*, 1992; Goliger and Paul, 1994; Choudhry *et al*, 1997). Furthermore, the expression of Cx26 is selectively increased during the reepithelialization that accompanies wound healing also in rodents (Goliger and Paul, 1995).

Hence, a remarkable increase in Cx26, which is far from being matched by a comparable change of the multiple other connexins expressed in skin, appears to be required for the proper differentiation, migration, and/or proliferation of keratinocytes that become activated under a variety of *in vivo* and *in vitro* conditions and in several animal species. By providing a reproducible system, which is amenable to the experimental modification of various keratinocyte genes, including those coding for connexins, the model of epidermal equivalents will be most useful for investigating the reason(s) for this requirement in humans and for elucidating the underlying molecular mechanism(s). In this context, the expression of Cx26 at the very beginning of the stratification of the epidermal equivalents, at a time when Cx43 was not yet detectable, and its persistence throughout the growth of the tissue, favor a role of the former connexin in both early and later stages of the differentiation program of keratinocytes, rather than in their proliferation. This tentative conclusion, under conditions of a *de novo*, *in vitro* formation of a multilayered epithelium, has also been reached from observations on native skin samples (Lucke *et al*, 1999).

An implication of this conclusion is that alterations in Cx26 expression may be associated with abnormal skin phenotypes. Recent support for this possibility has been provided by the findings that mutations of Cx26 are associated with human skin diseases (Richard *et al*, 1998b; Maestrini *et al*, 1999; Heathcote *et al*, 2000). Two observations do not readily fit in this scheme, however. First, patients featuring the 30delG mutation of Cx26, which is associated with the DFNB1 form of nonsyndromic deafness and results in the functional deletion of the protein similar to an experimental knockout condition, do not feature an obvious cutaneous phenotype (Kelsell *et al*, 1997; Zelante *et al*, 1997). Second, no obvious change in dye transfer was observed in this and other studies during the stratification of keratinocytes, in spite of major changes in the differentiation and connexins of these cells. Presumably, keratinocytes can variably use channels made of different types of connexins at least for some basic, essential functions. Indeed, in spite of subtle biophysical, permeability, and regulatory characteristics that are specific features of channels made by distinct connexin species (Goldberg *et al*, 1999), all connexins form channels with similar generic properties, such as permeability to multiple current-carrying ions, a variety of second messengers, metabolites, nutrients, and other low molecular weight species. Thus, full understanding of the role of a given connexin in the biology of keratinocytes requires a full elucidation of its hierarchic interactions with the multiple other connexins that are expressed, probably at minimal protein levels, in human epidermis (Richard *et al*, 1998a). The model we describe here provides a unique system in which the function of these multiple proteins is amenable to direct experimental testing, using human cells that feature control or mutated patterns of one or more connexins.

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REFERENCES

Brissette J, Kumar N, Gilula N, Hall J, Dotto G: Switch in gap junction protein expression is associated with selective changes in junctional permeability during keratinocyte differentiation. *Proc Natl Acad Sci USA* 91:6453–6457, 1994

Choudhry R, Pitts JD, Hodgins MB: Changing patterns of gap junctional intercellular communication and connexin distribution in mouse epidermis and hair follicles during embryonic development. *Dev Dyn* 210:417–430, 1997

Ebling FJG, Eayd RA, Leigh IM: Anatomy and organisation of human skin. In: Champion RH, Burton JL, Ebling FJG, (eds). *Textbook of Dermatology*, 5th edn. Oxford: Blackwell Scientific Publications, 1992, pp. 49–123

Fishman GI, Spray DC, Leinwand LA: Molecular characterization and functional expression of the human cardiac gap junction channel. *J Cell Biol* 111:589–598, 1990

Goldberg GS, Lampe PD, Nicholson BJ: Selective transfer of endogenous metabolites rough gap junctions composed of different connexins. *Nat Cell Biol* 1:457–459, 1999

Goliger JA, Paul DL: Expression of gap junction proteins Cx26, Cx31.1, Cx37, and Cx43 in developing and mature rat epidermis. *Dev Dyn* 200:1–13, 1994

Goliger JA, Paul DL: Wounding alters epidermal connexin expression and gap junction-mediated intercellular communication. *Mol Biol Cell* 6:1491–1501, 1995

Grinnell F: Wound repair, keratinocyte activation and integrin modulation. *J Cell Sci* 101:1–5, 1992

Guo H, Acevedo P, Parsa FD, Bertram JS: Gap-junctional protein connexin 43 is expressed in dermis and epidermis of human skin: differential modulation by retinoids. *J Invest Dermatol* 99:460–467, 1992

Heathcote K, Syrris P, Carter ND, Patton MAA: Connexin 26 mutation causes a syndrome of sensorineural hearing loss and palmoplantar hyperkeratosis. *J Med Genet* 37:50–51, 2000

Kasper M, Traub O, Reimann T, Bjermer L, Grossmann H, Muller M, Wenzel KW: Upregulation of gap junction protein connexin43 in alveolar epithelial cells of rats with radiation-induced pulmonary fibrosis. *Histochem Cell Biol* 106:419–424, 1996

