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Dynamic changes in connexin expression correlate with key events in the wound healing process

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Abstract

Wound healing is a complex process requiring communication for the precise co-ordination of different cell types. The role of extracellular communication through growth factors in the wound healing process has been extensively documented, but the role of direct intercellular communication via gap junctions has scarcely been investigated. We have examined the dynamics of gap junction protein (Connexins 26, 30, 31.1 and 43) expression in the murine epidermis and dermis during wound healing, and we show that connexin expression is extremely plastic between 6 hours and 12 days post-wounding. The immediate response (6 h) to wounding is to downregulate all connexins in the epidermis, but thereafter the expression profile of each connexin changes dramatically. Here, we correlate the changing patterns of connexin expression with key events in the wound healing process.

Keywords: Wound healing; Connexin; Gap junctions; Epidermis; Dermis; Skin; Communication

1. Introduction

Cutaneous wound healing is a complex process that requires the co-ordinated efforts of multiple cell types to repair any discontinuity in the skin. The process involves a series of overlapping phases: haemostasis, inflammation, re-epithelialisation, granulation and tissue remodelling. Many of these stages are tightly regulated by a careful balance between cell proliferation and programmed cell death, such that disruption of these processes leads to delayed wound healing and excessive scarring.

An increasing amount of evidence suggests that the precise co-ordination of cell types and events in the wound healing process are brought about by gap junctional communication between cells. These specialised clusters of plasma membrane channels, which are composed of connexin proteins, permit the exchange of ions and metabolites between adjacent cells. Gap junctional communication has been implicated in many events, such as inflammation (Beyer and Steinberg, 1991; Oviedo-orta et al., 2000, 2001), differentiation (Kumar and Gilula, 1996), proliferation (Lucke et al., 1999) migration (Kwak et al., 2001; Oviedo-orta et al., 2002; Pepper et al., 1992) and tissue contraction (Bowman et al., 1998; Ehrlich and Rittenberg, 2000).

In rodents, at least four different connexins are differentially expressed in skin, resulting in an elaborate gap junctional network, compartmentalising the epidermis and dermis (Goliger and Paul, 1995; Risek et al., 1992). In keratinocytes of the epidermis, different connexins are present in single or multiple layers. For example, Connexin43 (Cx43) is localised in the basal cell layer, while Cx26 and Cx31.1 are expressed in the granular and upper spinous layers (Goliger and Paul, 1994; Kamibayashi et al., 1993). In the dermis, single or multiple connexins are expressed in dermal fibroblasts, the vascular system and skin appendages. In the case of hair follicles in the first postnatal hair cycle, high levels of Cx26 and Cx43 are present in specific cell layers, while Cx40 and Cx31 are barely detectable (Guo et al., 1992). These expression profiles suggest that gap junctions composed of different connexins have unique functional roles.

Wounding results in a cascade of events that is accompanied by changes in connexin expression patterns in the skin. Haemostasis occurs immediately after

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injury and involves the constriction of blood vessels at an injury site and the formation of a fibrin clot that releases a cocktail of cytokines that generate an inflammatory response (2 h–7 d post-wounding (pw)). Previous wound healing studies in rodents that have examined the epidermis at limited time points following trauma have shown that Cx43 and Cx31.1 are downregulated in epidermis at the wound site, while Cx26 is elevated in differentiated cells at the same site 24 h after injury (Goliger and Paul, 1995).

Re-epithelialisation, the process of wound closure, overlaps with the timing of inflammation and occurs between 1–4 d pw. In situations where hair follicles remain intact after injury, the stem cells from the follicles (the outer root sheath or ORS cells) often proliferate to aid re-epithelialisation (Jahoda and Reynolds, 2001; Taylor et al., 2002). However, connexin expression patterns in hair follicles have not previously been studied during wound healing. As re-epithelialisation ends, the granulation process begins (between 3–14 d pw) with the introduction of new fibroblasts, blood vessels and contractile myofibroblasts. The final stage of wound healing involves the conversion of wound granulation tissue into a scar, and is called remodelling. This process, which involves apoptosis of cell types that are no longer needed, begins within 2 weeks pw but may last several years. Previous studies have shown that the terminal differentiation of keratinocytes that occurs in wound epidermis is also a form of apoptosis (Polakowska et al., 1994). Interestingly, studies in mouse primary keratinocyte cultures (Brissette et al., 1994) suggest that Cx31.1 expression is induced on terminal differentiation, a program similar to that observed in the upper epidermis in vivo, where growth arrest, stratification and cornification of dead cells occur. Most of the connexins known to be expressed in rodent skin have not been examined in detail during the full time course of the wound healing process. With the ongoing discovery of new connexins, it is possible that other, as yet unidentified, proteins may also be present in mouse skin.

In the present study, we examine the dynamics of connexin expression in mouse neonatal epidermis, dermis and hair follicles during the wound healing process (6 h–12 d pw). We show that the different stages of wounding are accompanied by striking changes in connexin expression in both the epidermis and dermis, and that individual connexins can be correlated with key events in the wound healing process.

2. Methods

2.1. Tissue

Full thickness incisional wounds measuring 3 mm in length were made with a scalpel on the backs of 2-day-old ICR neonatal mice (Halen UK Ltd, Oxford, UK) that had been anaesthetised by cooling on ice. At 6 h, 1 d, 2 d, 4 d, 7 d and 12 d pw time points, animals (six per time point) were sacrificed by a Schedule 1 method, according to Home Office regulations. Wounded skin tissues were excised, and positioned either transversely (ts) or longitudinally (ls) (to examine longitudinal sections of hair follicles; Paus et al., 1999) in O.C.T and fast frozen in liquid N_2. Serial sections of 10 μm thickness were cut and mounted on to poly-L-lysine coated slides (BDH) before treatment with acetone (10 min, RT) to permeabilise and fix skin tissues. Cryosections were processed for wax staining, to examine the morphology of wounds during wound healing, or for immunostaining, to examine the changes in connexin expression and proliferation during the wound healing process.

2.2. Immunostaining

Double-label immunohistochemistry was performed with one of the following polyclonal connexin antibodies: Cx26 (Monaghan et al., 1994) (1:200), Cx30 (Zymed, 1:1000), Cx31.1 (Wright et al., 2001) (1:10,000) or Cx43 (Becker et al., 1995) (1:100), and the monoclonal antibody to the cell proliferation marker, Ki67 (DAKO, 1:25), to correlate changes in connexin expression with proliferation during wound healing. Most of these antibodies, with the exception of Cx30 and Cx31.1, were raised against short peptide sequences of connexins, as described in Becker et al. (1995), while Cx31.1 (Wright et al., 2001) was the kind gift of Professor Colin Green (University of Auckland). In general, slides were incubated overnight at 4 °C in primary antibodies diluted in PBS. Slides were subsequently washed three times in PBS (5 min each) and incubated for 2 h at RT in a mixture of CY3 goat anti-rabbit and Alexa 488 goat anti-rat secondary antibodies (Molecular Probes, Oregon, USA), both diluted to 1:200 in PBS. Slides were then washed in PBS (3 × 5 min), counterstained with Hoescht (1:50,000 for 10 min) and washed again, prior to mounting with Citifluor (Chem. Lab, Canterbury, UK) and coverslips. The specificity of all four connexins was checked by comparing staining patterns without the primary antibody (negative control), and staining a variety of tissues known to express high levels of each connexin (positive control), as described in Wright et al. (2001).

All the wounds examined in this study were essentially symmetrical about the long axis of the wound, with regard to morphology and connexin staining. Staining was examined on a Leica SP UV confocal microscope. A 488 laser line was used to excite Alexa 488 and a 543 laser line to excite CY3. A Z series of images (4 μm deep) was taken at intervals of 0.5 μm. All parameters of laser power, pinhole, PMT settings were

