Abnormal Connexin Expression Underlies Delayed Wound Healing in Diabetic Skin

Chiuhui Mary Wang, Jill Lincoln, Jeremy E. Cook, and David L. Becker

OBJECTIVE—Dynamically regulated expression of the gap junction protein connexin (Cx)43 plays pivotal roles in wound healing. Cx43 is normally downregulated and Cx26 upregulated in keratinocytes at the edge of the wound as they adopt a migratory phenotype. We have examined the dynamics of Cx expression during wound healing in diabetic rats, which is known to be slow.

RESEARCH DESIGN AND METHODS—We induced diabetes with streptozotocin and examined Cx expression and communication in intact and healing skin.

RESULTS—We found that diabetes decreased Cx43 and Cx26 protein and communication in the intact epidermis and increased Cx43 protein and communication in the intact dermis. Diabetes also altered the dynamic changes of Cxs associated with wound healing. Within 24 h, Cx43 was upregulated in a thickened bulb of keratinocytes at the wound edge (rather than downregulated as in controls, which formed a thin process of migratory cells). Cx43 decline was delayed until 48 h, when reepithelialization began. Although Cx26 was upregulated as normal after wounding in diabetic skin, its distribution at the wound edge was abnormal, being more widespread. Application of Cx43-specific antisense gel to diabetic wounds prevented the abnormal upregulation of Cx43 and doubled the rate of reepithelialization, which exceeded control levels.

CONCLUSIONS—Cx expression in diabetic skin is abnormal, as is the dynamic response of Cx43 to injury, which may underlie the delayed healing of diabetic wounds. Preventing the upregulation of Cx43 in diabetic wounds significantly improves the rate of healing and clearly has potential therapeutic value. ***Diabetes* 56:2809–2817, 2007**

Skin forms a protective barrier against the outside world, and any breach in it must be rapidly repaired to prevent infection and maintain normal function and homeostasis. Most adult skin wounds heal, without problems, through a series of overlapping events: hemorrhage and blood clot formation, inflammation, reepithelialization, and granulation tissue formation and tissue remodeling (1,2). Closure is normally rapid, but it can be slow in diabetic patients and the elderly, resulting in chronic wounds that are prone to infection (3).

Gap junction proteins (connexins [Cxs]), which form intercellular channels, are tightly regulated after wounding of the skin, at the transcriptional, translational, and post-translational levels. Signaling via gap junctions within communication compartments of the advancing epithelium is proposed to play a central role in the repair process (4–10). Cx43 is the most ubiquitous of the Cxs in skin; it is localized in cutaneous vasculature, fibroblasts, dermal appendages, and the basal and lower spinous cell layers of the epidermis. Cx26 has a different pattern of expression and is found only in the upper layers of the epidermis undergoing differentiation (11–14).

In response to skin lesioning, dynamic changes in the expression of different Cxs have been found to correlate with specific events during wound closure (4,8). Cx43 is downregulated in keratinocytes at the wound edge in the first 24–48 h. Indeed, wound repair is enhanced if this downregulation is accelerated by antisense oligodeoxynucleotide (ODN) knockdown of Cx43 mRNA (5) or when Cx43 is absent, as in the inducible Cx43 knockout mouse (6). However, Cx26 is upregulated in the leading-edge cells as they transform into a migratory phenotype, using lamellipodia to crawl forward and close the wound. Once the wound is closed, Cx26 downregulates within the epidermis as it remodels and Cx43 expression returns (8).

Interestingly, in human chronic diabetic wounds, Cx43 protein can still be found at high levels in the epidermis at the edge of the wound (10). As the dynamics of epidermal Cx43 expression are clearly central to the process of wound closure, we have examined Cx43 and Cx26 protein expression in intact skin and in skin undergoing wound healing in a rat model of diabetes induced by streptozotocin (STZ), in which delayed wound healing is known to occur.

