Altered connexin expression and wound healing in the epidermis of connexin-deficient mice

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Accepted 28 April 2003
Journal of Cell Science 116, 3443-3452 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00638

Summary

To analyze the effect of connexin loss on the repair of wounded tail skin, we have studied the following transgenic mouse mutants: *connexin30*$^{-/-}$, *connexin31*$^{-/-}$ and *connexin43*$^{Cre\text{-}ER(T)/fl}$ (for inducible deletion of the connexin43 coding region). *Connexin43* and *connexin31* are expressed in the basal and spinous layers of wild-type epidermis, whereas connexin31 and small amounts of connexin30, as well as connexin26 proteins, were found in the granulous layer. Connexin43 was downregulated in *connexin31*-deficient mice, whereas mice with reduced connexin43 exhibited an upregulation of connexin30. During wound healing, connexin30 and connexin26 proteins were upregulated in all epidermal layers, whereas connexin43 and connexin31 protein expression were downregulated. In *connexin31*$^{-/-}$ mice, reduced levels of connexin30 protein were observed on days 1 and 2 after wounding. The closure of epidermal wounds in mice with decreased amounts of connexin43 protein occurred one day earlier. Under these conditions the expression profiles of *connexin30* and *connexin31* were also temporarily shifted by one day. Furthermore, dye transfer between keratinocytes in skin sections from *connexin43*-deficient mice was decreased by 40%. These results suggest that downregulation of connexin43 appears to be a prerequisite for the coordinated proliferation and mobilization of keratinocytes during wound healing.

Key words: Connexin, cx, Connexin-deficient mice, Gap junction, Epidermis, Wound healing

Introduction

Gap junction channels establish direct conduits between adjacent cells and permit the exchange of ions, secondary messenger molecules (like cyclic adenosine monophosphate, inositol 1,4,5-trisphosphate or calcium ions) and metabolites up to 1000 Da in molecular mass. Gap junction channels consist of two hemichannels, the connexons, which are synthesized by two apposed cells. Each connexon is built up by six connexin (cx) protein subunits (cf. Goodenough et al., 1996). Nineteen connexin genes in the mouse genome (Willecke et al., 2002) are cell type specifically expressed with overlapping specificity. Targeted deletion of several connexin genes has been used to show diverse functions of gap junctions in different organs (White and Paul, 1999; Willecke et al., 2002).

The epidermis, a stratified surface epithelium, protects the organism against external influences like mechanical, chemical or thermal stress and serves as a barrier to water. Keratinocytes pass through a program of differentiation which is accompanied by differential expression of several proteins like keratins and cadherins. The basal layer, the undermost layer of the epidermis, contains nondifferentiated, proliferating keratinocytes. When keratinocytes migrate into the spinous layer they loose their ability to proliferate and start the program of terminal differentiation. When they reach the cornified layer, keratinocytes peel off the epidermis as scurf (for a review, see Fuchs et al., 1994).

To date, nine different connexins have been described in the murine epidermis (reviewed by Richard, 2000a). The connexin expression pattern in rodents is altered during embryogenesis and after birth (Risek et al., 1992; Choudry et al., 1997). In the epidermis of adult mice, Cx43 and Cx40 are expressed in the basal layer and Cx43 and Cx31 are expressed in the spinous layer. In the granulous layer, Cx43 expression no longer occurs, so that besides weak expression of Cx26, only Cx31 appears in this layer (Kamibayashi et al., 1993; Butterweck et al., 1994) (reviewed by Richard, 2000a). Transcripts of Cx30, Cx30.3, Cx31.1 and Cx57 have been found in mouse epidermis but the epidermal expression pattern of the corresponding proteins has not yet been analyzed (Manthey et al., 1999; Hennemann et al., 1992; Dahl et al., 1996). In the epidermis of adult rats, expression of Cx43 and Cx31.1 has been detected in the basal and spinous layers, Cx37 is localized in all epidermal layers besides the stratum corneum, and Cx26 has been found in the granulous and – at low concentrations – in the spinous layer (Risek et al., 1992; Goliger and Paul, 1994).

Mutations in human connexin genes are known to cause several skin diseases. For example, erythrokeratodermia variabilis, an autosomal dominant genodermatosis, is characterized by the appearance of transient erythema and localized or generalized hyperkeratosis that can be due to mutations in the *Cx31* or *Cx30.3* gene (Richard et al., 1998; Richard, 2000a; Richard et al., 2000b; Macari et al., 2000; Gottfried et al., 2002). Different mutations in the *Cx26* gene

can lead to Vohwinkel's syndrome which implies hearing loss in conjunction with palmoplantar keratoderma (Maestrini et al., 1999; for reviews see White, 2000, and Kelsell et al., 2001). Mutations in the *Cx26* gene can also cause the Keratitis-ichthyosis-deafness syndrome (Richard et al., 2002).

Normal skin can regenerate after wounding or damaging. Wound healing involves proliferation, migration and differentiation of keratinocytes (Martin, 1997; Jacinto et al., 2001). The expression of connexins in rat skin was shown to be altered in response to the repair process (Goliger and Paul, 1995). The healing process of an incision wound in rat tail skin takes about five days (Goliger and Paul, 1995). To study the effects of connexin loss on residual connexin expression in the mouse epidermis, we have analyzed the expression of Cx43, Cx31, Cx30 and Cx26 protein in intact and wounded tail skin of the transgenic mouse mutants $Cx43^{Cre\text{-}ER(T)/fl}$, $Cx31^{-/-}$, $Cx30^{-/-}$ and wild-type control mice.

Materials and Methods

Animals

Mice were kept in accordance with local governmental and institutional animal care regulations and maintained under a 12/12 hours light/dark cycle. For our experiments we used the $Cx31^{-/-}$ (Plum et al., 2001) and $Cx30^{-/-}$ mouse lines (Teubner et al., 2003) in which a *LacZ* reporter gene (in the case of $Cx30^{-/-}$ mice with a nuclear localization signal) replaced both alleles of the connexin coding region. For wild-type controls for $Cx31^{-/-}$ or $Cx30^{-/-}$ mice we used litter mates obtained by interbreeding of the corresponding heterozygous animals. The influence of induced Cre-mediated deletion of Cx43 was studied in adult $Cx43^{Cre\text{-}ER(T)/fl}$ mice (D. Eckardt, M. Theis, J. Degen et al., unpublished). Briefly, these animals were generated as follows: one Cx43 coding region in HM1 mouse embryonic stem cells was replaced by Cre-ER(T), a fusion construct of the Cre recombinase and the mutated ligand binding domain of the human estrogen receptor (Feil et al., 1996). The homologously recombined HM1 cells were injected into mouse blastocysts to generate $Cx43^{Cre\text{-}ER(T)/+}$ mice, which were mated to previously published $Cx43^{fl/fl}$ mice. In these animals, the Cx43 alleles were flanked by loxP sequences, i.e. the recognition sites for Cre recombinase (Theis et al., 2001).The resulting $Cx43^{CreER(T)/fl}$ mice were used for phenotypic analyses. In cells expressing Cre-ER(T), treatment with 4-hydroxytamoxifen (4-OHT) induced the translocation of the Cre-ER(T) protein to the nucleus where Cre could excise the floxed Cx43 allele. Quantitative western blot analysis of total skin lysates revealed a decrease of Cx43 protein in 4-OHT treated $Cx43^{Cre\text{-}ER(T)/fl}$ mice by up to approximately 85% compared with $Cx43^{fl/fl}$ controls (data not shown). All transgenic mice were of mixed genetic background (at least 75% C57BL/6 and at most 25% 129ola). To minimize the effects of genetic background, we performed all analyses with littermates of heterozygote breedings, including control animals that lacked the Cre allele.