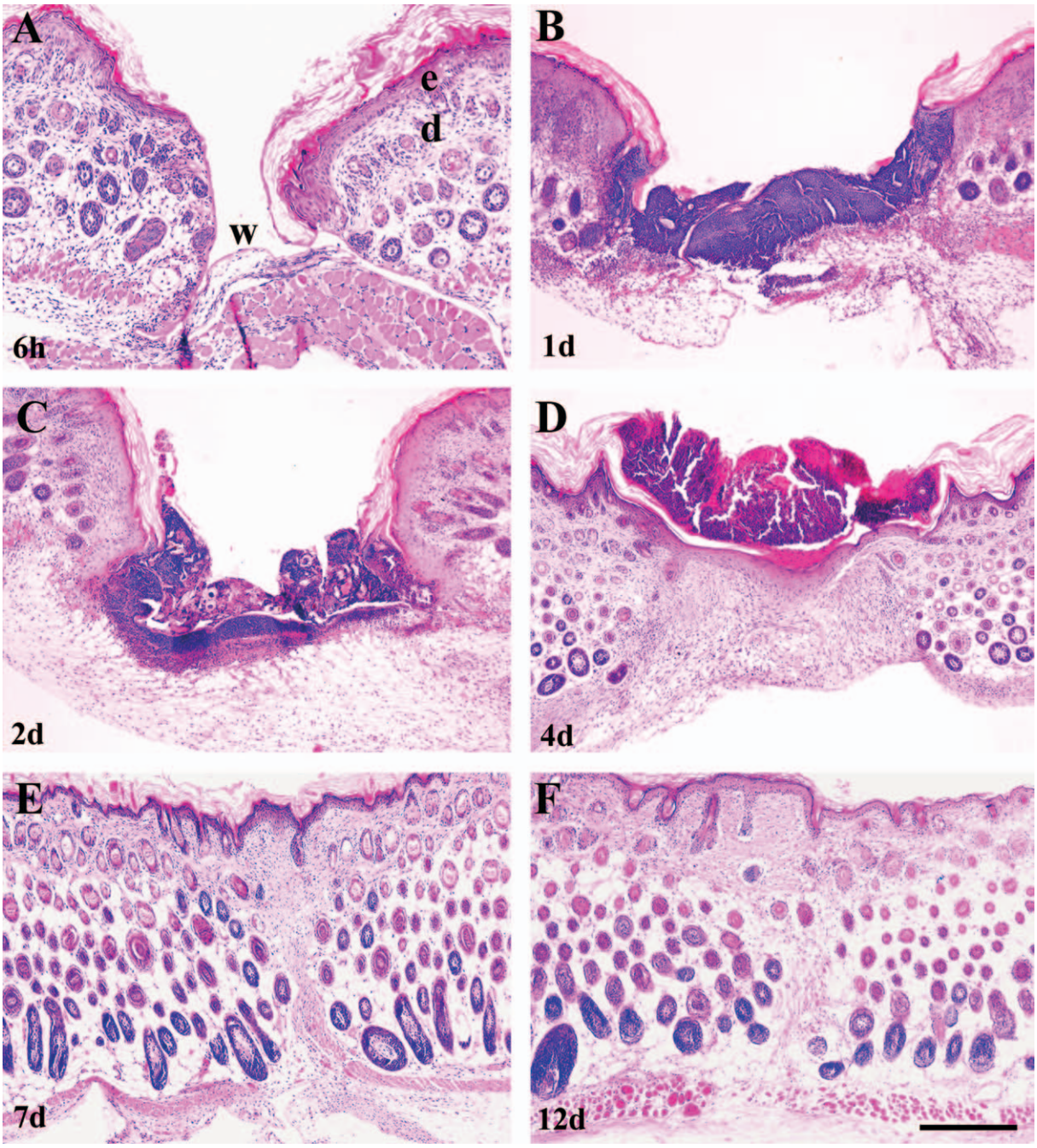
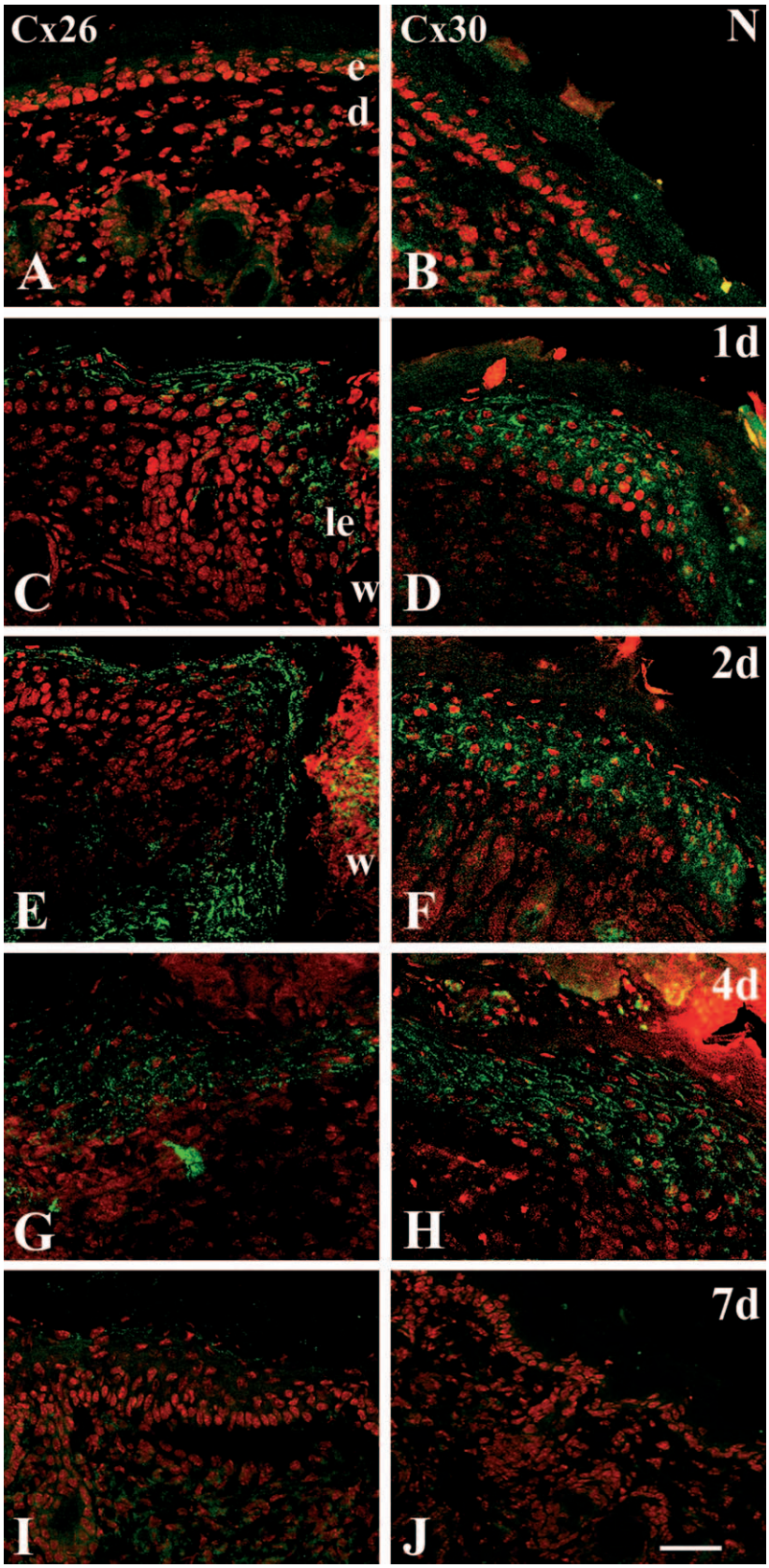


Fig. 1. Haematoxylin and Eosin stained wax sections through wounded tissue at 6 h (a), 1 d (b), 2 d (c), 4 d (d), 7 d (e) and 12 d (f) post-wounding. The wound site, leading edge and granulation tissue are indicated. Scale=200 μm.

kept constant at sessions where comparisons were being made. The 3D data set was projected into a single 2D image, collected and stored digitally for subsequent analysis. Images were finally imported into Photoshop 6.0 for reproduction. Where quantitation of immunohistochemistry was required, raw data files were imported into Image J. The number and areas of gap junction plaques were measured in demarcated blood vessels from at least three animals at each time point. Similarly, numbers and areas of gap junction plaques were compared in control and 7 d wound epidermal regions spanning 150 μm in length. In both tissue types, one threshold was applied to all blood vessel images and another to all epidermal regions, to enable a direct comparison of expression levels with controls.

To compare levels of proliferation during the wound healing process, numbers of Ki67 positive cells were counted in three regions of injured skin (from 1 d–12 d pw), 350 μm wide. Epidermal regions examined included the wound site, the region adjacent to the wound, and a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region far from it. Blind counts were performed by three individuals and data pooled. Counts of proliferating cells in hair follicles were not performed, as variations in the stage of hair follicle cycling were apparent.

3. Results

3.1. Wound morphology

H&E staining of sections through wounded tissue from 6 h–12 d is shown in Fig. 1. At 6 h–1 d pw, the wound appears as a discontinuity in the epidermis. However, by 2 d pw, keratinocytes at wound edges appear to be actively migrating under the scab, towards one another. This migration, which facilitates re-epithelialisation or wound closure, is complete at 4 d pw, with a newly formed epidermis beneath the dead scab tissue. By 7–12 d after injury, the location of the wound site is marked by a vertical strip of granulation tissue through the dermis, an absence of hair follicles and a break in the panniculus carnosus muscle layer beneath the dermis.

3.2. Connexin expression in the epidermis

In the normal unwounded epidermis, Cx26 and Cx30 are present at extremely low levels and separate compartments of the differentiating granular layer (Fig. 2A and B), while Cx31.1 is detected at low levels in the granular layer (Fig. 3A), and Cx43 at moderate levels in the proliferating basal layer (Fig. 3B). On wounding, both the amount and location of connexin expression in mouse neonatal skin changes (Figs. 2 and 3). In general, the temporal and spatial expression patterns of Cx26 and Cx30 are similar, but very different to those of Cx31.1 and Cx43.