RESEARCH DESIGN AND METHODS

All of the rats were supplied from a longstanding breeding colony in University College London's Biological Services Unit. (The original stock came from Harlan [Loughborough, U.K.]). Animals were maintained according to the U.K. Home Office Animals (Scientific Procedures) Act 1986 Code of Practice. They were housed in 12-h light and dark cycles in a climatically controlled room with free access to water and food. In total, 89 rats were used in these studies. Diabetes was induced in adult male Sprague-Dawley rats (350–400 g) by a single intraperitoneal injection containing 65 mg/kg STZ in citrate buffer (n = 6 diabetic and 6 control animals per time point) (15). Diabetes was confirmed 3–5 days after STZ injection using urinary glucose strips (Clinistix; Bayer, Newbury, U.K.). Specific blood glucose readings were also taken at the time of killing, and all STZ-injected rats were found to be severely hyperglycemic (diabetic rat blood glucose level 27.07 ± 1.09 mmol/l). Most diabetic wound-healing studies have been carried out 2 weeks after diabetes induction (16,17); this time point was used for our wound-healing study. However, we also examined Cx expression in the skin of diabetic rats at 8 weeks (n = 6 diabetic and 6 control animals) to confirm that the changes

From the Department of Anatomy and Developmental Biology, University College London, London, U.K.

Address correspondence and reprint requests to David Becker, Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC1E 6BT, U.K. E-mail: d.becker@ucl.ac.uk.

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Cx, connexin; H&E, hematoxylin and eosin; ODN, oligodeoxynucleotide; STZ, streptozotocin.

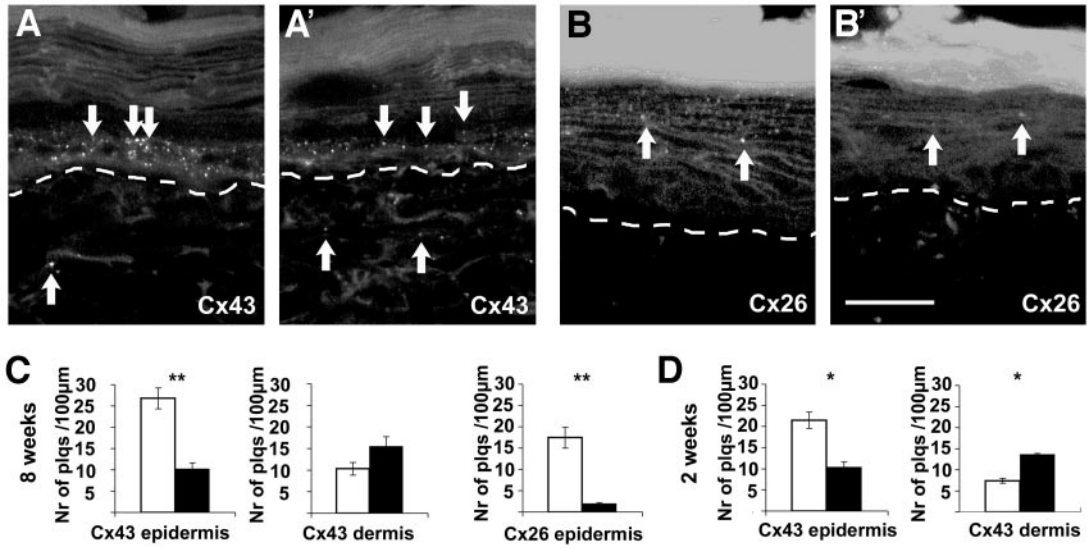

FIG. 1. Cx43 (*A* and *A'*) and Cx26 (*B* and *B'*) staining (arrows) in normal (*A* and *B*) and STZ-induced diabetic (*A'* and *B'*) intact rat skin, 8 weeks after induction of diabetes. The dashed line marks the boundary between the epidermis (above) and dermis (below). Scale bar = 25 μm. *C* and *D*: Graphs show the numbers of Cx plaques in the epidermis and dermis at 8 (*C*) and 2 (*D*) weeks after induction of diabetes (■) and in normal rats (□). Cx43 and Cx26 staining are significantly reduced in the diabetic epidermis, whereas Cx43 staining is increased in the dermis as early as 2 weeks after induction of diabetes, with no further change at 8 weeks. *$P < 0.05$; **$P < 0.01$.

we had detected at 2 weeks remained the same. Normal back skin was excised, cryosectioned, and immunostained for Cxs; imaged by confocal microscopy; and the staining quantified as described previously (18).

