trans-dominant inhibition of connexin-43 by mutant connexin-26: implications for dominant connexin disorders affecting epidermal differentiation

Fatima Rouan[1], Thomas W. White[2], Nkecha Brown[1], Aileen M. Taylor[3], Thomas W. Lucke[4], David L. Paul[2], Colin S. Munro[4], Jouni Uitto[1], Malcolm B. Hodgins[5] and Gabriela Richard[1,*]

[1]Department of Dermatology and Cutaneous Biology, and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA, USA
[2]Department of Neurobiology, Harvard Medical School, Boston, MA, USA
[3]Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne, UK
[4]Department of Dermatology, Southern General Hospital, Glasgow, UK
[5]Department of Dermatology, University of Glasgow, UK
*Author for correspondence (e-mail: Gabriele.Richard@mail.tju.edu)

Accepted 28 February 2001
Journal of Cell Science 114, 2105-2113 (2001) © The Company of Biologists Ltd

SUMMARY

Dominant mutations of *GJB2*-encoding connexin-26 (Cx26) have pleiotropic effects, causing either hearing impairment (HI) alone or in association with palmoplantar keratoderma (PPK/HI). We examined a British family with the latter phenotype and identified a new dominant *GJB2* mutation predicted to eliminate the amino acid residue E42 (ΔE42) in Cx26. To dissect the pathomechanisms that result in diverse phenotypes of dominant *GJB2* mutations, we studied the effect of three Cx26 mutants (ΔE42, D66H and R75W) identified in individuals with PPK/HI, and another (W44C) present in individuals with non-syndromic HI on gap junctional intercellular communication. We expressed mutant Cx26 alone and together with the epidermal connexins Cx26, Cx37 and Cx43 in paired *Xenopus* oocytes, and measured the intercellular coupling by dual voltage clamping. Homotypic expression of each connexin as well as co-expression of wild-type (wt) Cx26/wtCx43 and wtCx26/wtCx37 yielded variable, yet robust, levels of channel activity. However, all four Cx26 mutants were functionally impaired and failed to induce intercellular coupling. When co-expressed with wtCx26, all four mutants suppressed the wtCx26 channel activity consistent with a dominant inhibitory effect. However, only those Cx26 mutants associated with a skin phenotype also significantly ($P<0.05$) inhibited intercellular conductance of co-expressed wtCx43, indicating a direct interaction of mutant Cx26 units with wtCx43. These results demonstrate, for the first time, a trans-dominant negative effect of Cx26 mutants in vitro. Furthermore, they support a novel concept suggesting that the principal mechanism for manifestation of dominant *GJB2* mutations in the skin is their dominant interference with the function of wtCx43. This assumption is further corroborated by our finding that Cx26 and Cx43 focally colocalize at gap junctional plaques in affected skin tissue of two carriers of ΔE42.

Key words: Connexins, Gap junctions, Deafness, Skin disorder, Epidermal differentiation

INTRODUCTION

In solid tissues of metazoans, most cells form gap junctions: clusters of gated intercellular channels that directly connect the cytoplasm of neighboring cells and thereby allow the passage of small ions, metabolites and other signaling molecules. In the plasma membrane, connexon hemichannels are assembled by hexameric array of gap junction proteins, which dock in the intercellular space with connexons of the adjacent cell to form functional channels. In the vertebrates connexons are formed from connexin proteins (Cx), a family of more than 20 members each encoded by a different gene. Cx share a common structure of four transmembrane segments, which extend into two extracellular and three cytoplasmic domains (Bruzzone et al., 1996). Most cell types express more than one Cx species; thus connexons may either stem from a single species (homomeric) or different Cx (heteromeric). Depending on the compatibility of interacting connexons, this diversity is amplified at the level of intercellular channels, which can be formed by similar (homotypic channels) or different homomeric connexons (heterotypic channels), or two heteromeric Cx (heteromeric channels) (White and Bruzzone, 1996).

Gap junctional intercellular communication (GJIC) fulfills a multitude of different functions, tailored to meet the specific needs of organs, tissues or groups of cells in which Cx are expressed. In the auditory system, intercellular channels formed predominantly by Cx26 but also Cx30 and Cx31 (Kelley et al., 1999; Lautermann et al., 1998; Xia et al., 2000) seem crucial for maintaining a high extracellular electrical potential in the cochlea by facilitating the local circulation of K^+ ions (Forge et al., 1999). Autosomal recessive and autosomal dominant mutations in the genes encoding each one of these Cx, *GJB2* (Cx26), *GJB3* (Cx31) and *GJB6* (Cx30),

cause hearing impairment (HI) and demonstrate the crucial role of GJIC in the auditory process (Denoyelle et al., 1998; Estivill et al., 1998; Grifa et al., 1999; Kelley et al., 1998; Kelsell et al., 1997; Liu et al., 2000; Morle et al., 2000; Xia et al., 1998). To date, over 36 different *GJB2* mutations have been identified, making them the leading cause of autosomal recessive non-syndromic HI (DFNB1, OMIM 220290) and of 10-40% of sporadic cases of congenital deafness (Cohn and Kelley, 1999; Green et al., 1999; Rabionet et al., 2000; Wilcox et al., 2000). The majority of these mutations are nonsense and frameshift mutations that are likely to result in loss of Cx26 expression or obliteration of its function. In contrast to these recessive mutations invariably resulting in HI, dominant non-conservative missense mutations of *GJB2*, *GJB3* and *GJB6* manifest with a spectrum of clinical phenotypes affecting hearing and the skin. Specifically, without any evidence for phenotypic overlap, autosomal dominant mutations in *GJB3* may cause either HI or the rare skin disorder erythrokeratodermia variabilis (OMIM 133200; Richard et al., 1998a; Xia et al., 1998). Similarly, mutations in *GJB6* underlie HI in some families and hidrotic ectodermal dysplasia (Clouston syndrome, OMIM 129500) in others (Grifa et al., 1999; Lamartine et al., 2000). Finally, *GJB2* mutations may manifest with HI alone (Denoyelle et al., 1998; Morle et al., 2000), or in association with thickening of the skin of palms and soles, diagnosed as palmoplantar keratoderma (PPK). The mutations R75W and G59A have been identified in families with co-segregation of congenital deafness and diffuse fissuring PPK, although one individual carrying R75W had no obvious cutaneous phenotype and unknown hearing status (Heathcote et al., 2000; Richard et al., 1998b). In contrast, HI was only mild to moderate in individuals from four unrelated families of diverse origins carrying mutation D66H (with exception of individuals compound heterozygous for other Cx mutations), while the skin in most individuals was severely affected with a mutilating type of PPK characterized by a honeycomb-like surface and development of circular constriction bands of the digits (Vohwinkel syndrome; OMIM 124500; Maestrini et al., 1999).

These observations indicate a striking similarity in the role of Cx for development and function of each epithelium in the skin and the inner ear, yet the mechanisms that determine the phenotypic outcome of dominant Cx mutations remain obscure. Recent functional in vitro studies in the paired *Xenopus* oocyte system have demonstrated a dominant inhibitory effect of the Cx26 mutant R75W on the function of co-expressed wtCx26, resulting in suppression of Cx26-mediated intercellular coupling between paired oocytes (Richard et al., 1998b). Such a dominant negative mechanism in vivo could be sufficient to account for the severe HI observed in carriers of this mutation. Nevertheless, the involvement of the skin remains unexplained, because even the complete loss of Cx26 function in individuals with recessive *GJB2* mutations has never been associated with any skin disorder.

We now report a novel *GJB2* mutation (ΔE42) causing autosomal dominant HI that is associated with PPK, and we have examined its consequences on the expression pattern of Cx26 and other epidermal Cx. Based on the hypothesis that dominant *GJB2* mutations disturb the intercellular communication in human skin by interfering with the function of other epidermally expressed Cx, such as Cx43 and Cx37, we assessed the effect of four Cx26 mutants with distinct and differing phenotypes on cell-cell coupling mediated by co-expressed wtCx26, wtCx43 and wtCx37 in the paired *Xenopus* oocyte system.