At 6 h pw, Cx26 and Cx30 levels at the wound leading edge and in immediately adjacent regions dramatically decrease. By 1d pw, levels of Cx26 and Cx30 remain downregulated at the wound leading edge, but are greatly upregulated through many epidermal cell layers in the region behind the wound edge, and in regions up to 500 μm from the wound site (Fig. 2C and D). This upregulation response is slightly greater for Cx30, with more intense labelling in the spinous layer. At 2 d pw, the time of re-epithelialisation, levels of Cx26 and Cx30 are still elevated greatly above normal (Fig. 2E and F). By 4 d pw, the period of granulation tissue formation and remodelling, and upregulation of Cx26 and Cx30 is restricted to the region around the wound site, closely following the pattern of re-epithelialisation (Fig. 2G and H). By 7–12 d pw, when terminal differentiation and cell death occur during the remodelling phase, Cx26 and Cx30 are expressed at very low levels in the region of the wound, as in normal unwounded skin (Fig. 2I and J).

Like Cx26 and Cx30, Cx43 and Cx31.1 decrease at the wound edge by 6 h pw. By 1 d pw, Cx31.1 and Cx43 are both downregulated at sites of injury compared to normal (Fig. 3A–D). However, in regions some distance from the wound, Cx43 continues to be downregulated while Cx31.1 remains at normal levels in the granular layer (data not shown). At 2 d pw, expression of Cx43 protein in the basal layer appears to be returning to normal, while Cx31.1 levels in the granular layer at the wound site remain low (Fig. 3E and F). In contrast to very low Cx31.1 expression levels at the wound site at 4d pw (Fig. 3G), Cx43 levels increase dramatically and spread through many epidermal layers at the wound site and in adjacent regions (Fig. 3H), where cells are proliferating. Interestingly, at 7 d pw, Cx31.1 staining increases in intensity, particularly in the granular and spinous layers around the wound region where terminal differentiation occurs (Fig. 3I). Comparisons of epidermal staining in control and 7 d wound sections show a 10-fold increase in the number of Cx31.1 gap junction plaques and a 1.2-fold increase in the average size of plaques in the epidermis of wounded tissue. At this time, the pattern of Cx43 expression in the basal epidermal layer returns to nearly normal pre-wounding conditions (Fig. 3J), and the intensity and location of Cx31.1 expression also appears to return to near normal by 12 d pw. A summary of connexin expression patterns in the epidermis is shown in Table 1.

3.3. Connexin expression in hair follicles of the dermis

In the normal neonatal dermis, hair follicles in various stages of the hair follicle cycle express varying amounts of connexin protein. On wounding, the amount of connexins in hair follicles changes, as the follicles de-differentiate and migrate toward the basal epidermis to aid re-epithelialisation.

Fig. 2. Immunolocalisation of Cx26 and Cx30 (green) in normal and wounded mouse neonatal skin counterstained with Hoescht for cell nuclei (red) (A–J). In all images, the wound site (w) is located on the right hand side and the epidermis (e), dermis (d) and wound leading edge (le) are indicated. Cx26 and Cx30 are hardly detected in normal unwounded skin (A and B, respectively). One day after wounding, Cx26 and Cx30 are both upregulated at the wound leading edge (C and D). Expression levels remain upregulated at 2 d (E and F) and 4 d pw (G and H), but have returned to normal by 7 d pw (I and J). Scale=50 μm.

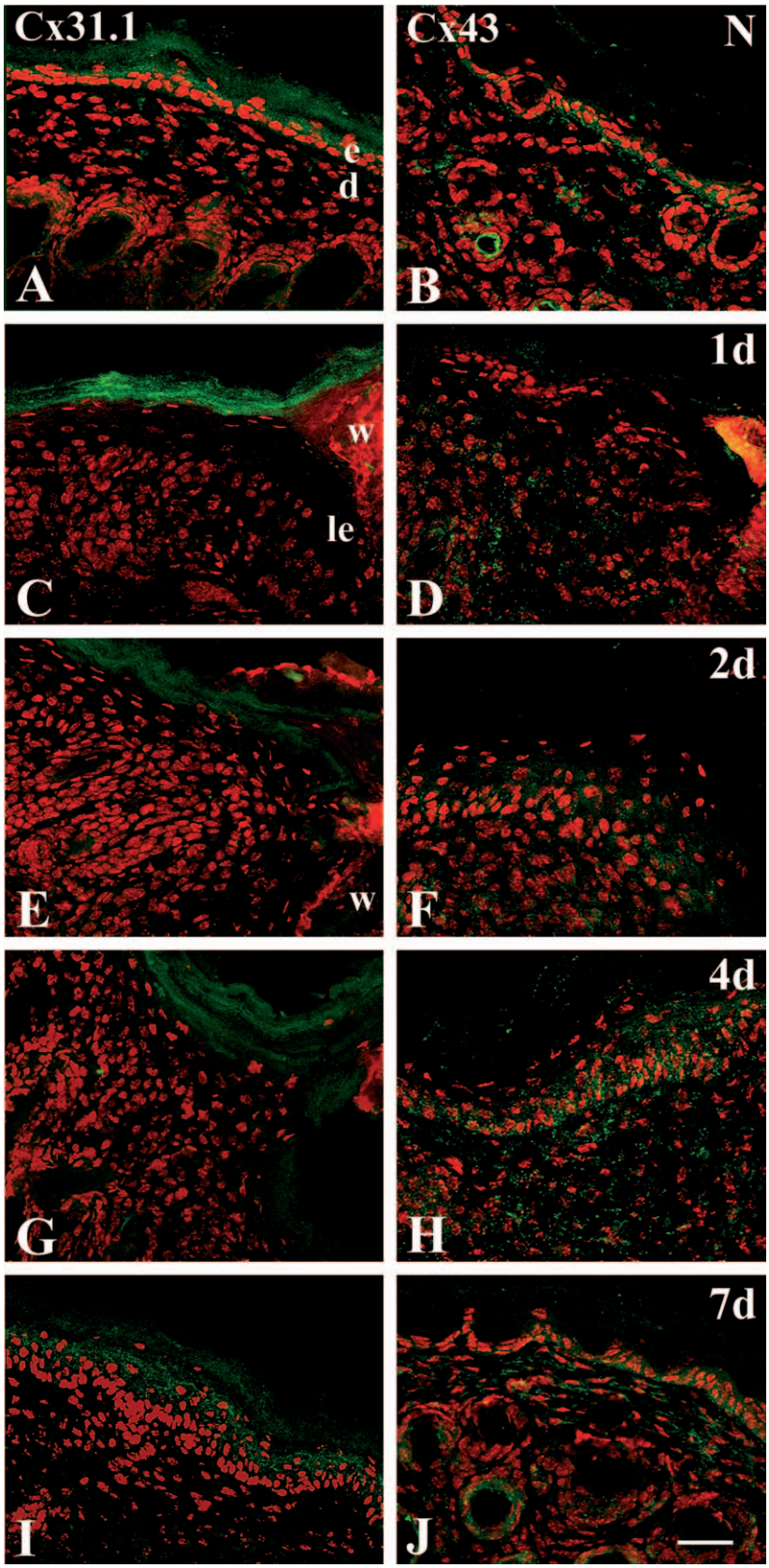
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Table 1
A summary of connexin expression in layers of injured epidermis (granular, upper spinous, lower spinous and basal) and dermis (bv-blood vessels, hf-hair follicles, ors-outer root sheath, irs-inner root sheath, md-medulla). Immunolabelling was scored as follows: −, no labelling; −/+, very weak labelling; +, weak labelling; ++, moderate levels of labelling; +++, high density of labelling. All connexins initially respond to injury by decreasing expression levels, but Cx26 and Cx30 are upregulated soon after the injury (1–2 d), while Cx43 is dramatically upregulated later (4 d), and Cx31.1 during terminal differentiation (7–12 d).