Excisional wound model. Rats were anesthetized with 5% halothane by inhalation and maintained with 1.5% halothane. Their backs were shaved and then wiped with 70% ethanol. Two pairs of 5 × 5-mm full-thickness excisional wounds were made, and 50 μl of 10 μmol/l Cx43-specific antisense ODN in 30% Pluronic F-127 gel (Sigma, Poole, U.K.) was applied to one wound and a matched dose of control (sense) ODN gel to the other (5). Cx43 antisense ODN, sequence GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC and control sense ODN, sequence GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC, were purchased from Sigma-Genosys (Poole, U.K.). Both ODN stocks and 30% Pluronic gels were dissolved in diethylpyrocarbonate-treated water before mixing. The ODNs are very stable inside the gel but have a half-life of ~30 min when in contact with sera or inside a cell. Slow release of the ODNs from the gel provides a sustained delivery of the antisense over time, which has proved to be a very effective delivery method (5,7). The ODN-gel mixtures and pipette tips were kept on ice until use to keep the gel in liquid form. Tissue was harvested on days 1, 2, 5, 10, and 15 after wounding ($n = 6$ diabetic and 6 control rats per time point) and cryosectioned at a thickness of 10 μm in preparation for Cx immunohistochemistry or hematoxylin and eosin (H&E) staining (8).

Intercellular communication was assessed by applying 10 μl of a 4% solution of Lucifer Yellow CH (Sigma) in a pledget of gelfoam into a fresh, full-thickness skin incision (4). Dye was allowed to diffuse for 5 min before removal of the gelfoam and fixation of the tissue in 4% fresh paraformaldehyde for 30 min. Several control studies were also included: a 10-kDa fluorescein isothiocyanate dextran, which will enter injured cells but not pass through gap junctions, was applied to the wound as described above and was found only in the injured cells of the wound margin. Application of the gap junction blocker octanol to a wound 5 min before applying Lucifer Yellow resulted in failure of the Lucifer Yellow to spread beyond the wound margin. In addition, a nonfixable form of Lucifer Yellow (Lucifer Yellow methoxycarbonyl, M-1341; Molecular Probes, Eugene, OR) revealed a similar pattern of dye spread to that of Lucifer Yellow CH. Tissues were cryosectioned and imaged on a Leica SP2UV confocal microscope (Leica, Milton Keynes, U.K.).

Microscopy and image analysis. Dye spread and Cx immunostaining were examined using the confocal microscope. Optimal gain and offset were set in advance and kept constant during image acquisition. A series of single optical section images was taken to generate a montage of the skin from the cut. Digital 8-bit images were analyzed using ImageJ software (National Institutes of Health). To assess dye spread, a 1,500 × 30-pixel region-of-interest box was placed with one short side aligned to the cut edge in the mid-dermis and an image intensity graph across the box was generated. A gray-level intensity drop to <50 was arbitrarily taken as the limit to which Lucifer Yellow had traveled. Similarly, in the epidermis, the distance from the cut to where the Lucifer Yellow signal dropped to <50 was recorded. At least three images were analyzed from each animal. Dye-spread data were tested for significance using the Mann-Whitney *U* test. All values were expressed as mean ± SE, and significance was taken at $P < 0.05$.

To compare levels of Cx protein, six single optical section images of dermis or epidermis were taken from different sections for intact tissue and for each wound. All parameters of laser power, pinhole, gain/offset, and objective were kept constant across both control and diabetic groups. Cx expression was quantified, as described previously (18), using ImageJ software. A threshold was set to detect gap-junction plaques with minimal background noise and was then kept constant for all images. The number and size of Cx plaques were recorded for each image and expressed per 100 μm length of epidermis or 10,000 μm^2 area of dermis. This approach has proved much more accurate than Western blot, as it generates information on protein expression at the cellular level. Western blots would be unable to distinguish between epidermal and dermal cells or detect effects of proximity to the wound edge. Using this approach, we were able to quantify Cx levels in keratinocytes in an adjacent zone at the wound edge and in a zone 500 μm away at 1, 2, and 5 days after wounding. At day 5 after wounding, we also imaged an additional zone of the leading edge of the nascent epidermis.