Dye transfer in acute thick sections of mouse ear skin

After sacrificing the mice, the ears were immediately dissected and transferred into Dulbecco's modified Eagle's Medium (Gibco, Karlsruhe, Germany), warmed to 35°C. Tissue slices (250 μm) of mouse ear were produced using a microtome (VT1000S, Leica, Wetzlar, Germany) with commercial razor blades. Sections were microinjected, submerged in a chamber at 35°C under constant flow of oxygenated Dulbecco's Modified Eagle's Medium, after an initial incubation period of 15 minutes to 2 hours at 35°C in carbogen (95% O_2 and 5% CO_2). Microinjection of the fluorescent dye Alexa Fluor 488 (Molecular Probes Europe BV, Leiden, The Netherlands) was performed by iontophoresis into single cells under visual inspection. The dye was injected according to Romualdi et al. (Romualdi et al., 2002) with 2 nA for 2 minutes. Electrodes were filled with 200 mM KCl. These conditions resulted in the expected membrane potential of –25 mV when starting the injection of Alexa Fluor 488. Injections at lower membrane potentials were excluded from the final analysis. The intercellular spreading of the microinjected dye was followed by visual inspection under UV light and phase contrast. Finally, gap junctional coupling was evaluated 10 minutes after injection by counting fluorescent cells under UV light, and micrographs were taken with a SONY CCD camera. Dye spreading experiments were carried out on two to three skin sections per mouse with the following numbers: $Cx43^{Cre\text{-}ER(T)/fl}$ induced: 6 mice, 28 injections; $Cx43^{Cre\text{-}ER(T)/fl}$ uninduced: 4 mice, 16 injections; $Cx43^{fl/fl}$ uninduced: 3 mice, 16 injections; $Cx43^{fl/+}$ induced: 2 mice, 17 injections; wild-type: 13 mice; 44 injections; $Cx31^{-/-}$: 5 mice, 31 injections; $Cx31^{+/+}$: 3 mice, 26 injections. The statistically significant decrease of dye transfer in skin sections from $Cx43^{Cre\text{-}ER(T)/fl}$ mice ($P<0.001$) has been evaluated by the paired t-test.

Immunoblot analysis

Wounded or uninjured pieces of tail skin were dissected on ice and immediately frozen in liquid nitrogen. Homogenized tissue was taken up in protein lysis buffer [60 mM Tris HCl, pH 7.4 and 3% sodium dodecyl sulphate (SDS)], supplemented with proteinase inhibitor 'Complete' (Roche, Mannheim, Germany) and sonicated three times for 20 seconds on ice. Protein concentration was determined using the bichinchoninic acid protein assay (Sigma, Taufkirchen, Germany). For electrophoresis, 50 μg of protein were separated on 12.5% SDS-polyacrylamide gels. Proteins were electroblotted on nitrocellulose membranes (Hybond ECL, Biosciences, Bucks, UK) for 2 hours at 100 V. Membranes were blocked for 1 hour with blocking solution [(20 mM Tris, HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (TBS-Tween)] and 5% skim milk powder (w/v) and afterwards incubated with primary antibodies over night at 4°C. The antibodies were diluted in blocking solution (anti-Cx43: 1:500 (Traub et al., 1994); anti-Cx31: 1:250 (Butterweck et al., 1994); anti-Cx30: 1:250 (Zytomed, Berlin, Germany); anti-Cx26: 1:500 (Zytomed)). After washing for 30 minutes in blocking solution, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour (Dianova, Hamburg, Germany), diluted 1:20000 for anti-Cx43 and 1:5000 for the other antibodies in blocking solution. Afterwards, membranes were washed for 1 hour in TBS-Tween and incubated with an ECL chemiluminescence detection system (Amersham Biosciences, Freiburg, Germany). In order to check equal loading in all lanes of immunoblot, we performed Ponceau or Coomassie staining of the blotting membranes, followed by densitometric analyses of the stained protein. Lysates of HeLa cells after stable transfection with the respective connexin and lysates of untransfected HeLa cells were used as positive and negative controls.

Immunofluorescence analyses

Cryosections (5 μm) of tail epidermis were stained with the following antibody solutions: rabbit anti-Cx43 (1:2000) (Traub et al., 1994), rabbit anti-Cx31 (1:100) (Butterweck et al., 1994), rabbit anti-Cx30 (1:300; Zytomed) and rabbit anti-Cx26 (1:500; Zytomed). Primary antibodies were detected with Alexa594 conjugated goat anti-rabbit immunoglobulin (1:2000; MoBiTech, Goettingen, Germany). For immunofluorescence analyses, cryosections were fixed in 100% ethanol (–20°C) for 5 minutes, blocked with 4% bovine serum albumin (BSA, PAA Laboratories GMBH, Linz, Austria) in phosphate buffered saline (PBS) and incubated with antibodies diluted in 0.4% BSA in PBS over night at 4°C. Afterwards, sections were washed with 0.4% BSA in PBS and incubated with Alexa conjugated antibodies for 1 hour at room temperature. Nuclear staining was performed by

15 minutes incubation with 0.2 μg/ml Hoechst 33258 fluorescent dye in PBS (Sigma B-2883). Slices were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark).

Antibody-stained tissue slices were analyzed using the photomicroscope Axiophot (Zeiss, Jena, Germany). Scoring of sections to quantify the extent of immunostaining was carried out under the microscope to ensure that only immunofluorescent and not background signals were counted. The size of immunofluorescent signals was taken into account as follows: images were digitally recorded at the same magnification and time of exposure. Immunosignals were analyzed by counting immunopositive pixels in distinct areas (approx. 30 μm^2). The unit 'immunosignals/field' matched the number of pixels counted per 30 μm^2 area. The mean values of the counted samples with the corresponding standard deviations were determined and Student's t-tests were performed.

Sections of the corresponding connexin deficient mice were used as negative controls. Stable transfected HeLa cells or sections of connexin expressing tissues were used as positive controls.

Injection of 4-hydroxytamoxifen in Cx43$^{Cre\text{-}ER(T)/fl}$ and control mice

Fifty milligrams of 4-hydroxytamoxifen (Sigma, H-6278) were suspended in 200 μl ethanol with a sonicator. Peanut oil (4.8 ml) was added, and the mixture was sonicated and mixed vigorously (final concentration: 10 mg/ml). Injections were performed intraperitonally with a 26 gauge needle. 4-Hydroxytamoxifen (3.5 mg) was injected five times (once every 24 hours) and wounds were cut 5 days after the last injection.