	Cx26					Cx30					Cx31.1					Cx43				
	N	1 d	2 d	4 d	7 d	N	1 d	2 d	4 d	7 d	N	1 d	2 d	4 d	7 d	N	1 d	2 d	4 d	7 d
Epidermis																				
Granular	−/+	++	+++	+	−/+	−/+	++	+++	+	−	+	−	−	−	++	−	−	−	−	−
U. Spinous	−	+	++	+	−	−	+	+++	++	−	−	−	−	−	+	−	−	−	+	−
L. Spinous	−	−	++	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−/+	++	+
Basal	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	++	−	+	+++	++
Dermis																				
BV	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+++	+	++	+
HF ors	+++	+	++	+++	+++	+	+	+	+	+	−/+	−/+	−/+	−/+	+++	++	+	+	++	++
HF irs	+++	−	++	+++	+++	+	+	+	+	+	−	−	−	−	++	+	−	+	+	+
HF md	++	−	++	+++	+++	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−

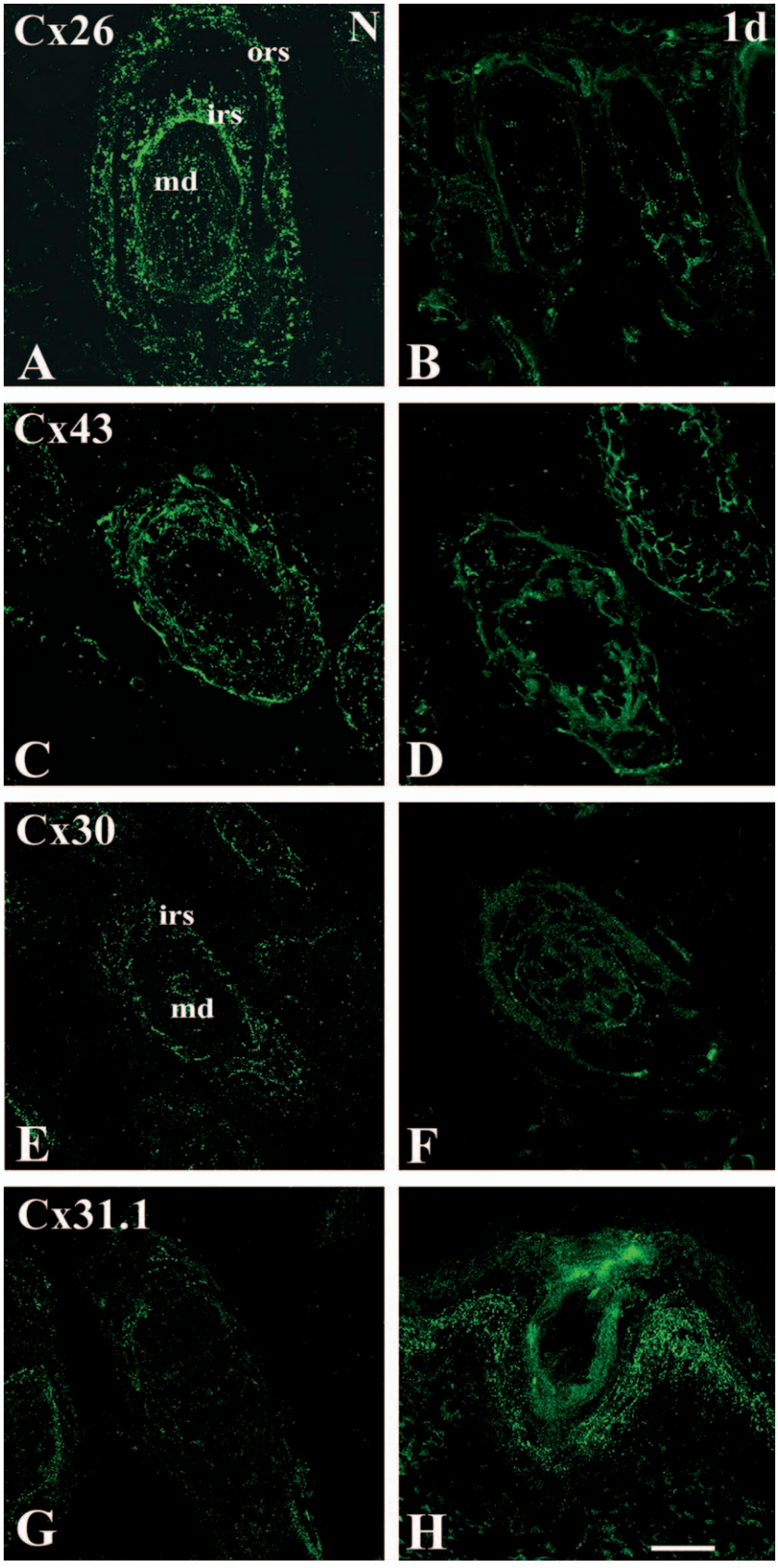
NB. Only as hair follicles incorporate into the basal epidermal layer do they upregulate Cx31.1 levels. The value assigned for hair follicles at 7 d pw in the table above is for hair follicles actively incorporating only rather than all hair follicles, which have very low, sporadic Cx31.1 expression levels.

Soon after wounding (6 h–1 d), hair follicles at the wound site and those migrating towards the wound leading edge exhibit little or no Cx26, in contrast to follicles in adjacent regions of dermis and control skin (Fig. 4A and B). This rapid downregulation of Cx26 in all cell layers of the hair follicle (ors; outer root sheath, irs; inner root sheath, md; medulla; Fig. 4B) occurs in the region where Cx26 is upregulated in the overlying epidermis. Outside the region of upregulation, normal levels of Cx26 are detected in other appendages. The expression profile of Cx43 in the dermis is similar to that of Cx26 dermal expression during wound healing, such that hair follicles around the wound have reduced Cx43 levels in the ors and irs cell layers (Fig. 4C) compared to follicles in control skin (Fig. 4D). While Cx30 is already expressed at very low levels in normal hair follicles, a slight reduction in expression levels is still detected at 1 d after injury compared to controls (Fig. 4E and F). After the initial early decline in Cx levels in hair follicles near the wound, expression appears to increase to control levels, and change little thereafter. Like Cx30, Cx31.1 is expressed at very low levels in the hair follicles of normal skin (Fig. 4G). However, at 7–12 d pw, there is a dramatic upregulation of Cx31.1 levels in suprabasal epidermis around hair follicles being incorporated into the epidermis (Fig. 4H).

3.4. Connexin expression in blood vessels of the dermis

Cx43 is the only connexin of those tested to be expressed in the blood vessels of skin. In normal blood vessels, only low levels of Cx43 are detected (Fig. 5A), but it is upregulated in blood vessels near a wound site within hours of injury (Fig. 5B). However, this upregulation response is short-lived, as Cx43 levels decline significantly by 2 d pw (Fig. 5C). There is a second increase at 4 d pw (Fig. 5D) as granulation tissue formation starts, but it declines again thereafter (Fig. 5D–F) and returns to normal by 12 d pw (Fig. 5F). A summary of connexin expression patterns in the dermis can be seen in Table 1. Quantitation of these results in Image J software indicates there is a change in the number and area of gap junction plaques in blood vessels after injury (Fig. 5G and H). At 6 h pw, there is a 3-fold increase in the number of gap junction plaques compared to controls. Thereafter, the number and area of plaques declines at 2 d, but again increases 2 fold at 4–7 d pw.

Fig. 3. Immunolocalisation of Cx31.1 and Cx43 (green) in normal and wounded mouse neonatal skin counterstained with Hoescht (red) (A–J). In all images, the wound site (w) is located on the right hand side and the epidermis (e), dermis (d) and wound leading edge (le) are indicated. (N.B. The cornified layer of skin often has high levels of autofluorescence associated with immunolabelling procedures.) Cx31.1 and Cx43 are present at low levels in normal unwounded skin (A and B, respectively). Cx31.1 and Cx43 are both downregulated 1d after wounding (C and D). While Cx31.1 levels remain downregulated until 7 d pw (C, E and G), Cx43 levels increase at 2 d (F) and upregulates significantly at 4 d pw (H). By 7 d pw, Cx43 levels have returned to normal (J), yet Cx31.1 upregulates dramatically (I). Scale=50 μm.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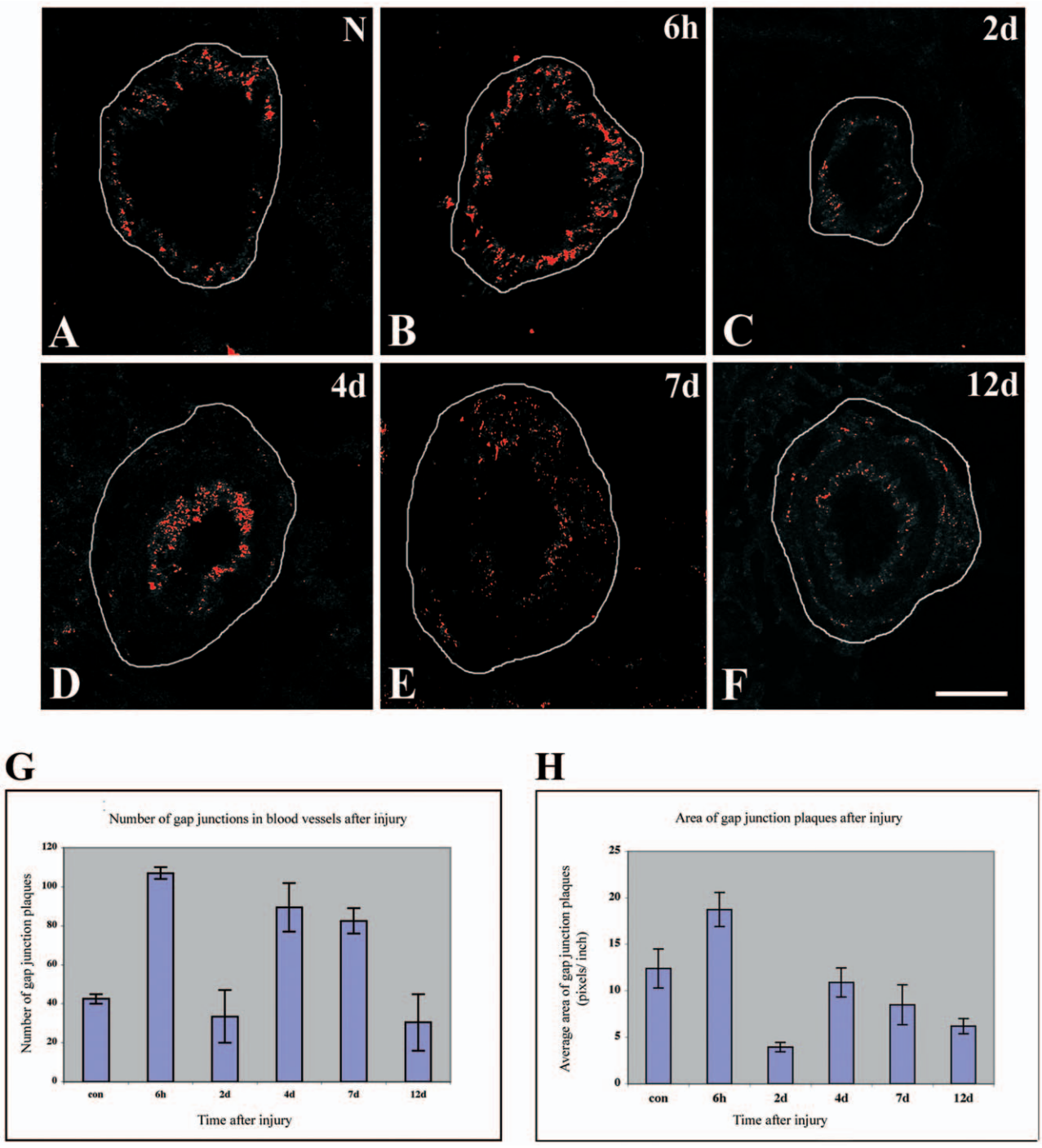