MATERIALS AND METHODS

Patients and biological material

We ascertained a three-generation British family with PPK associated with deafness (PPK/HI) (Fig. 1). DNA was collected from all family members using either buccal swabs or venous blood samples. Punch biopsy samples were obtained from lesional and/or normal-appearing skin from individuals II-1, II-2 and III-2. The studies were performed with informed consent of all family members.

Mutation analysis

We amplified a 963 bp fragment from genomic DNA encompassing the entire coding sequence of human connexin-26 (*GJB2*; GenBank Accession Number, M86849) (Lee et al., 1992), purified the DNA templates and subjected them to automated PCR cycle-sequencing on the ABI 377 sequencer system (PE Applied Biosystems) as previously described (Richard et al., 1998b).

Paired *Xenopus* oocyte expression and electrophysiology

We used the paired *X. laevis* oocytes expression system as previously described to test the gap junctional activity of wild-type (wt) and mutant (mt) Cx by double voltage clamp (Richard et al., 1998b). The coding regions of wtCx26, wtCx37 and wtCx43, as well as of Cx26 mutants ΔE42, W44C, D66H and R75W, were amplified by PCR from genomic DNA with primers containing *Bam*HI linkers:

Cx26 (sense) 5′-TGTTGTGGATCCATGGATTGGGGCACGC-TGCAGACG-3′

(antisense) 5′-TGTTGTGGATCCTTAAACTGGCTTTTTTGAC-TTCCCAG-3′

Cx37 (sense) 5′-TGTTGTGGATCCATGGGTGACTGGGG-CTTCCTGGAG-3′

(antisense) 5′-TGTTGTGGATCCCTATACATACTGCTTCTT-AGAAGCA-3′

Cx43 (sense) 5′-TGTTGTGGATCCATGGGTGACTGGAGCG-CCTTAGGC-3′

(antisense) 5′-TGTTGTGGATCCCTAGATCTCCAGGTCATCA-GGCCGA-3′.

The PCR products were gel purified using the QIAquik gel extraction kit (QIAGEN, Valencia, CA), digested with *Bam*HI and subcloned into the pSP64T or pCS2+ expression vectors (Krieg and Melton, 1984; Rupp et al., 1994; Turner and Weintraub, 1994). All constructs were sequenced in both directions, then linearized with *Eco*RI, and capped mRNAs were transcribed in vitro (mMessage mMachine, Ambion, Austin, TX). *X. laevis* eggs were collected and processed for the paired oocyte expression assay as previously described (Swenson et al., 1989). Prior to electrophysiological studies, defolliculated *X. laevis* oocytes were microinjected with the Cx38 antisense oligonucleotide 5′-CTGACTGCTCGTT-CTGTCCACACAG-3′ to prohibit contribution of endogenous Cx38 intercellular channels to the measured conductance (Bruzzone et al., 1993). For each of the wild-type Cx, oocytes were injected with an empirically determined amount of RNA to yield an electrical conductance level within a reliably measurable range (0.5 to 50 μS). Although injection of 2 ng RNA per oocyte was sufficient for wtCx37 and wtCx43, a higher RNA concentration (8 ng/oocyte) was required for Cx26

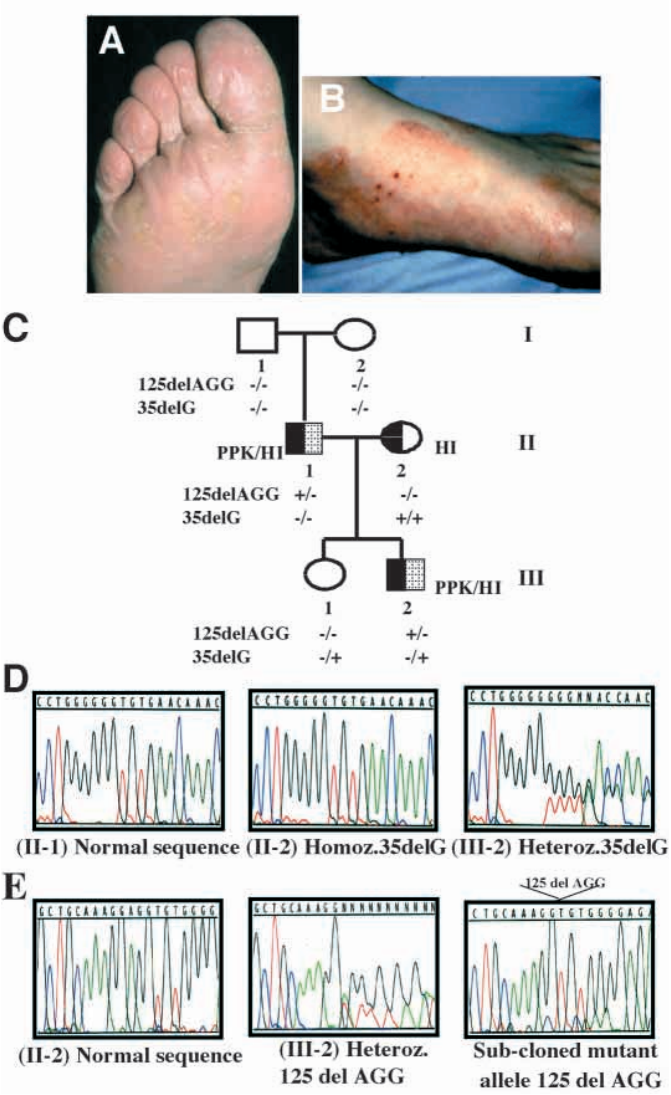

Fig. 1. Palmoplantar keratoderma associated with deafness in a family carrying mutation ΔE42 in *GJB2*. (A) Mild, diffuse hyperkeratosis of the sole with accentuated skin markings and marked callus formation over the pressure points (II-1 in C). (B) Transgradient, erythematous, hyperkeratotic plaques on the left foot (III-2 in C). (C) Pedigree of the three-generation family. Black symbols indicate HI; spotted symbols indicate PPK; white symbols represent unaffected individuals. Individual genotypes for both mutations, 35delG and 124delAGG, are shown below symbols. (D) Sequence chromatograms for 35delG in II-1, II-2 and III-2 (left to right). (E) Sequence chromatograms for 125delAGG in II-2, III-2 and the sub-cloned mutant allele (left to right).

to produce coupling levels that could be accurately measured. For co-expression studies of wtCx26 with different mutant forms of Cx26, RNAs were mixed in a 1:1 ratio, injecting 8 ng of each RNA. For co-expression of wt or mtCx26 with wtCx37 or wtCx43, equal amounts of RNA with respect to the single injections were used for each of the two Cx species, e.g. 8 ng Cx26 plus 2 ng wtCx43. To determine if a lower amount of mtCx26 still could interfere with the function of co-expressed wtCx43, we decreased the amount of injected mRNA for ΔE42-Cx26 and W44C-Cx26 from 8 ng to 2 ng (changing the RNA ratio mtCx26:wtCx43 from 5:1 to 1:1). 24 hours after RNA injections, *X. laevis* oocytes were manually paired, and the intercellular conductance as a measure of gap junctional communication was determined by dual voltage clamp. The electrophysiological data were analyzed using the Clampex 7.0 program, while the statistical analyses (average, s.e.m. and the Student's *t*-test) were made using Origin 4.0.