Preparation of skin sections and histochemistry

Animals were killed by cervical dislocation. The tail skin was dissected, immediately frozen in liquid nitrogen and stored at –70°C. Tail epidermis was cut lengthwise (5 μm sections) with a cryostat. For 5-bromo-4-chloro-3-indolyl-β-galactoside staining, sections were processed as described previously (Theis et al., 2001). Tissue used for paraffin embedding was fixed with 100% ethanol overnight at 4°C. Tissue infiltration was performed in an infiltration machine (TP1020; Leica, Bensheim, Germany) with the following protocol: 30 minutes H_2O; 1 hour 70% ethanol; 30 minutes 80%; 30 minutes 96%; 45 minutes 96%; 30 minutes 100%; 30 minutes 100% ethanol; 30 minutes xylol; 30 minutes xylol; paraplast 2 hours; paraplast 2 hours. Embedding was carried out with a paraffin embedding station (EG 1140 H; Leica). Slices of 5 μm were produced with a rotation microtome (HM 360; Microm). Deparaffination was performed with the following protocol: Xylol 3 minutes; 100% EtOH 1 minute; 96% EtOH 1 minute; 80% EtOH 1 minute; 60% EtOH 1 minute. Afterwards slices were stained with haematoxylin and eosin and mounted with mounting medium (DAKO).

Wounding and isolation of mouse tail skin

Incision wounds into mouse tail were cut with a scalpel as previously described for rat tail (Goliger and Paul, 1995). Six to eight transverse sections through the tail skin with a length of 1 cm were performed per mouse. Two of them were processed for immunofluorescence, two for histological analysis and four were used for immunoblot analyses. At each time point after wounding, connexin-deficient and control mice were analyzed.

Results

Distribution of connexins in wild-type and connexin-deficient mouse tail epidermis

Immunofluorescence analyses using Cx31 and Cx30 antibodies revealed the distribution of Cx31 and Cx30 proteins in mouse tail epidermis. Cx31 was strongly expressed in the granulous and spinous layers, as already shown in back skin epidermis (Butterweck et al., 1994). Additionally, patchy expression of Cx31 in the basal layer of tail epidermis was found (Fig. 1A). This result was confirmed by the finding that β-galactosidase staining of Cx31-deficient mice showed strong signals in the suprabasal tail epidermis and a patchy distribution of β-galactosidase in the basal layer (Fig. 2A). Thus, Cx31 expression in stratum basale was verified at the transcript and protein level.

Cx30 protein was barely detectable in the granulous layer of normal tail epidermis. In other epidermal layers, Cx30 protein was not found in nonirritated skin (Fig. 1B). In contrast to these results, β-galactosidase staining of Cx30$^{-/-}$ tail epidermis yielded signals in all epidermal layers (Fig. 2B). Thus, Cx30 transcripts were probably abundant in all epidermal layers but Cx30 protein was only barely detected in the granule layer of mouse tail epidermis, suggesting post-transcriptional control of Cx30 expression.

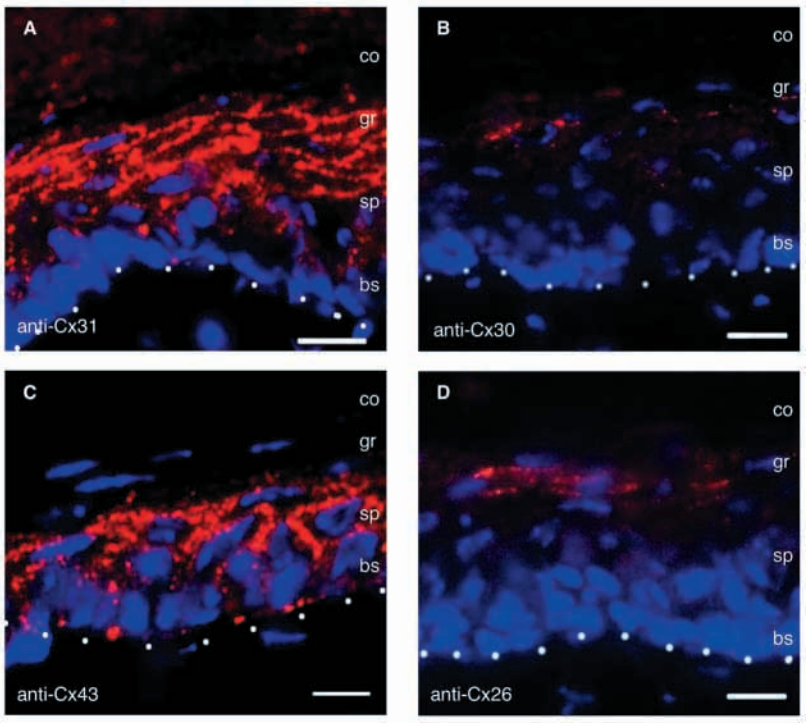

Fig. 1. Immunofluorescence analysis of connexins in cryosections of wild-type epidermis. Cx31 immunoreactivity was mainly detected in the granulous layer and the upper part of the spinous layer (A). In 20% of all specimen analyzed, Cx30 (B) and Cx26 (D) were weakly expressed in the granulous layer. Cx43 expression occurred in the basal as well as in the spinous layer (C). Nuclei were stained with Hoechst 33258 fluorescent dye. The basal lamina is marked by white dots. Bar, 25 μm. Epidermal layers: bs, basal; co, cornified; gr, granulous; sp, spinous.

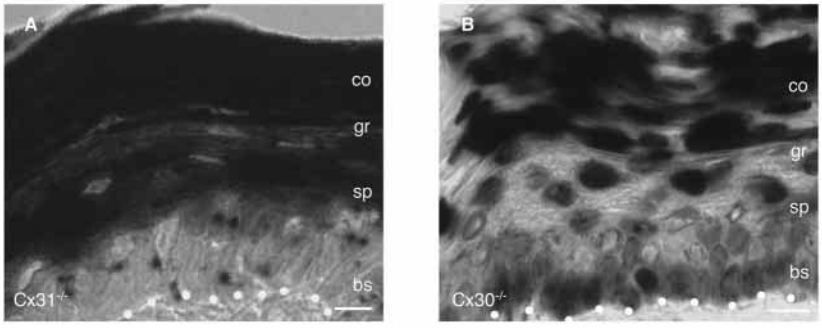

Fig. 2. β-Galactosidase staining of Cx31$^{-/-}$ (A) and Cx30$^{-/-}$ (B) tail epidermis. In Cx31$^{-/-}$ mice (no nuclear localisation signal in the *lacZ* gene) granulous and upper spinous layers were strongly stained. The innermost spinous layers were labelled more weakly and the basal layer showed punctuate staining. Cx30-deficient mice (B) showed β-galactosidase staining in all epidermal layers. The basal lamina is marked by white dots. Bars, 25 μm. Epidermal layers: bs, basal; co, cornified; gr, granulous; sp, spinous.

Cx43 expression pattern in mouse epidermis of the back was already found in the stratum basale and lowest layers of the stratum spinosum (Butterweck et al., 1994). This corresponds to the expression found in wild-type tail epidermis (Fig. 1C). In Cx31$^{-/-}$ mice, the level of Cx43 protein was significantly reduced (Fig. 3A) compared with wild-type tail epidermis (Fig. 1C). We observed by immunoblot analyses of *Cx31*$^{-/-}$ mice only about one third of the Cx43 protein found in wild-type controls (Fig. 3B), which is consistent with the above finding.