Fig. 5. Cx43 expression in blood vessels of normal and wounded skin. Cx43 is expressed at low levels in normal blood vessels (A), but after injury, Cx43 expression upregulates dramatically at 6 h pw in the dilated blood vessels near a wound site (B). Cx43 levels transiently decline at 2 d pw (C), but increase again at 4 d pw (D), then levels in blood vessels gradually decrease (E–F). Red indicates thresholded images. Semi-quantitation of Cx43 plaque numbers and areas in blood vessels are indicated in histograms in G and H, respectively. Scale=100 μm.

Fig. 4. Cx26, Cx30, Cx31.1 and Cx43 expression in hair follicles of normal (A, C, E and G) and injured skin (B, D, F and H). On wounding, hair follicles in the injured dermis express less Cx26 (B) Cx43 (D) and Cx30 (F), compared to controls (A, C and E, respectively). In contrast to these connexins, Cx31.1 is dramatically upregulated in hair follicles incorporating into the epidermis at 7 d pw (H), in contrast to control follicles (G). Scale bar=50 μm.

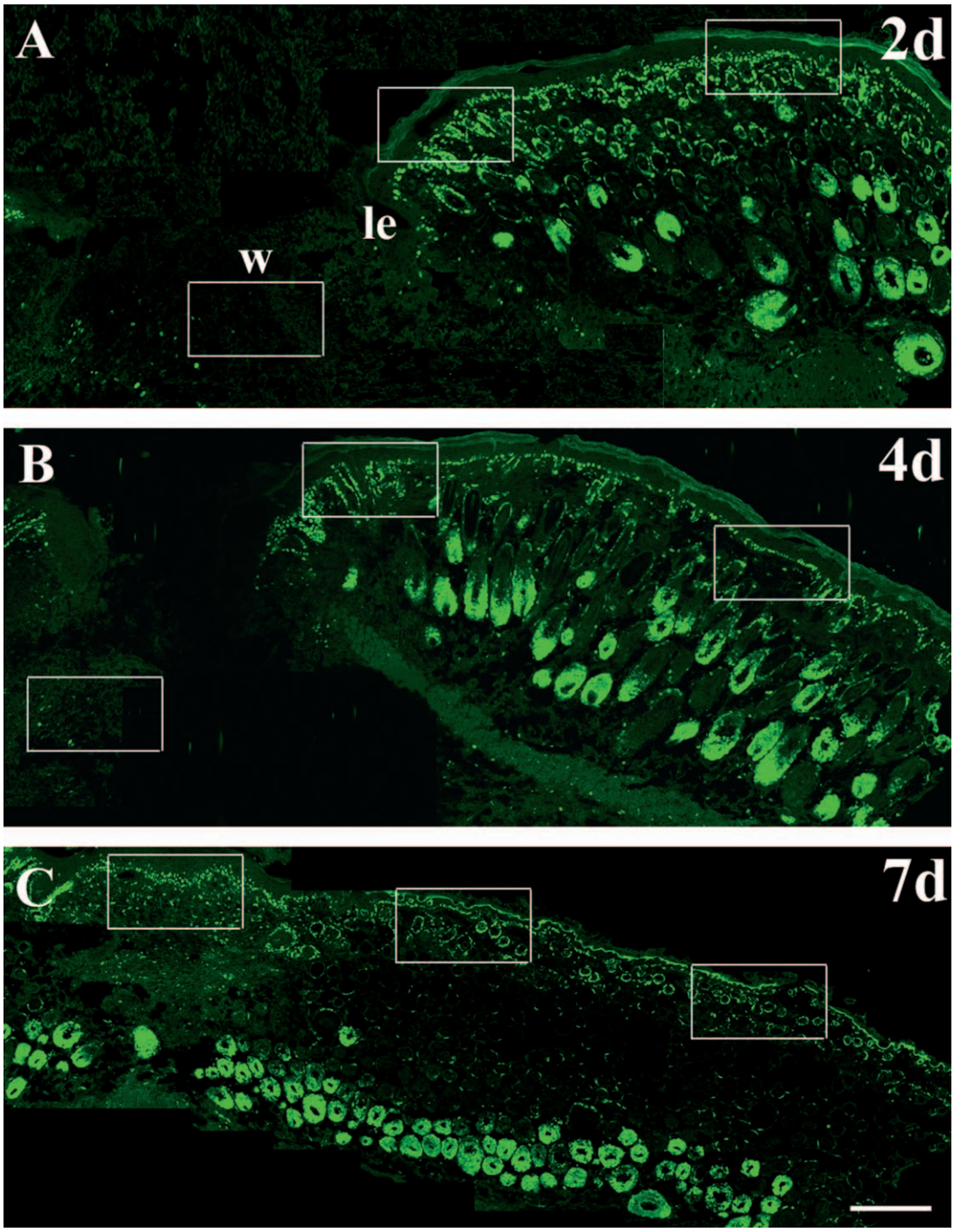


Fig. 6. Cell proliferation in the injured skin. Low magnification montages showing Ki67 labelling in injured skin at 2 d (A), 4 d (B) and 7 d (C) after injury. The wound site (w) is located on the left hand side and the migrating wound leading edge (le) is indicated. Numbers of Ki67 positive cell nuclei were counted in 3 regions of injured skin (indicated by white boxes): the wound site, the epidermis adjacent to the wound and the epidermis far from the wound. Ki67 labelling peaks in the wound epidermis at 2 d pw. Scale bar for montages =200 μm.

3.5. Ki67 expression in the epidermis

In normal skin, low levels of Ki67 positive cells are scattered in the proliferating basal layer of the epidermis. On wounding, their number and distribution changes (Figs. 6 and 7). Numbers of proliferating cells have been measured in three regions of the epidermis: the wound site, adjacent skin and distantly located skin, between 1 d and 12 d pw. In general, the results show that proliferation increases in the region behind the migrating wound leading edge during the early stages of healing, in order to aid the process of re-epithelialisation. However, proliferation increases at the wound site during the remodelling phase.