Immunohistochemistry

Sections of snap-frozen skin biopsies of lesional skin (5 μm; plantar, II-1; lateral foot, III-2) and non-lesional skin (arm, II-1; arm, II-2), as well as normal control skin, were subjected to immunohistochemical analyses as previously described (Lucke et al., 1999). The primary polyclonal anti-Cx43 antibody (rabbit) (provided by Dr E. Rivedal, Institute for Cancer Research, The Norwegian Radium Institute, Oslo, Norway) was detected with Texas Red-conjugated anti-rabbit immunoglobin, while two different monoclonal anti-Cx26 antibodies (mouse; 13-8100 and 33-5800 from Zymed Laboratories, San Francisco, CA), one of which (33-5800) does not crossreact with Cx30, were detected with fluorescein-conjugated anti-mouse immunoglobin. The analyses were performed on a BioRad MRC 600 confocal microscope.

Protein extraction and western blot analysis

X. laevis oocyte proteins were extracted using a lysis buffer (10 mM Tris, 5 mM EDTA, pH 8) including protease inhibitors chymostatin, leupeptin and pepstatin (BioRad Laboratories, CA). The cell extracts were then homogenized and centrifuged at 2,000 ***g*** for 5 minutes at 4°C. The resulting supernatants were centrifuged at 10,000 ***g*** for 30 minutes at 4°C, and the pellets were resuspended in Laemmli sample buffer (Laemmli, 1970). The extracted proteins were resolved on 12% SDS polyacrylamid gels, transferred to a nitrocellulose membrane and processed for western blot analysis. The membranes were incubated either with a rabbit anti-Cx26 polyclonal antibody or a mouse anti-Cx43 monoclonal antibody. A mouse anti-α-tubulin monoclonal antibody was used as a control. The blots were washed with 1× phosphate-buffered saline (PBS) containing 0.1% Tween 20. After incubation with the corresponding secondary IgG antibodies for 1 hour at room temperature, the blots were washed again with PBS and developed with an enhanced chemiluminescence system (ECL, Amersham Pharmacia, Inc).

RESULTS

Clinical features of a family with palmoplantar keratoderma and hearing impairment

We studied a non-consanguineous British family, in which two individuals had profound hearing loss confirmed by audiometry and thickening of the skin of palms and soles (Fig. 1). The father (II-1), 41 years of age, had developed a mild, diffuse PPK with accentuated skin markings and fine scaling during adolescence. In addition, a recent dermatological examination revealed multiple callus-like hyperkeratotic plaques around the heads of the metacarpals (Fig. 1A) and scaling plaques below the corners of the mouth. Hair, nails and mucous membranes were unremarkable. His 15-year-old son (III-2) had presented at 8 years of age with shallow pits and horizontal ridges of the nails, some of which were slightly thickened, as well as with transgradient, diffuse and sharply demarcated keratoderma of the soles. The hyperkeratosis progressively worsened causing deep fissures, and also involved the palms (Fig. 1B). The mother (II-2), 38 years of age, had also prelingual hearing loss, which was originally thought to result from a fetal rubella infection. Her skin was

Table 1. Effect of dominant Cx26 mutants on intercellular coupling of paired *X. laevis* oocytes expressing different connexins

$RNA_{injected}$	Gj (μS)*	±s.e.m.	Number of pairs	*P*‡	*P*§
Deionized H_2O	1.7×10^{-3}	1.7×10^{-3}	16	n.a.	n.a.
wtCx26/wtCx26	7.06	1.65	63	n.a.	n.a.
W44C-Cx26/W44C-Cx26	0.05	0.01	25	4.05×10^{-5}	n.a.
DelE42-Cx26/DelE42-Cx26	0.03	0.01	25	3.86×10^{-5}	n.a.
D66H-Cx26/D66H-Cx26	0.03	0.03	7	3.89×10^{-5}	n.a.
R75W-Cx26/R75W-Cx26	0.08	0.02	25	3.58×10^{-5}	n.a.
W44C-Cx26/WtCx26	0.59	0.19	13	1.50×10^{-4}	n.a.
delE42-Cx26/WtCx26	0.02	0.01	19	3.81×10^{-5}	n.a.
D66H-Cx26/WtCx26	1.43	0.80	15	n.s.	n.a.
R75W-Cx26/WtCx26	0.07	0.05	14	4.22×10^{-5}	n.a.
wtCx43/wtCx43	9.52	1.71	39	n.a.	n.a.
wtCx26/wtCx43	19.48	3.38	34	n.a.	5.77×10^{-3}
W44C-Cx26/wtCx43	10.18	2.09	31	0.012	n.s.
DelE42-Cx26/wtCx43	1.05	0.26	25	2.54×10^{-6}	8.78×10^{-6}
D66H-Cx26/wtCx43	3.75	2.77	9	5.34×10^{-4}	0.05
R75W-Cx26/wtCx43	2.86	0.73	10	1.41×10^{-5}	4.22×10^{-4}
wtCx37/wtCx37	39.35	3.31	27	n.a.	n.a.
wtCx26/wtCx37	27.42	4.74	22	n.a.	2.30×10^{-3}
W44C-Cx26/wtCx37	5.64	1.54	11	9.61×10^{-5}	4.20×10^{-11}
DelE42-Cx26/wtCx37	2.32	0.66	23	1.51×10^{-5}	6.08×10^{-12}
D66H-Cx26/wtCx37	30.15	3.75	7	n.s.	0.04
R75W-Cx26/wtCx37	50.67	8.91	8	0.02	n.s.

*Intercellular gap junctional conductance.
‡Relative to wtCx26.
§Relative to wtCx26/wtCx43 or wtCx26/Cx37.
n.s., not statistically significant ($P>0.05$).
n.a., not applicable.

normal. Finally, both parents of the affected father as well as his 18-year-old daughter (III-1) were unaffected and had normal skin and hearing. A biopsy of lesional skin of II-1 revealed histopathological features consistent with non-epidermolytic PPK, including marked orthokeratotic hyperkeratosis with small patches of parakeratosis as well as acanthosis of the epidermis.

Mutation analysis reveals two sequence variants of *GJB2*

Direct DNA sequence analysis of *GJB2* from amplicons of genomic DNA disclosed that two distinct sequence variants segregated in our family. The deaf individual II-2 (mother of III-2) was homozygous for the deletion of a guanine nucleotide at position 35 of the coding sequence, a mutation designated 35delG (Fig. 1C,D), which results in frameshift and premature stop codon immediately downstream of the mutation site. Consistent with this finding, both children (III-1 and III-2) were carriers of 35delG. In addition, II-1 and III-2, both affected with HI/PPK, carried a heterozygous 3 bp deletion mutation starting at nucleotide 125 of the coding sequence of *GJB2* (125delAGG) (Fig. 1C,E). This in-frame deletion eliminates a glutamic acid residue at position 42 of Cx26 (mutation designated as ΔE42), which is predicted to reside at the boundary between the first transmembrane segment and the first extracellular domain. Subcloning of *GJB2* of II-2 confirmed the presence of 35delG on the maternal and 125delAGG on the paternal allele (Fig. 1). The latter deletion was not detectable by direct DNA sequencing in either parent of II-1, suggesting that 125delAGG has arisen de novo. Parentage of the father of II-1 (I-1) was confirmed by the analysis of eight informative microsatellite markers from different chromosomes (99.99998% probability of paternity assuming a prior probability of 50%). Furthermore, 125delAGG was excluded from 102 alleles of unrelated Caucasian individuals without a history of HI and skin disorders, thus eliminating the possibility that it represents a frequent sequence polymorphism. In contrast, four control alleles carried 35delG, reflecting a frequency of 0.04 of this mutant allele in our control cohort.

Functional studies in *X. laevis* oocytes

Cx26 mutants dominantly inhibit the function of gap junctional channels formed by wtCx26

ΔE42-Cx26 is the fourth reported heterozygous mutation of *GJB2* that results in HI as well as PPK (Heathcote et al., 2000; Maestrini et al., 1999; Richard et al., 1998b). To elucidate the role of this and other dominantly inherited Cx26 mutants in the pathophysiology of PPK, we expressed wtCx26 and/or three different mutants with a skin phenotype (ΔE42-Cx26, R75W-Cx26, D66H-Cx26) in paired *X. laevis* oocytes and assessed the Cx-mediated intercellular communication by dual voltage clamp. The results were compared with those obtained for mutant W44C-Cx26, which causes dominant hearing loss without affecting the skin (Denoyelle et al., 1998).