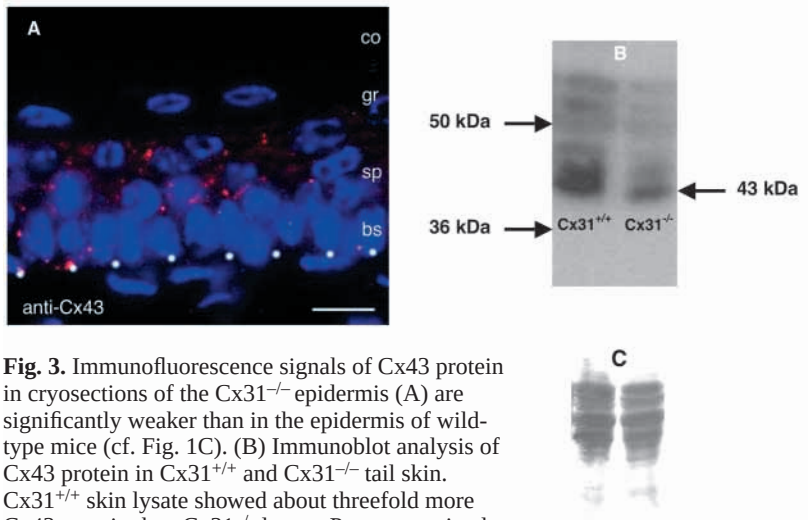

Fig. 3. Immunofluorescence signals of Cx43 protein in cryosections of the Cx31$^{-/-}$ epidermis (A) are significantly weaker than in the epidermis of wild-type mice (cf. Fig. 1C). (B) Immunoblot analysis of Cx43 protein in Cx31$^{+/+}$ and Cx31$^{-/-}$ tail skin. Cx31$^{+/+}$ skin lysate showed about threefold more Cx43 protein than Cx31$^{-/-}$ lysate. Ponceau-stained nitrocellulose membranes (C) indicated equal protein loading of the lanes shown in (B). Nuclei were stained with Hoechst 33258 fluorescent dye. The basal lamina in A is marked by white dots. Bar, 25 μm. Epidermal layers: bs, basal; co, cornified; gr, granulous; sp, spinous.

Cx30 and Cx26 proteins were barely detectable in the granulous layer of wild-type tail epidermis (Fig. 1B,D). By contrast, Cx30-specific immunoreactivity was much stronger in the granulous and spinous layers in induced *Cx43*$^{Cre-ER(T)/fl}$ mice (Fig. 4A), compared with wild-type controls (Fig. 4B). Thus, our results show an altered expression of Cx43 protein in *Cx31*-deficient mice and of Cx30 protein in *Cx43*$^{Cre-ER(T)/fl}$ mice.

Expression of Cx43, Cx31, Cx30 and Cx26 during wound healing of wild-type mouse tail epidermis

To study wound healing in mice we adapted the method described by Goliger and Paul (Goliger and Paul, 1995) for wounding of rat tail skin. Fig. 5 shows that the healing process in mouse skin took 5 days, similar to that reported for rat skin (Goliger and Paul, 1995). In the first 12 to 24 hours after wounding, the incision wound could be recognized on micrographs as cut-through epidermis and dermis of the mouse tail (cf. Fig. 5A). On day 1-2 of the healing process, the epidermis at the wound edges was thickened and rounded keratinocytes began to migrate under the coagulum (cf. Fig. 5B). Three days after wounding, the migrating keratinocytes closed the epidermal wound under the coagulum and began their process of terminal differentiation (Fig. 5C). Four to five days after wounding, the epidermis was again completely stratified (Fig. 5D).

The expression pattern of Cx43 and Cx26, which had been studied in wounded tail epidermis of 10-day-old rats (Goliger and Paul 1995), was largely consistent with our results on Cx43 and Cx26 in mouse tail epidermis. Furthermore, we found that the expression patterns of Cx26 and Cx30 after wounding were remarkably similar (Fig. 6A,C). Both Cx26 and Cx30 were barely expressed in the granulous layer of uninjured tail epidermis. After wounding, the expression of

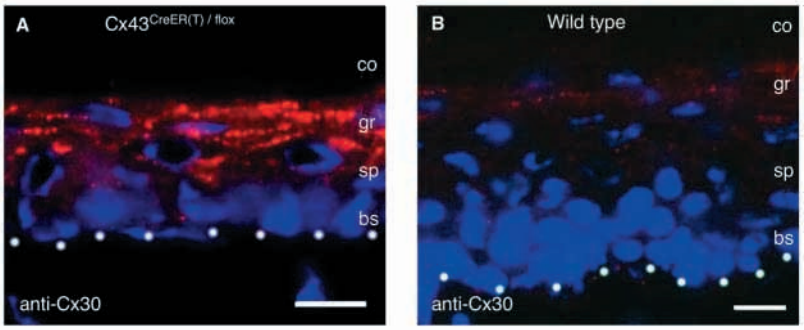

Fig. 4. Immunofluorescence labelling of Cx30 in cryosections of 4-hydroxytamoxifen-induced Cx43$^{Cre-ER(T)/fl}$ mouse tail epidermis (A) showed prominent immunoreactivity of Cx30 in the granulous and spinous layers, in contrast to barely visible Cx30 immuno signals in wild-type epidermis (B). Nuclei were stained with Hoechst 33258 fluorescent dye. The basal lamina is marked by white dots. Bars, 25 μm. Epidermal layers: bs, basal; co, cornified; gr, granulous; sp, spinous.