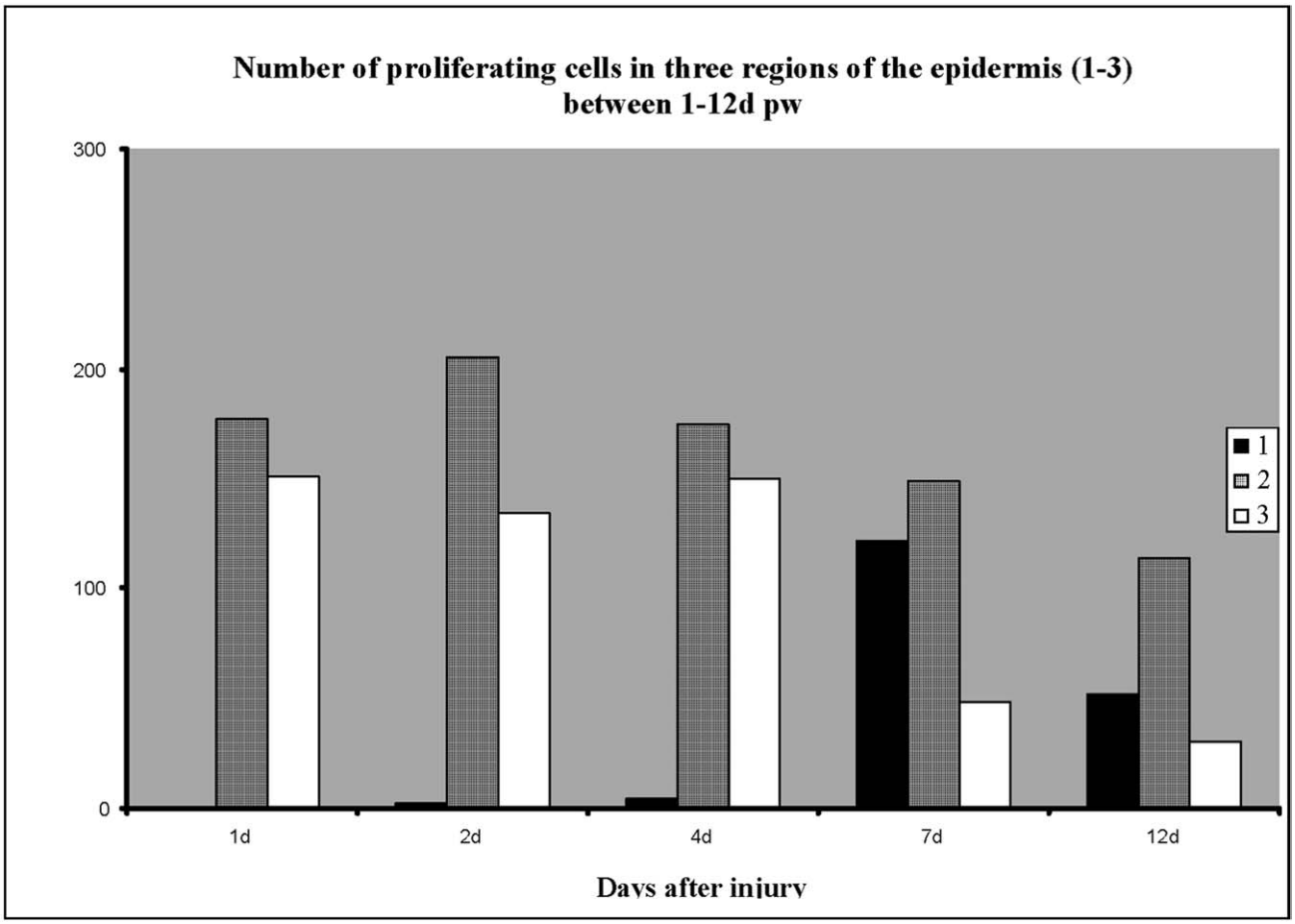


Fig. 7. Bar charts showing the numbers of proliferating cells in three regions of the wounded epidermis from 1–12 d pw. The three regions examined are: the wound site (1, black), the region adjacent to the wound (2, grey) and the region far from the wound site (3, white). Bar charts represent numbers of proliferating cells counted in three regions of epidermis spanning a 350 μm region, in sections from two animals per time point.

At 1 d after injury, no proliferating cells are detected in epidermis at the wound site (Fig. 7). However, high numbers of Ki67 positive cells are detected in regions adjacent to, and far from, the wound site (Fig. 7). At 2 d pw, numbers of Ki67 positive cells peak in the epidermal region behind the migrating wound leading edge (Fig. 6A and Fig. 7). However, by 4 d pw, when re-epithelialisation is usually complete, the numbers of proliferating cells in the regions behind the migrating zone decrease (Fig. 6B and Fig. 7), compared to earlier time points. This decline continues from 7–12 d pw (Fig. 6C and Fig. 7), while numbers of proliferating cells increase at the wound site at 7 d pw (Fig. 6C and Fig. 7).

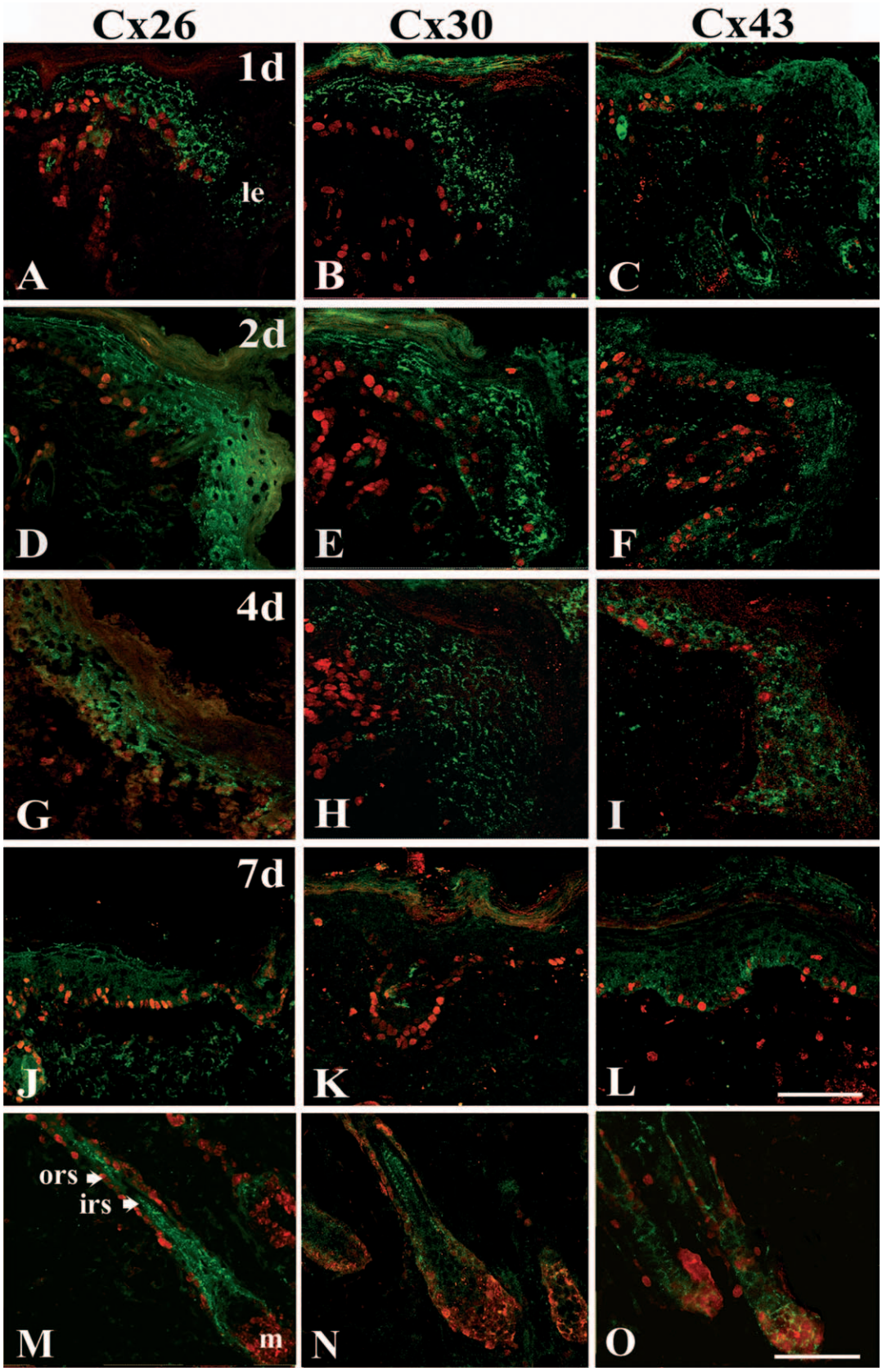
3.6. Ki67 expression in the dermis

In normal skin, Ki67 positive cells are present at moderate levels in ors cells and at high levels in the hair matrix of hair follicles. After injury, the distribution of Ki67 positive cells in the dermis appears to change (Fig. 6). In general, during the early stages of wound healing, numbers of proliferating cells decline in hair follicles near the wound site, compared to follicles in adjacent dermal regions. However, at later time points, numbers of proliferative cells increase in wound granulation tissue, as active remodelling occurs.

Between 1 and 4 d pw, low numbers of proliferating cells are detected in hair follicles at the wound site, but very high numbers are seen in dermal hair follicles outside the wounded region (Fig. 6). The majority of Ki67 labelling is found at the base of hair follicles in the bulb region, while moderate labelling is detected in the ors cells (Fig. 6). The peak of proliferative activity in the dermis, however, appears to occur in the wound site at 7 d pw, when remodelling occurs. Subsequently, numbers of proliferating cells appear to decrease in the wound site and also in dermal regions outside the wound (data not shown).