WtCx26 induced robust levels of electrical conductance between opposing cells, indicating the formation of functionally active intercellular channels. In contrast, all four Cx26 mutants were non-functional by themselves (Table 1, Fig. 2A). When co-expressed with wtCx26 at a mRNA ratio of 1:1, ΔE42-Cx26 and R75W-Cx26 almost completely blocked the channel activity of wtCx26 (99.6% and 99.0%, respectively). D66H-Cx26 and W44C-Cx26 were slightly less efficient, suppressing only 80% to 92% of wtCx26 function

(Table 1, Fig. 2B). These results suggest a dominant inhibitory mechanism of these *GJB2* mutations.

Cx26 mutants with a skin phenotype exert a selective, trans-dominant inhibitory effect on Cx43 channels

To test whether mutant Cx26 can interfere with the function of other epidermal Cx in our experimental in vitro system, we co-expressed wtCx43 or wtCx37 with each of the 4 mutants, W44C-Cx26, ΔE42-Cx26, R75W-Cx26 and D66H-Cx26 (Table 1, Fig. 2C,D). The electrical conductance between paired oocytes induced by homotypic wtCx43 or wtCx37 channels was consistently higher than that obtained with homotypic wtCx26 channels as has been previously reported (White et al., 1995).

Co-injection of identical amounts of wtCx26 and wtCx43 mRNAs as used for single injections resulted in synthesis of both Cx (western blot analysis, data not shown) and a significant increase in intercellular communication (P=0.0058), suggestive of an additive effect. When the mutant W44C-Cx26 (HI phenotype) was co-expressed with wtCx43, the registered conductance was similar to that of wtCx43 expression alone, probably because the mutant Cx26 channels were functionally inactive and did not contribute to the intercellular communication. In contrast, the presence of the PPK/HI mutants ΔE42-Cx26, R75W-Cx26 and D66H-Cx26 had a deleterious effect on the function of co-expressed wtCx43. Each mutant profoundly reduced the intercellular conductance, between 95% (ΔE42-Cx26) and 81% (D66H-Cx26), when compared with wtCx26/wtCx43 expression. The intercellular communication mediated by homotypic wtCx43 channels was significantly inhibited (P<0.05), with the residual activity ranging between 39% (D66H-Cx26) and 11% (ΔE42-Cx26). This significant trans-dominant effect could be elicited even when the amount of microinjected ΔE42-Cx26 mRNA was reduced by 75% (2 ng instead of 8 ng ΔE42-Cx26 mRNA co-injected with 2 ng wtCx43 mRNA).

In the presence of wtCx26, the functional activity of co-expressed wtCx37 channels was significantly reduced (P=0.0023), yet it was still 2- to 3-fold higher than the conductance of homotypic wtCx26 or wtCx43 channels. Based on the established conductance values for wtCx26/wtCx37, each of the four tested Cx26 mutants yielded a different effect independent of their phenotype. Co-expressed with wtCx37, Cx26-D66H was neutral and did not change the level of intercellular communication. While expression of R75W-Cx26/wtCx37 resulted in a significant increase of conductance (P=0.02), the activity of ΔE42-Cx26/wtCx37 and W44C-Cx26/wtCx37 was significantly inhibited (P<0.0001).

In summary, our results demonstrated that the Cx26 mutants ΔE42, D66H and R75W, all of which manifest with HI and PPK, not only impaired function of wtCx26 gap junctional channels but also trans-dominantly inhibited the function of gap junctional channels formed by wtCx43. In contrast, the Cx26 mutant W44C without a skin phenotype did not alter wtCx43-mediated intercellular communication.

Increased and aberrant expression of Cx26 and focal colocalization of Cx26 and Cx43 in lesional skin

If the trans-dominant interaction of mutant Cx26 with wtCx43 observed in vitro is relevant for the pathogenesis of PPK, then these two Cx should be co-expressed in vivo in the affected skin. To address this question, we performed immunohistochemical analysis of plantar skin from a control individual and both affected individuals with PPK/HI, including II-1 (who is heterozygous for ΔE42) and III-2 (who is compound heterozygous for this mutation and 35delG). In control plantar skin, Cx43 was predominantly expressed in interfollicular

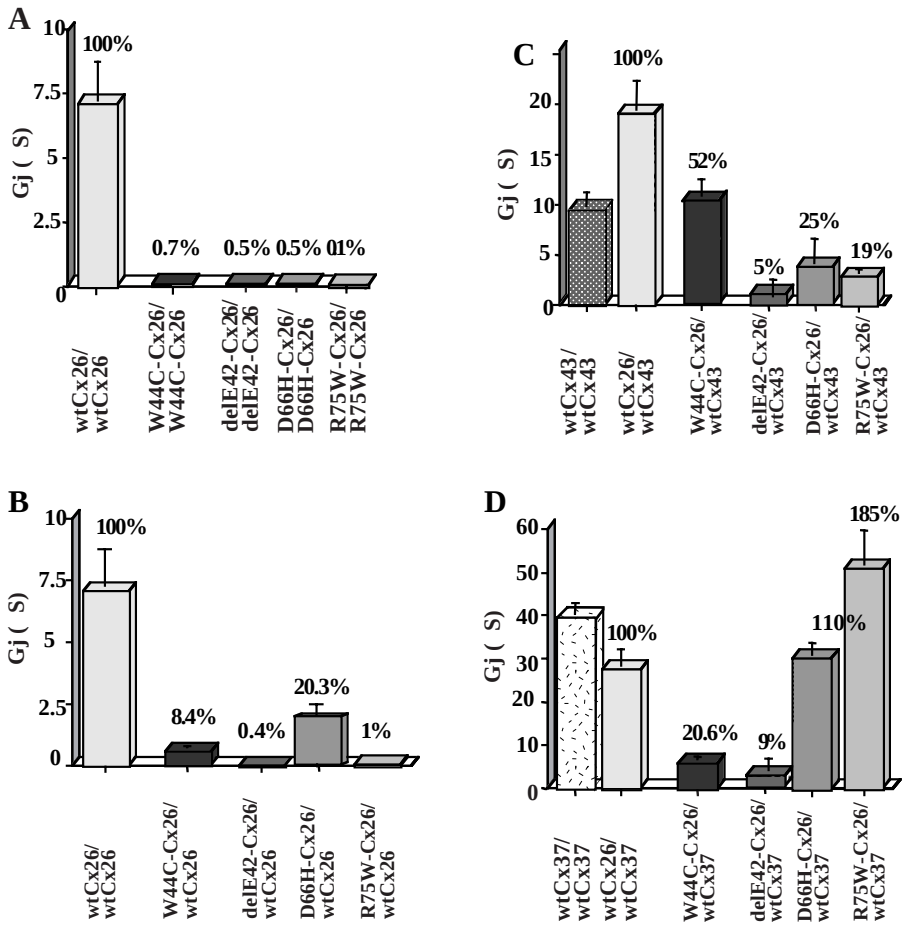

Fig. 2. Functional analysis of wild-type and/or mutant Cx26 co-expressed with wtCx43 or wtCx37 in *Xenopus* oocytes. The bars represent the junctional conductance established between two cells (mean±s.e.m.). The relative functional activity of gap junctional channels is given in percent. (A) Cx26 mutants are functionally inactive. (B) Cx26 mutants dominantly inhibit channel function of co-expressed wtCx26. (C) Cx26 mutants causing PPK/HI suppress channel activity of co-expressed wtCx43 (trans-dominant effect). (D) Cx26 mutants exert variable effects on the function of wtCx37 channels.