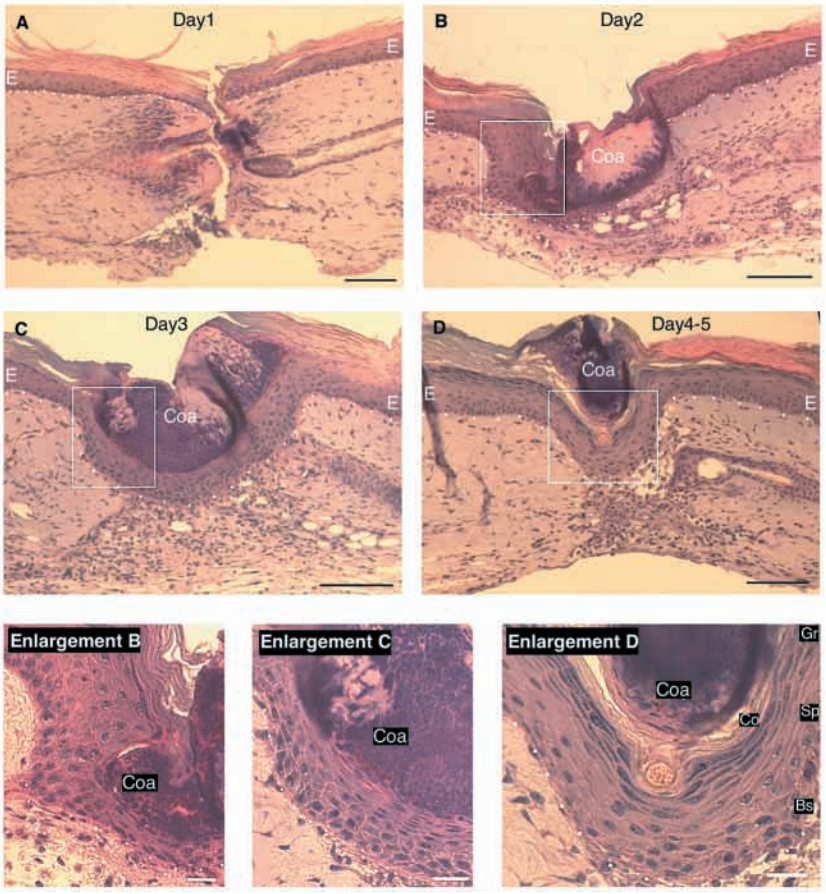

Fig. 5. Paraffin sections (5 μm) of wounded epidermis of mouse tail, stained with haematoxylin and eosin. During the first 12 to 24 hours after wounding (A), the incision wound could be recognized as a cut through the epidermis and dermis. On the second day of the healing process (B), the epidermis at the wound edges was thickened and the rounded keratinocytes (see enlargement of boxed area in B) began their migration under the coagulum. Three days after wounding (C), the migrating keratinocytes met under the coagulum, while closing the epidermal wound and beginning with their process of terminal differentiation (see enlargement C). After 4 to 5 days of wound healing (D), the epidermis was again completely stratified (enlargement D). Bars in main images, 250 μm; bars in inserted micrographs, 25 μm. Bs, basal; Co, cornified; Coa, coagulum; E, epidermis; Gr, granulous; Sp, spinous.

both connexins was upregulated in all epidermal layers, reaching a maximum on day 3 after wounding and declining afterwards to the expression level in uninjured epidermis.

Cx31 was strongly expressed in suprabasal layers and weakly in the stratum basale of uninjured tail epidermis. After wounding, Cx31 expression in suprabasal layers was drastically downregulated, whereas expression in the basal layer increased to a maximum on day 3 postwounding (Fig. 6D). Cx31 expression in suprabasal layers started again to increase on day 3 after wounding, in contrast to the basal layer, where it returned to the low expression level of uninjured epidermis.

In uninjured tail epidermis, immunofluorescence analysis of Cx43 protein revealed its expression in basal and spinous layers. Between days 1 and 2 after wounding, the amount of Cx43 protein was clearly reduced at the wound edges and in the periphery of the wounds, a region where proliferation has been described (Fig. 6B). At day 3 postwounding, Cx43 expression started to increase in basal and suprabasal layers to the expression level in uninjured epidermis. Thus, localization of Cx43 protein did not change during wound-healing process.

Expression of Cx43, Cx31, Cx30 and Cx26 during epidermal wound healing in connexin-deficient mice

Mice with reduced levels of Cx43 protein showed premature fusion of tail epidermis on day 2 after wounding in seven out of eight cases. Thus, in contrast to wild-type mice, where the epidermis under the coagulum fused on day 3 after wounding in 14 of 16 cases (Figs 7, 8), Cx30 and Cx31 showed an earlier peak of expression in tail epidermis of mice with decreased amounts of Cx43 (Fig. 6E,F). Immunofluorescence analyses revealed a peak in Cx30 protein expression in the tail epidermis of mice, with reduced Cx43 levels already at day 2 postwounding, whereas wild-type controls displayed maximal Cx30 protein on day 3 (Fig. 7). In addition, the total amount of Cx30 protein on days 2 and 3 of the wound-healing process was clearly higher in tail epidermis of Cx43-deficient mice than in wild-type controls. Cx30 expression decreased in the basal layer of mice with reduced levels of Cx43 one day earlier (on day 3) compared with wild-type epidermis.

Immunostained Cx31 protein was clearly more abundant on days 1 and 2 postwounding in tail epidermis of mice with decreased amounts of Cx43 protein than in wild-type controls (Fig. 8). Cx31 expression already increased on day 2 in the spinous and granulous layers, whereas it occurred only on day 3-4 in wild-type epidermis. On day 3 there was a reduction of the Cx31 protein in the basal layer. By contrast, Cx31 protein did not decrease until day 4 after wounding of wild-type tail epidermis.

In Cx31-deficient mice, only traces of immunoreactive Cx30 protein were found in the epidermis on days 1 and 2 after wounding (data not shown). By contrast, Cx30 protein in wild-type epidermis was increased on day 1 and 2 postwounding compared with intact epidermis. In wounded wild-type tail epidermis, the highest amount of Cx26 and Cx30 protein was detected on day 3 postwounding (Fig. 9). By contrast, $Cx30^{-/-}$ and $Cx31^{-/-}$ mice showed maximal expression of Cx26 on day 4 after wounding, together with maximal expression of Cx30 in *Cx31*$^{-/-}$ mice (data not presented).

Dye transfer in acute thick sections of mouse ear epidermis

Injection of the fluorescent dye Alexa Fluor 488 was performed in thick sections of the ear and not in tail epidermis