3.7. Correlation of proliferation marker Ki67 and connexin in the epidermis

In normal skin, Cx43 is the only connexin that co-localises with Ki67 positive proliferating cells in the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basal cell layer. However, this pattern of expression changes after injury (Fig. 8A–L). In general, Cx26 and Cx30 have a negative correlation with proliferating cells in the zone behind the wound leading edge, although a small degree of overlap between Ki67 and Cx expression is detected at the wound leading edge. Cx31.1 also has a negative correlation with proliferation and is never detected in proliferating cells, not even those at the wound edge (data not shown). In contrast, Cx43 has a positive correlation with proliferating cells, particularly in the later stages of wound healing.

One day after injury, Ki67 positive cells are detected in the basal epidermal layer behind the migrating wound leading edge. Double labelling studies indicate that a few of these Ki67 positive cells at the wound edge also express Cx26 and, to a lesser extent, Cx30 at their cell–cell interfaces (Fig. 8A and B). However, the majority of Ki67 positive cells in the region behind the migrating leading edge are separated from Cx26 and Cx30 proteins, which are confined to more differentiated layers above the basal layer (Fig. 8A and B). This negative correlation between Cx26 and Cx30 with Ki67 expression is exaggerated by 2 d pw, when peak levels of Ki67 are detected in the region behind the migrating wound epidermis and connexins are upregulated in the migrating wound edge (Fig. 8D and E). From the time of re-epithelialisation (4 d pw) onwards, there is a clear negative correlation between Cx26 and Cx30 expression and Ki67 positive nuclei (Fig. 8G and H, J and K).

Decrease in basal layer Cx43 expression at the wound site during the early stages of injury shows little co-localisation with Ki67 (Fig. 8C). By the time some patchy Cx43 staining has returned to the region behind the migrating leading edge (2 d pw), co-localisation with ki67 is just apparent (Fig. 8F). This positive correlation between Cx43 and Ki67 continues when the former is upregulated at the wound leading edge and in adjacent skin at 4 d pw (Fig. 8I). However, the correlation is more apparent in the region behind the leading edge than at the wound site itself. By 7–12 d pw, Cx43 becomes restricted to its normal basal cell layer compartment, where Ki67 labelled cells are predominantly found (Fig. 8L).

3.8. Correlation of proliferation marker Ki67 and connexin in the dermis

In the normal dermis, the apical portions of hair follicles have moderate levels of Ki67, but low levels of Cx26 and Cx43, and very low levels of Cx30. However, as the depth of the dermis is traversed, the basal portions of hair follicles, i.e. the hair bulb, appear to express a little more connexin and more Ki67 (Fig. 8M–O). Regions of follicles that are Ki67 positive tend to be Cx26 negative (Fig. 8M), suggesting an inverse relationship between Cx26 expression and proliferation. In contrast, regions of follicles that are positive for Cx30 (Fig. 8N) or Cx43 (Fig. 8O) have a positive correlation with cell proliferation.

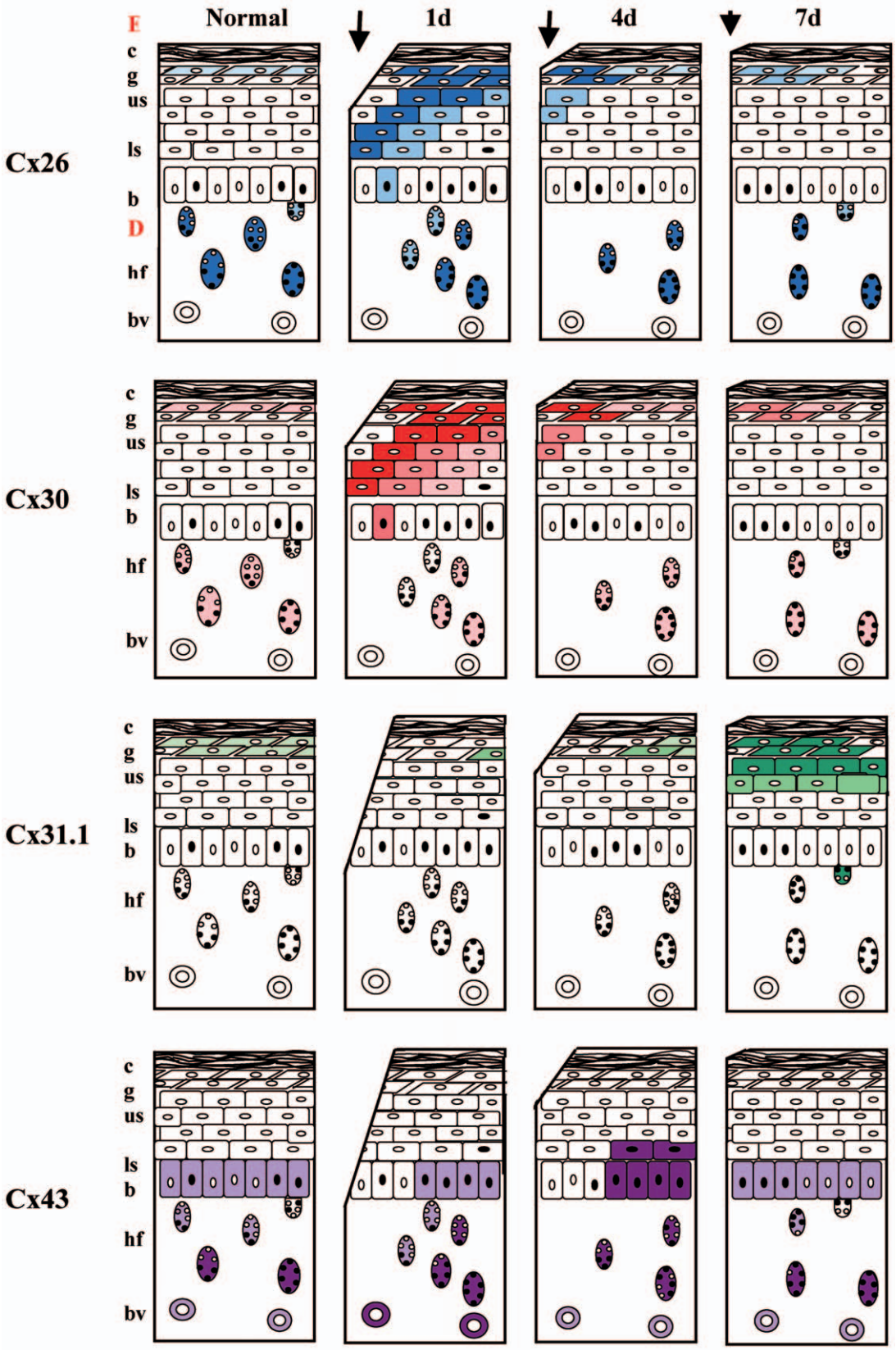
After wounding, hair follicles at the wound site express little Cx26 or Cx43, but high levels of Ki67. However, in dermal regions far from the wound site, hair follicles express abundant Ki67, moderate levels of Cx26 and Cx43, and low levels of Cx30, as in normal, unwounded skin. Although differences in connexin and Ki67 expression are detected in hair follicles within the first day after injury, in general the expression patterns in dermal hair follicles change little during the course of healing.

Whilst most of the connexins studied showed some differences in expression during the proliferative phase of wound healing (Fig. 8A–L and Fig. 9), Cx31.1 instead showed differences during the period of cell death or terminal differentiation (7–12 d pw). This is because levels of Cx31.1 increase dramatically at 7 d pw, particularly in the epidermal regions around hair follicles that have contributed to the re-epithelialisation process (Fig. 9).

4. Discussion

In this study, we show that the expression patterns of four gap junction proteins change dramatically in the epidermis and dermis in response to wounding, and we correlate these changes with key stages in the wound healing process: migration, proliferation and differentiation. Wound healing is a complex process that requires the combined efforts of numerous cell types, many of which communicate with one another via gap junctions. The key stages are inflammation, which involves migration of inflammatory cell types to the injury site, re-epithelialisation, which involves the migration of leading edges to close the wound (a process supported by proliferation), granulation tissue formation, which includes differentiation and proliferation, and tissue remodelling, which involves contraction.

Fig. 8. Correlation between connexin expression and cell proliferation. Double labelled immunostaining of wounded mouse neonatal skin (at 1, 2, 4 and 7 d pw) (A–L) and dermal hair follicles (hf) of normal skin (M–O) with either Cx26, Cx30 or Cx43 (green) and the cell proliferation marker, Ki67 (red). The wound site is always located on the right hand side of each image (A–L). In the epidermis, Cx26 and Cx30 have a negative correlation with proliferation in the migrating wound leading edge (le) (2 d pw), while Cx43 has a positive correlation with proliferation from 4 d pw onwards (A–L). In the dermis, there appears to be a negative correlation between Cx26 expression and proliferation (M). In the case of Cx30 (N) and Cx43 (O), a positive correlation with proliferation is detected in the ors cells, but a negative correlation in the irs cells. ors=outer root sheath cells, irs=inner root sheath cells. Scale bar in A–L=50 μm, bar in M–O=100 μm.