epidermis throughout the spinous and granular layers, but also showed a weak staining in the basal epidermis. Cx26 staining was restricted to basal keratinocytes (Fig. 3A). In contrast, double immunofluorescence analysis of Cx26 and Cx43 in hyperkeratotic sole skin of II-1 demonstrated a distinct but overlapping expression pattern of both connexins. Although Cx43 expression appeared normal, Cx26 showed not only punctate plasma membrane staining of basal keratinocytes, but was also strongly expressed in the upper spinous and granular cell layers of the epidermis. At multiple foci, both Cx colocalized in cells of the basal and suprabasal layers of the epidermis (Fig. 3B), in particular around the ducts of eccrine sweat glands. Essentially similar results were obtained for individual III-2 (Fig. 3C**)**. Specifically, Cx26 was expressed in a patchy distribution in basal as well as suprabasal keratinocytes, but most abundantly by the epithelium of the eccrine sweat ducts with a distinct, punctate staining of the cell surface indistinguishable from that of specimens from II-1. Preferentially in keratinocytes surrounding the eccrine sweat ducts, both Cx43 and Cx26 were found to colocalize. For comparison, a section of a common wart, stained and imaged along with patient material, is shown in Fig. 3F to illustrate the strong expression and overlapping distribution of both Cx26 and Cx43 in the suprabasal layers of hyperproliferative epidermis. In comparison with affected sole skin, the normal-appearing arm skin of II-1 showed less intense Cx26 staining, which was, nevertheless, more pronounced than in normal controls (Fig. 3E; see also Lucke et al., 1999). Staining for Cx26 was completely absent in the arm skin of II-2 (including the eccrine sweat ducts which normally stain strongly for Cx26), who was homozygous for the 35delG mutation in *GJB2*, thus confirming the in vivo 'knock-out' of Cx26 in this individual (Fig. 3D).

DISCUSSION

Skin has an elaborate gap junction network comprising at least eight different Cx species in rodent epidermis, which are expressed in overlapping, spatial and temporal patterns (Butterweck et al., 1994; Choudhry et al., 1997; Goliger and Paul, 1994; Kam et al., 1986; Risek et al., 1992; Salomon et al., 1994). In human skin, only the expression patterns of Cx43 and Cx26 have been studied in detail to date. Cx43 is expressed in interfollicular epidermis, particularly in the spinous and granular cell layers, in sebaceous glands and in hair follicles. Cx26 is present in hair follicles, and in eccrine sweat glands and ducts, but is much less abundant in normal epidermis, being seen mainly in the skin of palms and soles (Lucke et al., 1999; Salomon et al., 1994). Other Cx known to be expressed in human epidermis include Cx30, Cx30.3, Cx31, Cx31.1 and Cx37 (Lamartine et al., 2000; Macari et al., 2000; Richard et al., 1998a). Their exact distribution, level of expression and interactions as well as the specific contributions of each Cx to the intercellular communication of keratinocytes, however, remain enigmatic. As pathogenic mutations in *GJB3* (Cx31), *GJB4* (Cx30.3) and *GJB6* (Cx30) may also manifest with hyperkeratosis as part of their clinical phenotype (Lamartine et al., 2000; Macari et al., 2000; Richard, 2000), it appears that faulty Cx interfere with specific, yet unidentified functions of intercellular signaling in the epidermis leading to a disturbed differentiation of keratinocytes and thus epidermal hyperkeratosis.

In the present report, we have identified a new dominant *GJB2* mutation (ΔE42) that causes deafness and PPK. In addition, the results of our molecular analysis of *GJB2* in the tested family allowed us to draw several interesting conclusions. First, deafness of the probands mother (II-2) was heritable and caused by homozygosity for the single, most common *GJB2* mutation in Caucasians, 35delG. Second, the absence of a skin phenotype

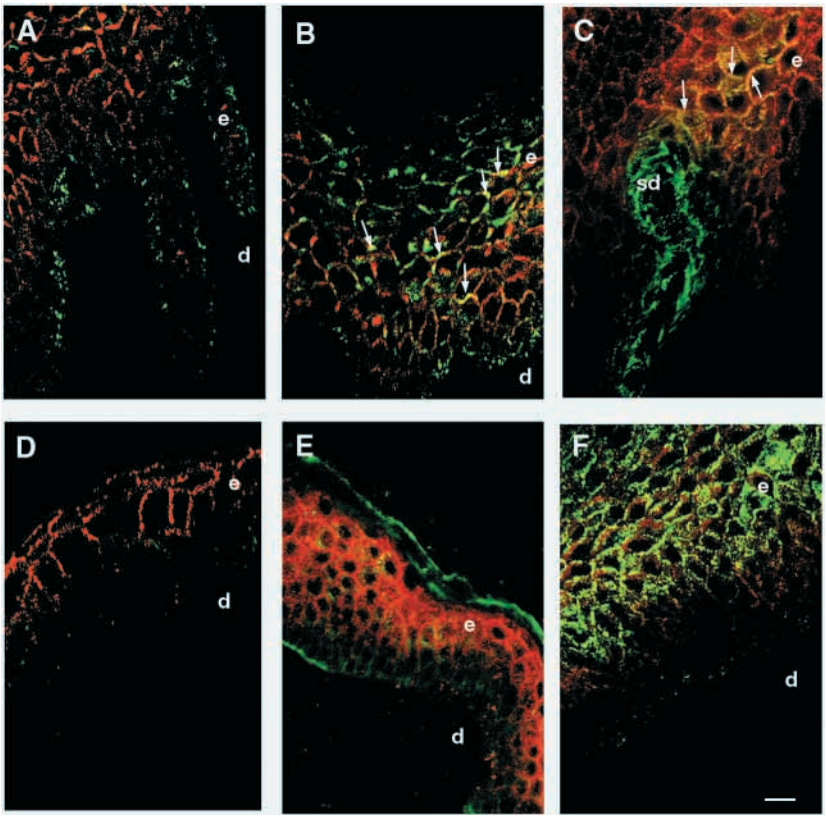

Fig. 3. Double immunofluorescence of normal and lesional skin with Cx26 (green) and Cx43 (red) antibodies. (A) Normal plantar skin. (B) Lesional plantar skin of II-1 (carrier of ΔE42). Areas of focal co-localization of Cx26 and Cx43 are shown in yellow. (C) Lesional skin of the lateral foot of III-2 (compound heterozygous for ΔE42/35delG). Normal expression of Cx26 by the epithelium of an eccrine sweat duct with punctate staining of the plasma membranes. Note the focal co-localization of Cx26 and Cx43 in suprabasal keratinocytes surrounding the sweat duct. (D) Normal-appearing skin (arm) of II-2 (homozygous 35delG mutation carrier) with absent immunostaining of Cx26. (E) Normal-appearing skin (arm) of II-1 (carrier of ΔE42) demonstrating basal expression of Cx26 with extension into the lower suprabasal cells which results occasionally in co-localization of Cx26 and Cx43. (F) Viral wart. Epidermal hyperproliferation is associated with strong expression of Cx26 throughout the upper epidermis and extensive co-localization of Cx26 and Cx43.e, epidermis; d, dermis; sd, sweat duct. Bar, 40 μm (A,B,C,F); 24 μm (D); 120 μm (E).

of II-2, despite the complete loss of Cx26 confirmed by immunofluorescence, suggests that either Cx26 is not crucial for epidermal function or other epidermally expressed Cx (e.g. Cx30, Cx30.3, Cx31, Cx31.1 or Cx37) can compensate for the loss of Cx26. A likely candidate could be Cx30, which shares about 77% identity in amino acid sequence with Cx26 (Kelley et al., 1999) and closely mirrors its expression pattern in the inner ear and skin (Kelley et al., 1999; M. B. H., unpublished). Third, both parents transmitted a mutant *GJB2* allele to their son (III-2), who was compound-heterozygous for the recessive null allele (35delG, maternal allele), and a dominant *GJB2* mutation (125delAGG, paternal allele). Therefore, he is the first known individual to be in essence hemizygous for a dominant *GJB2* mutation. The absence of Cx26 has no obvious consequences for development and differentiation of the epidermis, as illustrated by II-2, and many other individuals who are homozygous or compound heterozygous for recessive *GJB2* mutations. Therefore, the skin manifestation associated with ΔE42 in II-2 can be explained only by dominant interference of the mutant Cx26 protein with the function of other epidermal proteins, or alternatively, a gain-of-function effect. However, our results favor the former explanation. Finally, the nail dystrophy noted in III-2 has not been described in other individuals with PPK/HI and could potentially indicate an overlap with Clouston syndrome, which is characterized by PPK, primary nail dystrophy, and hypotrichosis, caused by dominant mutations in *GJB6* (Cx30) (Lamartine et al., 2000). However, nail dystrophy (especially secondary to trauma) is relatively common in the dermatological practice. Consequently, PPK and nail findings in III-2 could also be associated by chance.