Images of H&E staining were taken using a Leica DMLFS microscope with a DC300F digital camera. Measurements for reepithelialization rate (total length of nascent epidermis as a percentage of the length of the wound bed) have been described previously (5). Comparisons of Cx expression in intact skin, between control and diabetic rats, were made using the Mann-Whitney *U* test. The effect of diabetes on the dynamic changes in Cx expression during the wound-healing process was assessed using a two-way ANOVA. One-way ANOVA was used to assess the effect of the antisense treatment on the reepithelialization rate.

RESULTS

Cx expression in intact skin. STZ-induced diabetes in the rats was confirmed in the following week with a urine glucose test. In addition, the blood glucose level was measured when the diabetic rats were killed (average 27.07 ± 1.09 mmol/l). In diabetic skin, we found typically punctate Cx43 immunostaining in the basal layer of the epidermis and, in dermal fibroblasts, hair follicles, blood vessels, and appendages (Fig. 1). However, quantification of staining revealed that in 8-week diabetic skin, Cx43 staining was significantly reduced in the epidermis, in terms of the number of gap junction plaques (Fig. 1*A*; $P = 0.0039$, Fig. 1*C*), although there was no difference in plaque size distribution. Staining for Cx26 in the upper layers of

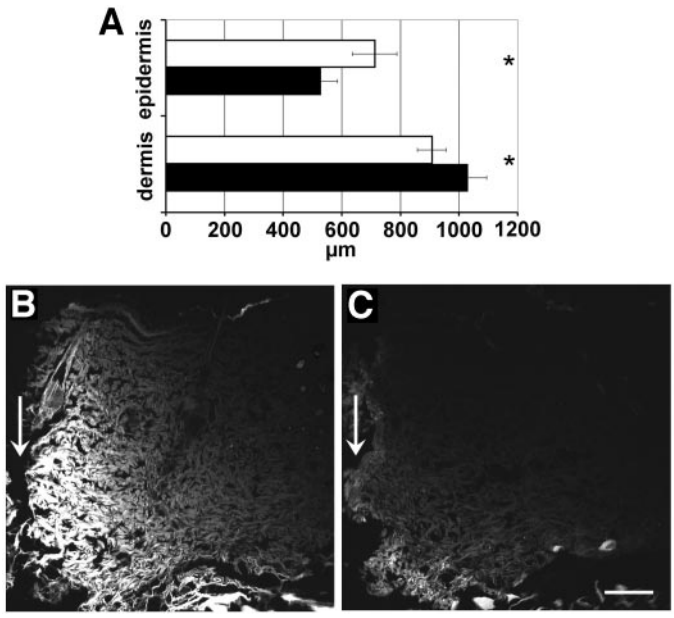

FIG. 2. *A*: Quantification of the distances that Lucifer Yellow dye migrated in 5 min in the epidermis and dermis of normal and diabetic rat skin, 2 weeks after induction of diabetes. In the epidermis of diabetic skin (■), dye spread was significantly (*$P < 0.05$) reduced compared with control skin (□), whereas in the dermis, dye spread was significantly (*$P < 0.05$) increased. *B*: Example of the spread of Lucifer Yellow CH through the skin of a normal rat, from the cut on the left side of the image (arrow). *C*: Octanol, a gap junction blocker, prevented the spread of Lucifer Yellow. Scale bar = 200 μm.