Normal
1d
4d
7d
Cx26
Cx30
Cx31.1
Cx43
E
c
g
us
ls
b
D
hf
bv

We find that in normal, uninjured skin, Cx26 and Cx31.1 are detected at low levels in the upper differentiating (granular and upper spinous) layers of the epidermis, while Cx43 protein is expressed at slightly higher levels in the lower proliferating (basal) layer of normal skin epidermis. These results are consistent with the findings of previous rodent skin studies (Goliger and Paul, 1994, 1995). In this study, we also document the expression pattern of another connexin isotype, Cx30, which has not been previously described. Like Cx26, Cx30 is expressed at low levels in the upper differentiating layers of the mouse epidermis. After injury, the expression and distribution of all connexins changes according to the events taking place.

After an initial downregulation response from all connexins at the wound leading edge, both Cx26 and Cx30 are dramatically upregulated at 1–2 d pw and expressed beyond their normal cellular compartments, particularly in the migrating wound leading edge. This increased connexin protein expression, particularly at 2 d pw, may be necessary for the synchronised movement of wound leading edge keratinocytes during migration. Double labelling studies with connexin antibodies and Ki67 show that, in regions where Cx26 and Cx30 expression starts to decline, Ki67 expression begins. Thus, through most of the migrating zone, there is a negative correlation between proliferation and Cx26 or Cx30. This inverse relationship between connexin expression and proliferation has been detected recently in hyper-proliferative skin conditions such as psoriasis and warts (Di et al., 2001; Labarthe et al., 1998; Lucke et al., 1999). Further evidence to support the negative role of Cx26 in proliferation comes from studies which suggest that Cx26 may function as an anti-proliferative tumour suppressor (Mesnil et al., 1997), as it is upregulated during benign epithelial hyper-proliferation, but decreases during tumour progression.

In contrast to the early upregulation of Cx26 soon after injury, Cx43 levels in the proliferating epidermal layer remain low until days 2–4. The early downregulation of Cx43 in the non-proliferative wound leading edge after injury may be related to the migration of the two wound edges. This migration is thought to occur via proliferation of keratinocytes behind the migrating leading edge cells. In this study, we confirmed this concept, showing that Ki67 and Cx43 labelling were absent at the wound leading edge, but present in the proliferative zone behind the wound leading edge. By 4 d pw, after re-epithelialisation is complete, Cx43 levels in the epidermis and dermis dramatically upregulate, as the onset of tissue remodelling and active proliferation in the granulation tissue of the dermis begins. These findings demonstrate a positive correlation between Cx43 expression and cell proliferation. This profile of strong Cx43 expression in proliferating cells has been observed previously in skin cultures (Lampe et al., 1998) and in the developing retina (Becker and Mobbs, 1999). By 7–12 d pw, when the proliferative phase of epidermal wound repair is over, the epidermis resumes its original thin appearance and Cx43 in the basal epidermal layer declines to pre-wounding levels.

In this study, we also showed for the first time that the intimate relationship between connexin expression and proliferation observed in the epidermis extends down to the dermis. After wounding, hair follicles near a wound are ki67 positive and connexin negative, while those follicles in adjacent and deep portions of the dermis express more connexin and very high levels of Ki67, as in normal, unwounded skin. These observations suggest that hair follicles may lose their Cx26 and Cx43 gap junctions as they migrate and proliferate to assist the epidermal wound healing process. However, hair follicles in both normal and wounded skin did share a common feature-an inverse correlation between connexin expression and cell proliferation. Another interesting observation was that neighbouring hair follicles in the dermis express varying amounts of Ki67 and connexin. This may simply be the result of hair follicles being at different stages of the postnatal follicle cycle, or may indicate that hair follicles in normal and wounded skin are in a state of flux. The dynamics of connexin and Ki67 expression in hair follicles are certainly complex, and require further study to distinguish the changes that occur following wounding from the normal pattern of events during follicle development.

Cx43 is expressed in multiple cell types in the skin and is involved in many stages of tissue repair. It has been shown that Cx43 is expressed on the surface of activated immune cells (Oviedo-orta et al., 2000, 2001) that mediate the inflammatory response, in blood vessels (Chaytor et al., 1998) that carry inflammatory cells to wound sites, and on fibroblasts (Gabbiani et al., 1978; Salomon et al., 1988) that play a critical role in collagen contraction (Ehrlich and Rittenberg, 2000) during granulation tissue

Fig. 9. Summary diagram showing the changes in connexin expression and cell proliferation in the epidermis (E) and dermis (D) following injury. Distribution of Cx26 (blue), Cx30 (red), Cx31.1 (green), Cx43 (purple) and $Ki67^{+}$ (black nuclei) cells in the normal and wounded epidermis and dermis are indicated. Three shades of each colour are used to indicate levels of expression, with the darker shades indicating high levels of expression. Expression of connexins in hair follicles is also indicated. In general, Cx26, Cx30 and Cx31.1 have a negative correlation with the proliferation marker Ki67 in the epidermis, while Cx43 has a positive correlation with proliferation. In the dermis, Cx26 has a negative correlation with proliferation, while Cx30 and Cx43 have a positive correlation with proliferation in the ors cells. (These correlations are not indicated on the diagram.) (c=cornified layer, g=granular layer, us=upper spinous layer, ls=lower spinous layer, b=basal layer, hf=hair follicles, bv=blood vessels.)

formation. In this study, we also described a modulation of Cx43 expression in the blood vessels of injured skin at 6 h and 4 d pw. While the upregulation response at 4 d pw may be consistent with the start of angiogenesis in wound tissue, the earlier increase at 6 h pw may support the view that increased communication and dilation of blood vessels promotes the recruitment of inflammatory cells to a wound site. In a parallel study, the authors have found that transient knockdown of Cx43 gap junctional communication promotes wound healing in mouse neonatal skin lesion models. The knockdown is thought to reduce the vascular response to injury, which in turn alters the number and distribution of inflammatory cells at the wound, resulting in accelerated wound closure. Thus, the Cx43 protein could serve as a future therapeutic target. Although the epidermal expression patterns of Cx43 in mouse and man are different (proliferative vs non-proliferative layers, respectively) (Salomon et al., 1988; Lucke et al., 1999), a number of other cell types critical to the wound healing process do share similar Cx43 expression profiles.

While most connexin expression profiles in this study returned to approximately normal levels after 7 d, Cx31.1 levels, which were normally low in the granular epidermal layer, peaked in wound epidermis and hair follicles incorporating into the epidermis at 7–12 d pw. Studies in injured guinea pig epidermis (Nagata et al., 1999) show that 7–12 d pw is the time when apoptosis peaks, implicating a role for Cx31.1 in terminal differentiation of granular keratinocytes to cornified squames. Upregulation of Cx31.1 at the wound sites in this study may, therefore, be required for terminal differentiation, and thereby eliminate the excess keratinocytes produced during earlier proliferative stages. Further supporting evidence for a link between Cx31.1 expression and cell death comes from studies by Wright et al. (2001), who showed that Cx31.1 was turned on in atretic follicles undergoing programmed cell death, while all other connexins were turned off. An alternative hypothesis is that Cx31.1 may be a marker for particular populations of keratinocytes. In addition, while keratinocytes may not express other connexins at 12 d pw, this may simply reflect the switch/interplay between different connexins.

The changes in epidermal connexin expression documented in this study up to 6 days following injury are in agreement with the study of Goliger and Paul (1995). However, we have found that expression continues to change at later time points during wound healing, that have not previously been studied. These variations may be the result of different time points being compared (6 h and 6 d post injury by Goliger and Paul, and 6 h through to 12 d pw in the current study) or different species and tissue being studied (rat tail by Goliger and Paul, or mouse back skin in the current study).

Increased cell proliferation during wound healing is a prerequisite for the replacement of eliminated cells and the generation of new tissue after injury. We have shown that a dynamic modulation of connexin expression in the epidermis and dermis of the skin accompanies proliferation, migration and differentiation during wound healing. These changes in connexin expression no doubt reflect the changing requirements of cell types involved in the wound healing process and implicate Cx26 and Cx30 in cell migration, Cx43 in proliferation and Cx31.1 in terminal differentiation. We show that, in the epidermis, Cx26 and Cx30 have a negative correlation with proliferation, while Cx43 has a positive correlation. In addition, there appears to be a negative correlation between Cx26 expression and proliferation in hair follicles of the dermis, but a positive correlation in ors cells and a negative correlation in irs cells in the case of Cx43. This study extends previous work on gap junctions during wound healing and implicates connexins as potential therapeutic targets.

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