ΔE42 is the fourth published autosomal dominant *GJB2* mutation with pleiotropic effects that impair the normal function of the inner ear and the skin. All these mutations result in amino acid substitutions clustering in the highly conserved extracellular domain (G59A and D66H) or at its predicted interface with the transmembrane domains (ΔE42 and R75W), suggesting that the character and location of these mutations could at least partially determine their phenotypic expression. In vitro site-directed mutagenesis and swapping of domains between different Cx has clearly demonstrated that the first extracellular loop is crucial for connexon-connexon interactions and voltage gating of Cx channels (Bruzzone et al., 1996). Moreover, most predicted boundaries of the transmembrane domains of Cx are marked by conserved, highly charged residues, including R, K, H (basic) or E (acidic) (Yeager and Nicholson, 1996). Distinct mutations changing the charge of these regions have been associated with disease, such as R75W and ΔE42 in *GJB2* (Richard et al., 1998b), R42P in *GJB3* (Richard et al., 2000; Wilgoss et al., 1999) or R75W, R75Q and E186K in *GJB1* (Bergoffen et al., 1993; Silander et al., 1997; Tan et al., 1996), probably by altering the conformation of the mutant Cx and/or the gating polarity of aberrant Cx channels (Wilgoss et al., 1999).

Our functional in vitro studies confirmed that all tested dominant *GJB2* mutations have a deleterious effect on Cx function, similar to recessive mutations, and render the mutant protein incapable of forming functional Cx channels (Martin et al., 1999; White, 2000). Studies of the fate of mutant Cx in mammalian cell culture systems have determined that this functional impairment may due to disruption of any of the steps in Cx synthesis, intracellular trafficking, and assembly to connexons and gap junctional channels, or due to interference with their functional properties, and thus may be different for each mutation (Martin et al., 1999; VanSlyke et al., 2000). To assess the direct consequences of ΔE42 on the Cx system in the skin, we compared the normal expression profile of Cx26 and Cx43 to that of skin samples of all three family members with different *GJB2* genotypes. The complete loss of Cx26 staining seen in a skin specimen of II-2 (35delG/35delG) implies that there is also no immunoreactive Cx26 protein produced from the 35delG allele of III-2. Consequently, the observed Cx26 immunostaining in III-2 reflects solely the expression of the mutant protein ΔE42-Cx26, thus, providing a unique insight into the effects of this Cx26 mutant with important implications. The presence, distribution and plasma membrane staining pattern of the mutant protein indicates that ΔE42 does not interfere with expression, synthesis and degradation of Cx26 in keratinocytes and the sweat duct epithelium. Moreover, this mutation obviously does not disturb the intracellular transport and targeting of Cx26-ΔE42 to the cell surface, as has been reported for other Cx mutations studied in vitro, e.g. G12S (Cx32) and W77R (Cx26) (Deschenes et al., 1997; Martin et al., 1999). These results strongly suggest that ΔE42 inhibits the normal Cx channel function by perturbing connexon-connexon interactions, altering the Cx compatibility code or modifying the passage of selected signals. The increased level of Cx26 staining observed, particularly in the sole skin of II-1, might reflect a compensatory overexpression that is due to compromised function of Cx26. Alternatively, this might be an indirect result of changes in the normal proliferation and differentiation program of keratinocytes, which are known to be associated with an induction of Cx26 expression, as shown for example, in response to trauma (tape stripping, wound healing) or in psoriasis (Goliger and Paul, 1995; Labarthe et al., 1998; Lucke et al., 1999).

Electrophysiological studies of the function of ΔE42 and other dominant Cx26 mutants expressed in *X. laevis* oocytes confirmed their dominant-negative effect, resulting in a significant inhibition of the function of co-expressed wtCx26. Although one can only speculate that the degree of interference of mtCx26 with the function of wtCx26 determines the severity of HI, this simple loss-of-function mechanism cannot explain the skin involvement. Therefore, our co-expression data provide the first experimental evidence to suggest that (1) pathogenic *GJB2* mutations may interfere with the function of other Cx species, and that (2) this trans-dominant effect could be the basis for the cutaneous manifestation of some dominant *GJB2* mutations. Selectively, all dominant Cx26 mutants causing PPK (ΔE42, D66H and R75W) sufficiently blocked the function of wtCx43, which we have demonstrated to colocalize with Cx26 in keratinocytes of affected skin tissue, while the mutant Cx26 without a skin manifestation (W44C) did not. Such a consistent result could not be elicited for co-expression with wtCx37. Although we observed loss- (ΔE42, W44C) as well as gain- (R75W) of-function perturbing the normal level of Cx37 signaling, there was no apparent correlation with the phenotypic expression of these mutants that suggested either random or biologically irrelevant effects. It remains to be elucidated if Cx37 and Cx43 colocalize under normal or pathological conditions in human skin when a specific antibody directed against human Cx37 becomes available.

Owing to differences in Cx biology between *X. laevis* oocytes and mammalian cells, our data obtained in this simple model system may not always reflect the much more complex situation in vivo as, for example, has been demonstrated for mutation M34T. The presence of this Cx26 mutant dominantly interfered with the function of wtCx26 in *X. laevis* oocytes, whereas M34T has now been recognized as a recessive allele associated with mild HI in human (Houseman et al., 2001; White, 2000). Nevertheless, the selective interference of dominant Cx26 mutations exhibiting a skin phenotype with Cx43 is remarkable, and similar interactions with other epidermal Cx (e.g. those associated with other skin disorders) are conceivable. Most of these Cx (e.g. Cx31, Cx31.1) could not be tested with Cx26 in paired *X. laevis* oocytes, owing to their inability to form functional homo- and/or heterotypic channels (Richard et al., 2000; White and Bruzzone, 1996), indicating a need for further investigations in other expression systems.

The significant inhibition of Cx43-mediated cell coupling by co-expressed mutant Cx26 points to their direct interaction, which is corroborated by the focal colocalization of both Cx in gap junctional plaques of keratinocytes in vivo, preferentially at sites of constitutive (e.g. palms and soles) or pathological (PPK) increased expression of Cx26. A similar overlapping expression was found in hyperproliferative skin disorders (Labarthe et al., 1998; Lucke et al., 1999), or in the vaginal and buccal epithelia (Lucke et al., 1999). The interactions and actual stoichiometric ratio of mutant and wild-type proteins are unknown. The mere presence of mtCx26 units in a mixed gap junction plaque might physically hinder Cx43 channel formation or prevent the plaque from exceeding a certain limiting size (Bukauskas et al., 2000). Alternatively, under normal conditions wtCx26 and wtCx43 might co-exist in gap junctional plaques without forming heterotypic channels, while distinct Cx26 mutants could gain the ability to interact with wtCx43, thereby locking up wtCx43 into nonfunctional channels. This mechanism could explain why different *GJB2* mutations that affect closely situated residues in the first extracellular loop of Cx26 have distinct phenotypic effects, depending on whether the mtCx26 can dock with other Cx hemichannels or not. Finally, wtCx26 might co-assemble in vivo with wtCx43 to heteromeric connexons as has been demonstrated for Cx43, Cx37 and Cx40 (Beyer et al., 2000). However, the incorporation of mtCx26 units might either prevent these heteromeric connexons from forming complete gap junctional channels, or these channels might acquire unique functional properties different from those of wild-type species. In turn, these changes might crucially interfere with GJIC mediated by other Cx that are simultaneously expressed in differentiating keratinocytes, which could explain why the phenotype is restricted to the epidermis despite the widespread expression of Cx43 and Cx26. Thus, it seems that the biological importance of the proposed pathomechanism can only be fully appreciated in the presence of other epidermal Cx.