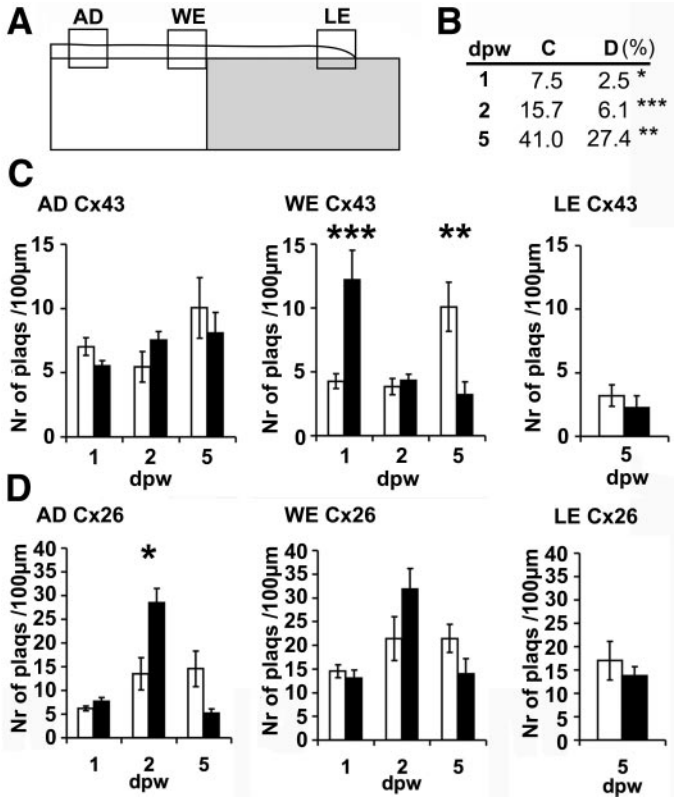

FIG. 3. Reepithelialization rates and the dynamic responses of Cx43 and Cx26 protein levels following injury in control and diabetic epidermis. *A*: Zones in which Cx staining was quantified by counting plaques at 1, 2, and 5 days after wounding. On days 1 and 2, quantification was carried out at the wound-edge (WE) and adjacent (AD) epidermis 500 μm away, and on day 5, an additional zone was included at the leading edge (LE) of the nascent epidermis. *B*: The rate of reepithelialization was significantly (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) reduced relative to controls in diabetic rats at all time points: 1, 2, and 5 days postwounding (dpw). *C*: The dynamic response of Cx43 protein expression to injury was found to be very different in diabetic (■) compared with normal (□) skin. Cx43 staining at the wound edge normally reduces in the first 24 h after injury, but in diabetic skin, it increased significantly (*$P < 0.001$), returning to near-control levels by 2 days postwounding, but Cx43 was found at significantly (**$P < 0.01$) reduced levels at the wound edge at 5 days postwounding. *D*: The normal response of Cx26 to injury is an elevation of staining at the wound edge, and this occurred in both control and diabetic animals at 1 and 2 days postwounding; however, the elevation was greater in diabetic skin.**

the epidermis was similarly significantly reduced in diabetic epidermis, with no changes in plaque size distribution ($P = 0.0039$, Fig. 1*C*). However, this was not a global downregulation of Cxs in response to diabetes, as a contrasting effect was observed in dermal fibroblasts; in these, we observed a distinct increase in the size and number of Cx43-positive plaques (Fig. 1*C*). Similar effects on Cx43 expression were seen in skin 2 weeks after STZ induction of diabetes (epidermis $P = 0.037$, dermis $P = 0.025$; Fig. 1*D*).

Effects of diabetes on gap junctional intercellular communication. To assess cell-cell communication in diabetic epidermis and dermis, we examined the extent of spread of the gap junction–permeant dye Lucifer Yellow through the tissue in 5 min (4). This revealed significantly reduced spread in the diabetic epidermis ($P = 0.0433$) but significantly enhanced spread in the diabetic dermis ($P < 0.0472$; Fig. 2*A*), consistent with the changes in Cx protein expression. In control experiments, a nonfixable, methoxycarbonyl version of Lucifer Yellow that is only retained during histological processing when it is inside nonpermeabilized cells showed the same distribution properties as Lucifer Yellow CH (Fig. 2*B*), whereas a gap junction–impermeant 10-kDa fluorescein isothiocyanate dextran, applied in the same way, was confined to the edge of the wound (data not shown). In addition, application of the gap junction blocker octanol to the wound for 5 min before application of Lucifer Yellow CH effectively prevented dye spread (Fig. 2*C*). These observations suggest that dye spread occurred through gap junctions rather than by diffusion through the extracellular space. Also, consistent with these findings, elevated expression of Cx43 protein and increased communication have been reported in human diabetic fibroblasts (19), and mixed responses of different Cxs to diabetes have been noted in the renal system (20) and bladder (21).