Kelsell DP, Dunlop J, Stevens HP, *et al*: Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* 387:80–33, 1997

Kumar NM, Gilula NB: The gap junction communication channel. *Cell* 84:381–388, 1996

Labarthe MP, Bosco D, Saurat JH, Meda P, Salomon D: Upregulation of connexin 26 between keratinocytes of psoriatic lesions. *J Invest Dermatol* 111:72–76, 1998

Lee SW, Tomasetto C, Paul D, Keyomarsi K, Sager R: Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *J Cell Biol* 118:1213–1221, 1992

Limat A, Mauri D, Hunziker T: Successful treatment of chronic leg ulcers with epidermal equivalents generated from cultured autologous outer root sheath cells. *J Invest Dermatol* 107:128–135, 1996

Limat A, Salomon D, Carraux P, Saurat JH, Hunziker T: Human melanocytes grown in epidermal equivalents transfer their melanin to follicular outer root sheath keratinocytes. *Arch Dermatol Res* 291:325–332, 1999

Lucke T, Choudhry R, Thom R, Selmer IS, Burden AD, Hodgins MB: Upregulation of connexin 26 is a feature of keratinocyte differentiation in hyperproliferative epidermis, vaginal epithelium, and buccal epithelium. *J Invest Dermatol* 112:354–361, 1999

Maestrini E, Korge BP, Ocana-Sierra J, *et al*: Missense mutation in connexin26, D66H, causes mutilating keratoderma with sensorineural deafness (Vohwinkel's syndrome) in three unrelated families. *Hum Mol Genet* 8:1237–1243, 1999

Masgrau-Peya E, Salomon D, Saurat JH, Meda P: *In vivo* modulation of connexins 43 and 26 of human epidermis by topical retinoic acid treatment. *J Histochem Cytochem* 45:1207–1215, 1997

Meda P, Pepper MS, Traub O, *et al*: Differential expression of gap junction connexins in endocrine and exocrine glands. *Endocrinology* 133:2371–2378, 1993

Pepper MS, Spray DC, Chanson M, Montesano R, Orci L, Meda P: Junctional communication is induced in migrating capillary endothelial cells. *J Cell Biol* 109:3027–3038, 1989

Pitts JD, Finbow ME, Kam E: Junctional communication and cellular differentiation. *Br J Cancer Suppl* 9:52–57, 1988

Ralfkiaer E, Wantzin GL, Stein H, Mason DY: Photosensitive dermatitis with actinic reticuloid syndrome: an immunohistological study of the cutaneous infiltrate. *Br J Dermatol* 114:47–56, 1986

Richard G, Smith LE, Bailey RA, *et al*: Mutations in the human connexin gene GJB3 cause erythrokeratodermia variabilis. *Nat Genet* 20:366–369, 1998a

Richard G, White TW, Smith LE, Bailey RA, Compton JG, Paul DL, Bale SJ: Functional defects of Cx26 resulting from a heterozygous missense mutation in a family with dominant deaf-mutism and palmoplantar keratoderma. *Hum Genet* 103:393–399, 1998b

Risek B, Klier FG, Gilula NB: Multiple gap junction genes are utilized during rat skin and hair development. *Development* 116:639–651, 1992

Risek B, Pozzi A, Gilula NB: Modulation of gap junction expression during transient hyperplasia of rat epidermis. *J Cell Sci* 111:1395–1404, 1998

Rivas MV, Jarvis ED, Morisaki S, Carbonaro H, Gottlieb AB, Krueger JG: Identification of aberrantly regulated genes in diseased skin using the cDNA differential display technique. *J Invest Dermatol* 108:188–194, 1997

Salomon D, Saurat JH, Meda P: Cell-to-cell communication within intact human skin. *J Clin Invest* 82:248–254, 1988

Salomon D, Masgrau E, Vischer S, Chanson M, Saurat JH, Spray D, Meda P: Gap-junction proteins and communication in human epidermis. *Prog Cell Res* 3:225–231, 1993

Salomon D, Masgrau E, Vischer S, *et al*: Topography of mammalian connexins in human skin. *J Invest Dermatol* 103:240–247, 1994

Sawey MJ, Goldschmidt MH, Risek B, Gilula NB, Lo CW: Perturbation in connexin 43 and connexin 26 gap-junction expression in mouse skin hyperplasia and neoplasia. *Mol Carcinog* 17:49–61, 1996

Tyner AL, Fuchs EE: Evidence for posttranscriptional regulation of the keratins expressed during hyperproliferation and malignant transformation in human epidermis. *J Cell Biol* 103:1945–1955, 1986

Werner R (ed.): *Gap Junctions*. Amsterdam: IOS Press, 1998

Wilgenbus KK, Kirkpatrick CJ, Knuechel R, Willecke K, Traub O: Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. *Int J Cancer* 51:522–529, 1992

Zelante L, Gasparini P, Estivill X, *et al*: Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. *Hum Mol Genet* 6:1605–1609, 1997