In summary, our data support a novel concept, which provides an explanation for the diverse clinical manifestations of dominant *GJB2* mutations. We believe that the skin may tolerate the loss of Cx26-mediated GJIC, while the additional trans-dominant interference of certain dominant *GJB2* mutations with the function of Cx43 or potentially other epidermal Cx might crucially impair the level or mode of epidermal intercellular signaling causing a disease phenotype. The zones of epidermal co-expression of Cx26 and Cx43 are limited and mainly involve the skin of palms and soles, and thus could explain the restricted phenotype (PPK). Future studies in mammalian cells and/or transgenic animals that express dominant *GJB2* mutations with different clinical manifestations will verify the biological significance of our concept in vivo.

We are grateful to the family members for their generous participation in our study. We wish to thank H. J. Alder for services in oligonucleotide synthesis, DNA sequencing and genotype analysis. This study was supported in part by NIH/NIAMS grants K08-AR02141, P01-AR38923 (G.R.), R21-AR47102 (T.W.W.), RO1-GM 37751 (D.L.P.), Dermatology Foundation Fellowship (F.R.), and The British Skin Foundation (M.B.H.).

REFERENCES

Bergoffen, J., Scherer, S. S. , Wang, S., Scott, M. O., Bone, L. J., Paul, D. L., Chen, K., Lensch, M. W., Chance, P. F. and Fischbeck, K. H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science* **262**, 2039-2042.

Beyer, E. C., Gemel, J., Seul, K. H., Larson, D. M., Banach, K. and Brink, P. R. (2000). Modulation of intercellular communication by differential regulation and heteromeric mixing of co-expressed connexins. *Braz. J. Med. Biol. Res.* **33**, 391-397.

Bruzzone, R., Haefliger, J. A., Gimlich, R. L. and Paul, D. L. (1993). Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. *Mol. Biol. Cell* **4**, 7-20.

Bruzzone, R., White, T. W., and Paul, D. L. (1996). Connections with connexins: the molecular basis of direct intercellular signaling. *Eur. J. Biochem.* **238**, 1-27.

Bukauskas, F. F., Jordan, K., Bukauskiene, A., Bennett, M. V., Lampe, P. D., Laird, D. W. and Verselis, V. K. (2000). Clustering of connexin 43-enhanced green fluorescent protein gap junction channels and functional coupling in living cells. *Proc. Natl. Acad. Sci. USA* **97**, 2556-2561.

Butterweck, A., Elfgang, C., Willecke, K. and Traub, O. (1994). Differential expression of the gap junction proteins connexin45,-43,-40,-31, and-26 in mouse skin. *Eur. J. Cell Biol.* **65**, 152-163.

Choudhry, R., Pitts, J. D. and Hodgins, M. B. (1997). Changing patterns of gap junctional intercellular communication and connexin distribution in mouse epidermis and hair follicles during embryonic development. *Dev. Dyn.* **210**, 417-430.

Cohn, E. S. and Kelley, P. M. (1999). Clinical phenotype and mutations in connexin 26 (DFNB1/GJB2), the most common cause of childhood hearing loss.*Am. J. Med. Genet.* **89**, 130-136.

Denoyelle, F., Lina-Granade, G., Plauchu, H., Bruzzone, R., Chaib, H., Levi-Acobas, F., Weil, D. and Petit, C. (1998).Connexin 26 gene linked to a dominant deafness. *Nature* **393**, 319-320.

Deschenes, S. M., Walcott, J. L., Wexler, T. L., Scherer, S. S. and Fischbeck, K. H. (1997). Altered trafficking of mutant connexin32. *J. Neurosci.* **17**, 9077-9084.

Estivill, X., Fortina, P., Surrey, S., Rabionet, R., Melchionda, S., D'Agruma, L., Mansfield, E., Rappaport, E., Govea, N., Mila, M., Zelante, L. and Gasparini, P. (1998). Connexin-26 mutations in sporadic and inherited sensorineural deafness. *Lancet* **351**, 394-398.

Forge, A., Becker, D., Casalotti, S., Edwards, J., Evans, W. H., Lench, N. and Souter, M. (1999). Gap junctions and connexin expression in the inner ear. *Novartis Found. Symp.* **219**, 134-150.

Goliger, J. A. and Paul, D. L. (1994). Expression of gap junction proteins Cx26, Cx31.1, Cx37, and Cx43 in developing and mature rat epidermis. *Dev. Dyn.* **200**, 1-13.

Goliger, J. A. and Paul, D. L. (1995). Wounding alters epidermal connexin expression and gap junction-mediated intercellular communication. *Mol. Biol. Cell.* **6**, 1491-1501.

Green, G. E., Scott, D. A., McDonald, J. M., Woodworth, G. G., Sheffield, V. C. and Smith, R. J. (1999). Carrier rates in the midwestern United States for GJB2 mutations causing inherited deafness. *J. Am. Med. Assoc.* **281**, 2211-2216.

Grifa, A., Wagner, C. A., D'Ambrosio, L., Melchionda, S., Bernardi, F., Lopez-Bigas, N., Rabionet, R., Arbones, M., Monica, M. D., Estivill, X., Zelante, L., Lang, F. and Gasparini, P. (1999). Mutations in GJB6 cause nonsyndromic autosomal dominant deafness at DFNA3 locus. *Nat. Genet.* **23**, 16-18.

Heathcote, K., Syrris, P., Carter, N. D. and Patton, M. A. (2000). A connexin 26 mutation causes a syndrome of sensorineural hearing loss and palmoplantar hyperkeratosis (MIM 148350). *J. Med. Genet.* **37**, 50-51.

Houseman, M. J., Ellis, L. A., Pagnamenta, A., Di, W. L., Rickard, S., Osborn, A. H., Dahl, H. H., Taylor, G. R., Bitner-Glindzicz, M., Reardon, W., Mueller, R. F. and Kelsell, D. P. (2001). Genetic analysis of the connexin-26 M34T variant: identification of genotype M34T/M34T segregating with mild-moderate non-syndromic sensorineural hearing loss. *J. Med. Genet.* **38**, 20-25.

Kam, E., Melville, L. and Pitts, J. D. (1986). Patterns of junctional communication in skin. *J. Invest. Dermatol.* **87**, 748-753.

Kelley, P. M., Harris, D. J., Comer, B. C., Askew, J. W., Fowler, T., Smith, S. D. and Kimberling, W. J. (1998). Novel mutations in the connexin 26 gene (GJB2) that cause autosomal recessive (DFNB1) hearing loss. *Am. J. Hum. Genet.* **62**, 792-799.

Kelley, P. M., Abe, S., Askew, J. W., Smith, S. D., Usami, S. and Kimberling, W. J. (1999). Human connexin 30 (GJB6), a candidate gene for nonsyndromic hearing loss: molecular cloning, tissue-specific expression, and assignment to chromosome 13q12. *Genomics* **62**, 172-176.