Effects of diabetes on dynamic responses of Cx expression to injury. Quantitative data on Cx43 and Cx26 expression in keratinocytes at the wound edge and in an adjacent zone 500 μm away 1, 2, and 5 days after wounding are shown in Fig. 3, together with data for leading-edge keratinocytes after 5 days. Representative micrographs illustrating Cx expression are shown in Fig. 4. We confirmed that reepithelialization is delayed in diabetic skin by measuring the length of nascent epidermis. After 2 days, there was 15.7% closure of wounds in control rats, whereas diabetic wounds were retarded (6.1% closed); the epidermis had only recently adopted a migratory morphology. The quantitative data were analyzed by two-way ANOVA with diabetes and time after injury as factors. This revealed that a highly significant interaction ($P = 0.0001$) existed between the effect of diabetes and that of time on Cx43 expression in the wound edge, indicating that diabetes was influencing the dynamic changes in Cx43 expres-

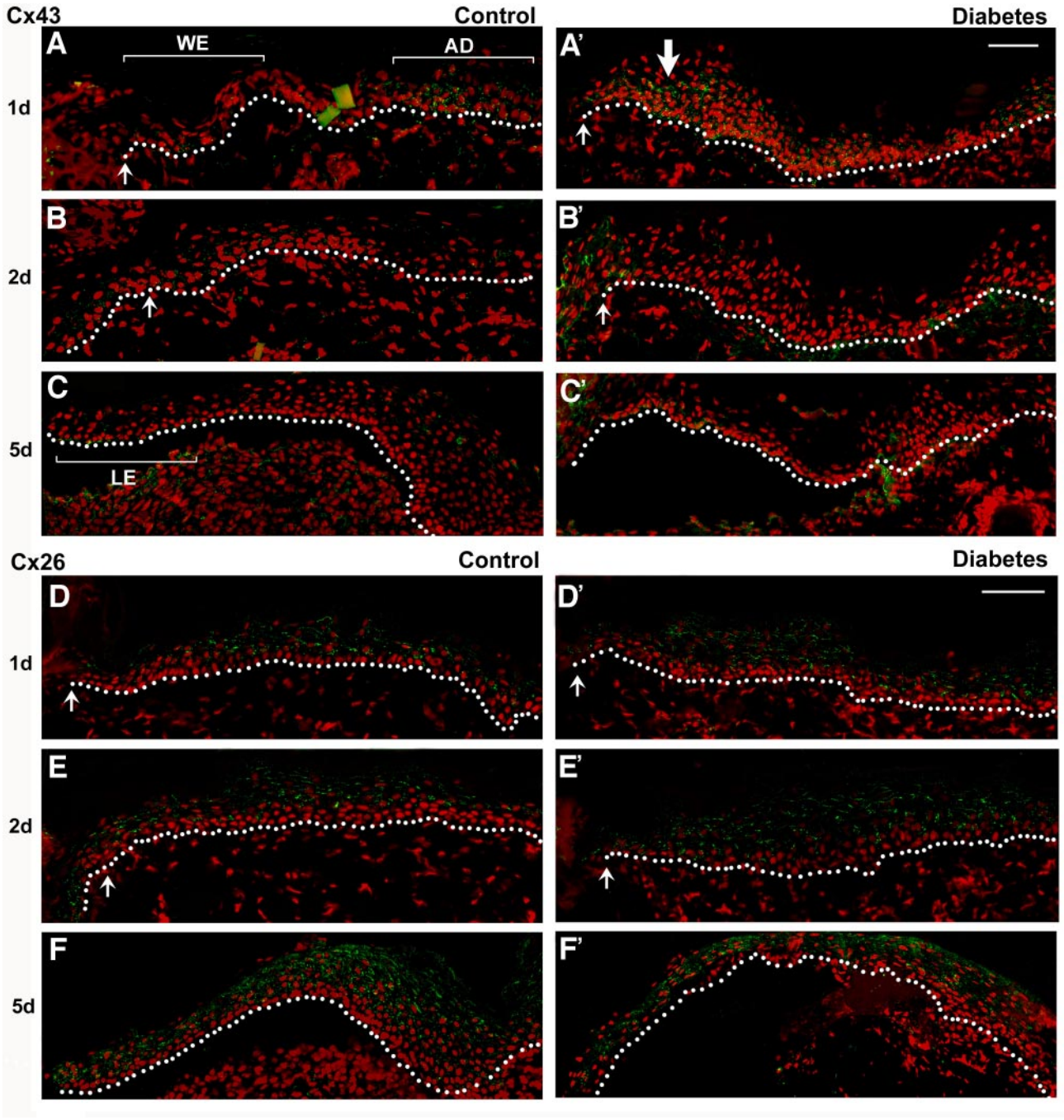

FIG. 4. Cx43 (*A*, *B*, and *C*) and Cx26 (*D*, *E*, and *F*) staining (green puncta) and nuclear staining (red) at the epidermal wound edge of control and diabetic skin during the wound-healing process. After 1 day, Cx43 protein expression is downregulated in the wound-edge (WE) keratinocytes of control rats (*A*) but has been upregulated in the corresponding region, which appears swollen, in diabetic animals (*A'*, thick arrow). Two days after wounding, Cx43 staining has reduced in the diabetic wound-edge keratinocytes (*B'*) and is similar to that of controls (*B*), while after 5 days, some Cx43 can be observed at the leading edge (LE) in control (*C*) but not diabetic (*C'*) keratinocytes. Cx26 can be seen to upregulate similarly in both control (*D*) and diabetic (*D'*) epidermis, while it continues to increase in all diabetic keratinocytes (*E'*) to significantly higher levels than those of controls (*E*). After 5 days, Cx26 is clearly observed in the differentiating keratinocytes (*F* and *F'*). Thin arrows mark the edge of the wound; the line of white dots represents basement membrane. Scale bar = 100 μm. (Please see http://dx.doi.org/10.2337/db07-0613 for a high-quality digital representation of this figure.)