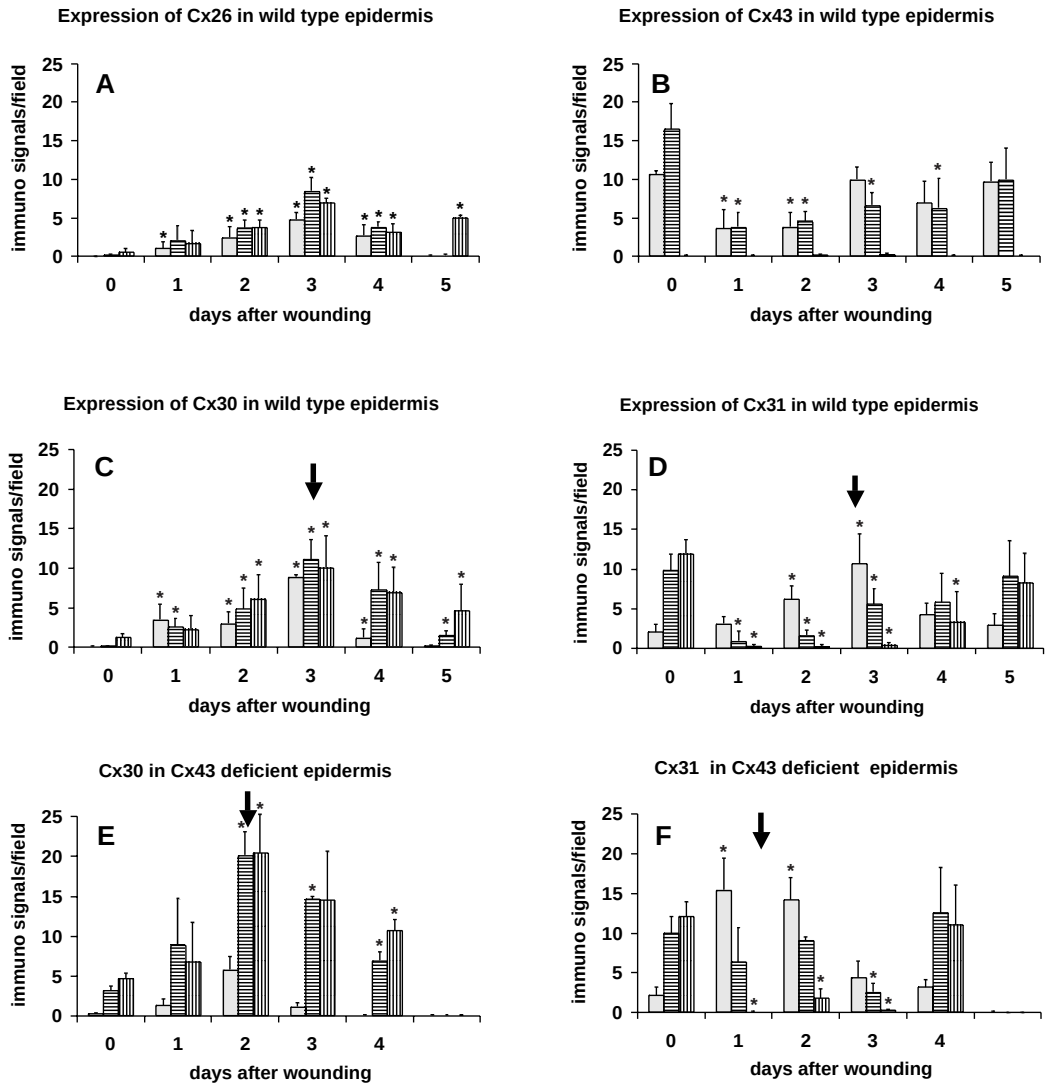

Fig. 6. Altered expression levels of Cx26, Cx30, Cx31 and Cx43 relative to the corresponding level in uninjured epidermis. (A,C) Cx26 and Cx30 immunosignals increased in all epidermal layers to maximal expression on day 3. (D) The Cx31 protein level decreased in the suprabasal layers accompanied by an increase in the basal layer until day 4. (B) Cx43 protein decreased within the first 4 days of wound healing. On day 5, all of the connexins analyzed were readjusted to the expression level of uninjured epidermis. Cx30 (E) and Cx31 protein (F) in mice with decreased amount of Cx43 protein showed an altered expression pattern which resembled that in wounded wild-type epidermis one day later. Labelling of the columns: grey, basal layer; horizontal lines, spinous layer; vertical lines, granulous layer. Arrows indicate the shifted maximal expression of Cx30 and Cx31. Asterisks above the lanes indicate significant changes of signal abundance (P<0.05) (Student's t-test) compared with corresponding uninjured epidermis.

because the stratum corneum of the tail was too thick to allow correct positioning of the injection needle in a cell of the basal layer. The microinjected, fluorescent dye spread only between epidermal cells, i.e. no dye spreading across the basal lamina into dermal cells was noticed (Fig. 10A,B). The epidermis of Cx31-deficient mice showed no statistically significant difference in dye transfer compared with wild-type skin from $Cx31^{+/+}$ litter mates of $Cx31^{-/-}$ mice or C57BL/6 mice for comparison (Fig. 10C). By contrast, epidermal sections from $Cx43^{Cre\text{-}ER(T)/fl}$-mice after induction with 4-hydroxytamoxifen yielded a 40% reduction in coupling compared with uninduced $Cx43^{Cre\text{-}ER(T)/fl}$-mice and wild-type mice (Fig. 10A-C).

Discussion

The changes in expression of the connexins analyzed during wound healing can be divided into two groups: one with a decreased expression and the other with increased expression after wounding. Between days 1 and 2 after wounding, the epidermis thickened due to migration of rounded keratinocytes, and the Cx43 and Cx31 protein levels were clearly decreased compared with normal epidermis. On day 3 the epidermis fused under the coagulum. At this time, the level of Cx43 and Cx31 protein increased again and afterwards were readjusted to expression in normal epidermis.

Our results suggested that the reduction in Cx43 and Cx31 protein may be associated with dedifferentiation and mobilization of keratinocytes at the wound edge during the first two days of wound healing. This notion was supported by the observation that mice with decreased Cx43 protein showed premature closure of the tail epidermis after wounding. Furthermore, in thick sections of the ear skin from Cx43-deficient mice, we found 40% decrease of dye transfer, in contrast to Cx31-deficient mice. Thus, the lack of Cx43 protein led to decreased gap junctional intercellular communication in epidermal cells of the skin. Therefore, the Cx43 protein seems to be important for maintaining epidermal gap junctional communication. Possibly, lack of Cx43 protein may accelerate the wound closure, as the amount of Cx43 did not have to be decreased before the keratinocytes started to migrate. For technical reasons dye transfer experiments were carried out with thick sections from ear. Connexin expression pattern in mouse ear epidermis was compared using immunofluorescence

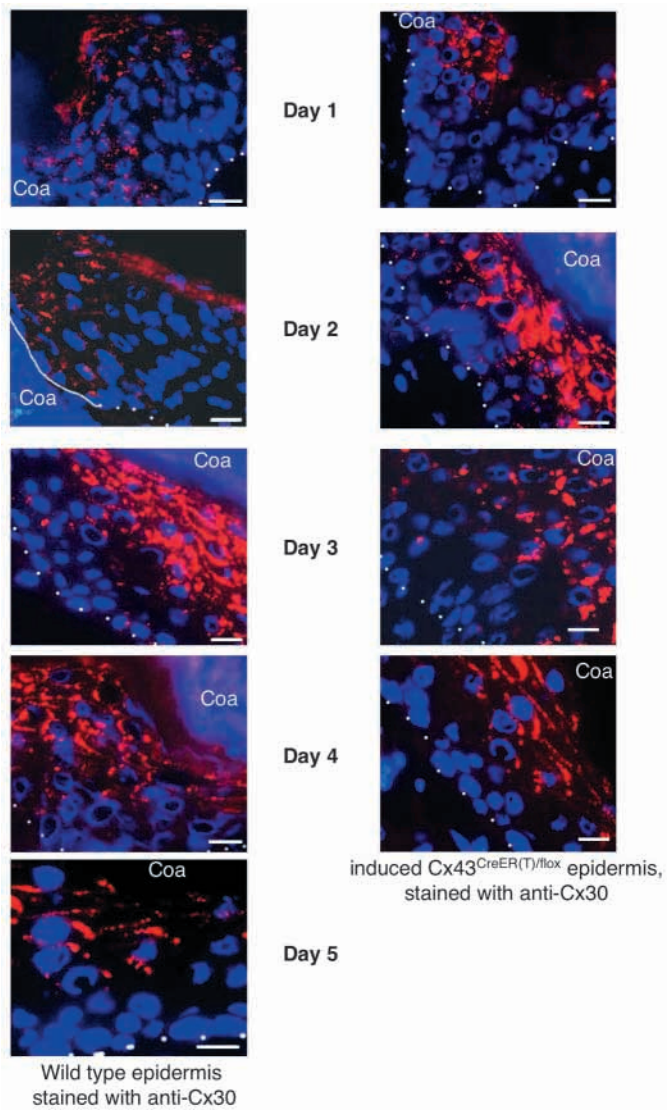

Fig. 7. Left panel: Immunostaining of Cx30 expression in wild-type epidermis on days 1-5 after wound healing. Cx30 protein increased after wounding to maximal expression on day 3. On days 4 and 5, Cx30 protein level decreased again. Right panel: Cx30 expression in tail epidermis of mice with decreased amounts of Cx43 protein. The maximal expression of Cx30 after wounding was shifted to one day earlier than in wild-type epidermis. On day 2 after wounding the epidermis was already closed under the coagulum, in contrast to wild-type epidermis, which fused on day 3 after wounding. Nuclei were stained with Hoechst 33258 fluorescent dye. The basal lamina is marked by white dots. Bars, 25 μm. Coa, coagulum.