Kelsell, D. P., Dunlop, J., Stevens, H. P., Lench, N. J., Liang, J. N., Parry, G., Mueller, R. F. and Leigh, I. M. (1997). Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* **387**, 80-83.

Krieg, P. A. and D. A. Melton. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* **12**, 7057-7070.

Labarthe, M. P., Bosco, D., Saurat, J. H., Meda, P. and Salomon, D. (1998). Upregulation of connexin 26 between keratinocytes of psoriatic lesions. *J. Invest. Dermatol.* **111**, 72-76.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamartine, J., Munhoz Essenfelder, G., Kibar, Z., Lanneluc, I., Callouet, E., Laoudj, D., Lemaitre, G., Hand, C., Hayflick, S. J., Zonana, J. et al. (2000). Mutations in GJB6 cause hidrotic ectodermal dysplasia. *Nat Genet* **26**, 142-144.

Lautermann, J., ten Cate, W. J., Altenhoff, P., Grummer, R., Traub, O., Frank, H., Jahnke, K. and Winterhager, E. (1998). Expression of the gap-junction connexins 26 and 30 in the rat cochlea. *Cell Tissue Res.* **294**, 415-420.

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

Liu, X. Z., Xia, X. J., Xu, L. R., Pandya, A., Liang, C. Y., Blanton, S. H., Brown, S. D., Steel, K. P. and Nance, W. E. (2000). Mutations in connexin31 underlie recessive as well as dominant non- syndromic hearing loss. *Hum. Mol. Genet.* **9**, 63-67.

Lucke, T., Choudhry, R., Thom, R., Selmer, I. S., Burden, A. D. and Hodgins, M. B. (1999). Upregulation of connexin 26 is a feature of keratinocyte differentiation in hyperproliferative epidermis, vaginal epithelium, and buccal epithelium. *J. Invest. Dermatol.* **112**, 354-361.

Macari, F., Landau, M., Cousin, P., Mevorah, B., Brenner, S., Panizzon, R., Schorderet, D. F., Hohl, D. and Huber, M. (2000). Mutation in the gene for connexin 30.3 in a family with erythrokeratodermia variabilis. *Am. J. Hum. Genet.* **67**, 1296-1301.

Maestrini, E., Korge, B. P., Ocana-Sierra, J., Calzolari, E., Cambiaghi, S., Scudder, P. M., Hovnanian, A., Monaco, A. P. and Munro, C. S. (1999). A missense mutation in connexin26, D66H, causes mutilating keratoderma with sensorineural deafness (Vohwinkel's syndrome) in three unrelated families. *Hum. Mol. Genet.* **8**, 1237-1243.

Martin, P. E., Coleman, S.L., Casalotti, S. O., Forge, A. and Evans, W. H. (1999). Properties of connexin26 gap junctional proteins derived from mutations associated with non-syndromal heriditary deafness. *Hum. Mol. Genet.* **8**, 2369-2376.

Morle, L., Bozon, M., Alloisio, N., Latour, P., Vandenberghe, A., Plauchu, H., Collet, L., Edery, P., Godet, J. and Lina-Granade, G. (2000). A novel C202F mutation in the connexin26 gene (GJB2) associated with autosomal dominant isolated hearing loss. *J. Med. Genet.* **37**, 368-370.

Rabionet, R., Zelante, L., Lopez-Bigas, N., D'Agruma, L., Melchionda, S., Restagno, G., Arbones, M. L., Gasparini, P. and Estivill, X. (2000). Molecular basis of childhood deafness resulting from mutations in the GJB2 (connexin 26) gene. *Hum Genet* **106**, 40-44.

Richard, G. (2000). Connexins: a connection with the skin. *Exp Dermatol* **9**, 77-96.

Richard, G., Brown, N., Smith, L. E., Terrinoni, A., Melino, G., Mackie, R. M., Bale, S. J. and Uitto, J. (2000). The spectrum of mutations in erythrokeratodermias–novel and de novo mutations in GJB3. *Hum. Genet.* **106**, 321-329.

Richard, G., Smith, L. E., Bailey, R. A., Itin, P., Hohl, D., Epstein, E. H., Jr, DiGiovanna, J. J., Compton, J. G. and Bale, S. J. (1998a). Mutations in the human connexin gene GJB3 cause erythrokeratodermia variabilis. *Nat. Genet.* **20**, 366-369.

Richard, G., White, T. W., Smith, L. E., Bailey, R. A., Compton, J. G., Paul, D. L. and Bale, S. J. (1998b). 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.

Risek, B., Klier, F. G. and Gilula, N. B. (1992). Multiple gap junction genes are utilized during rat skin and hair development. *Development* **116**, 639-651.

Rupp, R. A., Snider, L. and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8**, 1311-1323.

Salomon, D., Masgrau, E., Vischer, S., Ullrich, S., Dupont, E., Sappino, P., Saurat, J. H. and Meda, P. (1994). Topography of mammalian connexins in human skin. *J. Invest. Dermatol.* **103**, 240-247.

Silander, K., Meretoja, P., Pihko, H., Juvonen, V., Issakainen, J., Aula, P. and Savontaus, M. L. (1997). Screening for connexin 32 mutations in Charcot-Marie-Tooth disease families with possible X-linked inheritance. *Hum. Genet.* **100**, 391-397.

Swenson, K. I., Jordan, J. R., Beyer, E. C. and Paul, D. L. (1989). Formation of gap junctions by expression of connexins in Xenopus oocyte pairs. *Cell* **57**, 145-155.

Tan, C. C., Ainsworth, P. J., Hahn, A. F. and MacLeod, P. M. (1996). Novel mutations in the connexin 32 gene associated with X-linked Charcot-Marie tooth disease. *Hum. Mutat.* **7**, 167-171.

Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.

VanSlyke, J. K., Deschenes, S. M. and Musil, L. S. (2000). Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol. Biol. Cell* **11**, 1933-1946.

White, T. W. (2000). Functional analysis of human Cx26 mutations associated with deafness. *Brain Res. Rev.* **32**, 181-183.

White, T. W. and Bruzzone, F. (1996). Multiple connexin proteins in single intercellular channels: connexin compatibility and functional consequences. *J. Bioenerg. Biomembr.* **28**, 339-350.

White, T. W., Paul, D. L., Goodenough, D. A. and Bruzzone, R. (1995). Functional analysis of selective interactions among rodent connexins. *Mol. Biol. Cell* **6**, 459-470.

Wilcox, S. A., Saunders, K., Osborn, A. H., Arnold, A., Wunderlich, J., Kelly, T., Collins, V., Wilcox, L. J., McKinlay Gardner, R. J. et al. (2000). High frequency hearing loss correlated with mutations in the GJB2 gene. *Hum Genet* **106**, 399-405.

Wilgoss, A., Leigh, I. M., Barnes, M. R., Dopping-Hepenstal, P., Eady, R. A., Walter, J. M., Kennedy, C. T. and Kelsell, D. P. (1999). Identification of a novel mutation R42P in the gap junction protein beta-3 associated with autosomal dominant erythrokeratoderma variabilis. *J. Invest. Dermatol.* **113**, 1119-1122.

Xia, A. P., Ikeda, K. , Katori, Y., Oshima, T., Kikuchi, T. and Takasaka, T. (2000). Expression of connexin 31 in the developing mouse cochlea. *NeuroReport* **11**, 2449-2453.

Xia, J. H., Liu, C. Y., Tang, B. S., Pan, Q., Huang, L., Dai, H. P., Zhang, B. R., Xie, W., Hu, D. X., Zheng, D. et al. (1998). Mutations in the gene encoding gap junction protein beta-3 associated with autosomal dominant hearing impairment. *Nat. Genet.* **20**, 370-373.

Yeager, M. and Nicholson, B. J. (1996). Structure of gap junction intercellular channels. *Curr. Opin. Struct. Biol.* 6, 183-192.