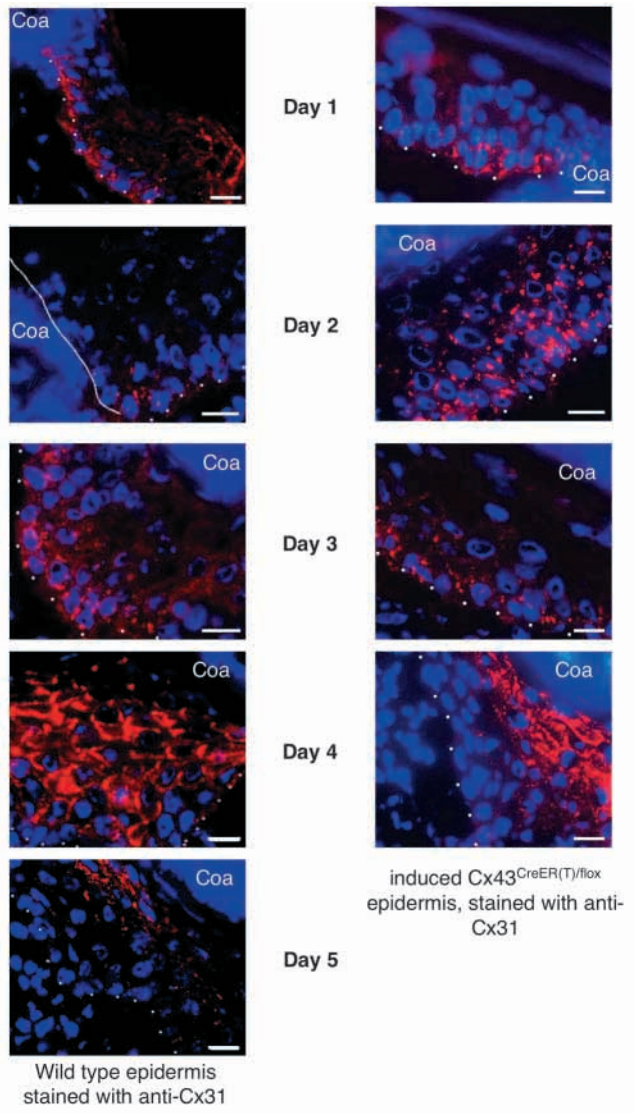

Fig. 8. Left panel: Immunostaining of Cx31 expression in tail epidermis on days 1-5 after wound healing. Cx31 protein amount decreased during the first 2 days postwounding and Cx31 protein was mainly localized in the basal layer. On days 4 and 5, the expression level increased again to the level of uninjured epidermis. Right panel: Cx31 expression in tail epidermis of mice with decreased amount of Cx43 protein ($Cx43^{CreER(T)/fl}$, induced). Maximal Cx31 expression after wounding occurred one day earlier than in wild-type epidermis. Nuclei were stained with Hoechst 33258 fluorescent dye. Basal lamina is marked by white dots. Bars, 25 μm. Coa, coagulum.

analyses with connexin expression in tail skin. Although, no obvious differences in connexin expression pattern were found between tail and ear epidermis, we cannot rule out the possibility that the intercellular coupling may be different.

Keratinocyte proliferation after wounding takes place in the periphery of the wound and not at the wound edge (Garlick et al., 1994). Because Cx43 was also decreased in the periphery on days 1 and 2 after wounding, Cx43 might have to be downregulated before extensive proliferation of keratinocytes could occur in the periphery of the wound. Because Cx43 protein was essential for maintaining the normal level of gap junctional communication, we conclude that decreased coupling between epidermal cells may be necessary for mobilization and proliferation of keratinocytes. Keratinocytes of induced $Cx43^{CreER(T)/flox}$ mice showed reduced coupling, and therefore cell migration and proliferation might have started earlier. Downregulation of Cx43 was also described after treatment of mouse back skin with tumor promoters or oncogenes (Brissette et al., 1991; Budonova, 1994). Ca^{2+}-mediated differentiation of cultured mouse keratinocytes also resulted in a decrease of Cx43 protein (Brissette et al., 1994).

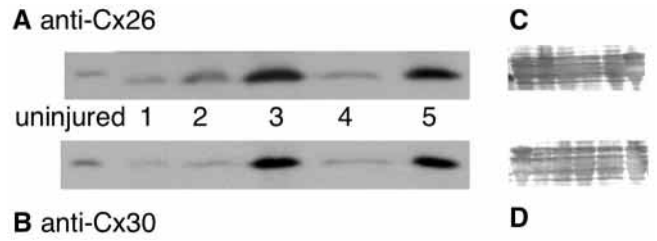

Fig. 9. Immunoblot analyses of connexin protein in uninjured and wounded tail epidermis from wild-type mice with antibodies to Cx26 (A) and Cx30 (B). Numbers indicate days after wounding. Ponceau stained nitrocellulose membranes (C, D) show equal protein loading of the lanes depicted in A and B. Cx26 and Cx30 expression was increased from day 1 and reached its peak on day 3 after wounding. On day 4, the level of Cx26 and Cx30 protein had clearly decreased and rose again on day 5 before it dropped to the level observed in uninjured epidermis.

Thus, Cx43-deficient mice might show an earlier onset of keratinocyte migration and/or proliferation compared with wild-type mice. This suggested function of Cx43 (i.e. downregulation of Cx43 appears to be necessary for mobilization and proliferation of keratinocytes) is a new feature that may be restricted to the skin. It was previously shown that the lack of Cx43 resulted in decreased migration of neural crest cells during development (Huang et al., 1998). Thus, Cx43-containing gap junctions, or at least the presence of functional Cx43 protein, might support the migration of neural crest cells. This could be explained by the finding that neural crest cells in mouse embryos appear to express Cx43 and Cx46 (Bannerman et al., 2000), in contrast to keratinocytes, which were shown to express nine different connexin isoforms.

The expression level of Cx31 in suprabasal epidermal layers appeared to depend on the state of keratinocyte differentiation: outer epidermal layers expressed more Cx31 protein. This notion was supported by the finding that the amount of Cx31 was strongly reduced in suprabasal layers of the wound edges during the first two days after wounding, when barely differentiated keratinocytes migrated under the coagulum to close the wounded epidermis.

In contrast to the Cx43 expression pattern, Cx31 expression was totally altered during wound healing. Cx31 protein, which was found in suprabasal layers of uninjured epidermis, was mainly expressed during the first two days after wounding in the stratum basale and only in small amounts in the suprabasal layers.

The function of Cx31 in the basal layer during the first three days of wound healing is not known. When Cx43 was decreased to a low level, Cx31 protein seemed to partially adopt the site of Cx43 expression during the first two days of wound healing. The correlation of Cx43 and Cx31 expression could be due to the observed downregulation of Cx43 in Cx31-deficient mice. Obviously, the expression level of Cx31 seemed to influence Cx43-containing gap junctions. Cx31 channels show only homotypic coupling in HeLa cells (Elfgang et al., 1995). Hence, the coupling of Cx43 hemichannels with those of Cx31 is unlikely. However, heteromeric channels, including Cx43 and Cx31 in the epidermal layers with overlapping expression, i.e. basal and innermost spinous layers, may form in wild-type mice. This might explain the decreased amounts of Cx43 protein in Cx31-deficient mice.

In contrast to the above-mentioned hypothesis, however, Cx43 and Cx31 showed expression patterns that were more or less separated from each other. In the overlapping expression areas, Cx31 was only present in small amounts. Thus, extensive formation of heteromeric channels is unlikely.

Taken together, Cx43 expression seemed to be dependent on the occurrence of Cx31 protein, because lack of Cx31 protein was associated with a reduced amount of Cx43 in the epidermis. There appears to be an inverse relationship between both connexins that led to the complementary distribution pattern in the mouse epidermis. This was supported by the observation that an increased amount of Cx31 in 60% of all observed cases was found in epidermis with a reduced Cx43 protein level. This could mean that expression of Cx43 affected the amount and site of Cx31 expression and vice versa. The mechanism of regulatory interactions between epidermal connexins remains to be clarified.

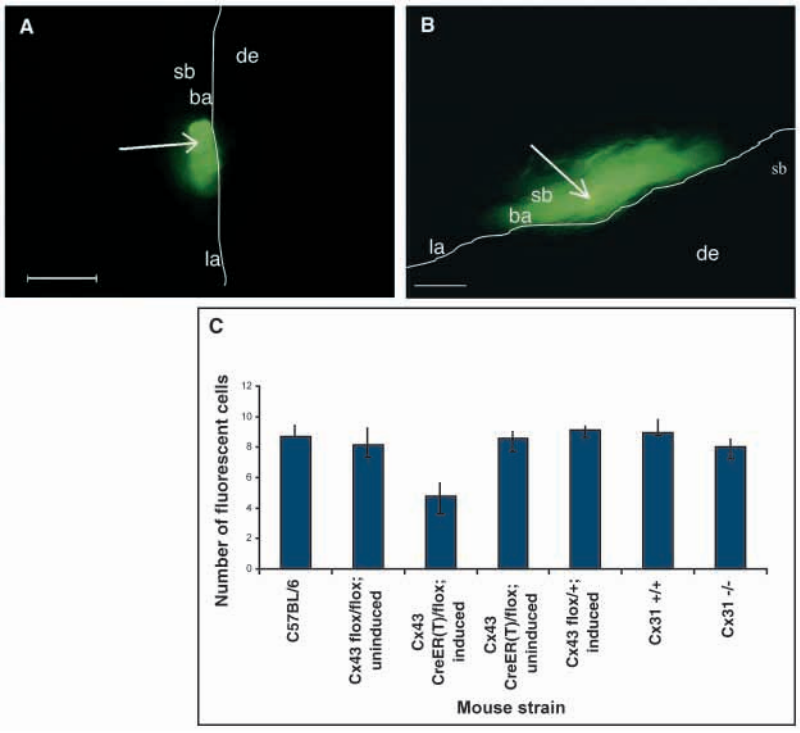

Fig. 10. Microinjection of Alexa Fluor 488 fluorescent dye into single cells in thick sections (250 μm) of ear epidermis. (A) Skin section derived from *Cx43*$^{Cre\text{-}ER(T)/fl}$ mouse after induction with 4-hydroxytamoxifen, photographed under UV light, indicating strongly decreased dye transfer compared with section of wild-type (C57BL/6) mouse (B). Arrows mark the injected cell. Bars, 20 μm. ba, basal layer; de, dermis; la, basal lamina; sb, suprabasal cell layers. (C) Dye transfer in *Cx43*$^{Cre\text{-}ER(T)/fl}$ epidermis was reduced by 40% compared with several controls (P<0.001). The numbers of mice and injections for each experiment are given in Materials and Methods.

In contrast to Cx43 and Cx31 expression, Cx30 and Cx26 proteins could barely be detected in intact epidermis. They responded to wounding with a slight increase in protein on days 1 and 2 postwounding and reached their peak of expression on day 3. At this time the epidermis fused under the coagulum and then the protein level decreased until the expression level of normal epidermis was reached again. Thus, Cx26 and Cx30 both showed the same immediate response to epidermal irritation. Cx26 upregulation has already been reported under hyperproliferative skin conditions (Labarthe et al., 1998). Epidermal wounding results in a loss of the epidermal Ca^{2+}. Premature regeneration of this gradient would complicate the restoration of the epidermal permeability barrier (Menon et al., 1992). The immediate increase of Cx26 and Cx30 expression in all epidermal layers could prevent the premature regeneration of the Ca^{2+} gradient (for Cx26 see Goliger and Paul, 1995). The hypothesis that Cx26 and Cx30 channels are responsible for fast gap junctional intercellular communication at the site of skin irritations is supported by the existence of an alternative, faster trafficking pathway for Cx26 (Martin et al., 2001; Ahmad et al., 2002). Cx26- and Cx30-containing channels could therefore be responsible for the transient formation of a network of cells for exchanging metabolites or ions. Cx30 and Cx26 protein in the epidermis seem to be partially redundant, as Cx30-deficient mice do not show obvious abnormalities in normal and wounded tail epidermis.

Overall, the expression level of Cx43, Cx31, Cx30 and Cx26 proteins was clearly increased on day 3 postwounding when the epidermis of wild-type mice had fused under the coagulum. After this, keratinocyte migration ended and the stratification of the epidermis under the coagulum began. Cx26 and Cx30, which were associated with a fast irritation response, were strongly increased in concentration at day 3. They could provide the required level of coupling for initiating restratification under the coagulum until the normal expression pattern of Cx43 and Cx31 was re-established and could contribute to normal epidermal function. Currently, we cannot explain the drop of Cx26 and Cx30 protein expression on day 4 after wounding. It might be due to a general change of expression in epidermis and dermis. Owing to the fibrin clot, the epidermis could not be dissected from the dermis by dispase treatment. Thus, for immunoblot analyses we used total skin lysates including dermis and epidermis. By contrast, our immunofluorescence analyses represent the epidermal changes of expression in the direct wound region.

There appears to be an extensive functional redundancy between connexins in the mouse epidermis. Analysis of double connexin-deficient mice or simultaneous ablation of several connexins using conditional gene targeting approaches or the use of transdominant connexin mutants might help to dissect this redundancy for the proper function of the skin. Primary cultures of keratinocytes which lack one or more connexins could be useful to analyse the function of gap junction channels between keratinocytes in response to various growth factors.

We are grateful to Dr Pierre Chambon (Illkirch, France) for providing the pCre-ER(T) vector. We thank Joana Fischer and Gaby Matern for genotyping transgenic mouse mutants. This work was supported by grants of the Deutsche Forschungsgemeinschaft (Wi 270/24-4), the Deutsche Krebshilfe, and the Fonds der Chemischen Industrie to K.W.

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