sion in the wound-edge region associated with wound healing. No such interaction was demonstrated for Cx43 expression in the intact region adjacent to the wound (Fig. 3*C*). In contrast, diabetes did not significantly affect the dynamic changes in Cx26 expression in the wound-edge region but did significantly interact ($P = 0.016$) with these responses in the intact adjacent region (Fig. 3*D*).

One day after wounding, Cx43 expression was reduced in the wound-edge region relative to the adjacent region (Fig. 4*A*), as has been reported previously (4,8). This has been associated with the change in the keratinocytes to a migratory phenotype. In marked contrast, and surprisingly in view of the decreased expression of Cx43 in intact skin in diabetes, Cx43 expression was greater in the wound-edge region than in the adjacent region in diabetic rats (Fig. 4*A*), and Cx43 expression in the wound-edge region from diabetic rats was more than double ($P < 0.001$) that in the wound-edge region from controls (Fig. 3*C*). Thus,

the initial wound-healing response of Cx43 in diabetic rats was the opposite of that observed in controls. Keratinocytes formed a thickened bulb at the wound edge in diabetic tissue, whereas they thinned out before crawling forward to close the wound in controls. After 2 days, Cx43 expression had fallen in the wound edge of diabetic tissue to levels similar to those of controls (Figs. 3*C* and 4*B*). There was an increase in Cx26 expression in the first 2 days following wounding in the wound-edge region in both control and diabetic tissue (Figs. 3*D* and 4*D* and *E*). However, the response to injury appeared to be more widespread in diabetes because Cx26 expression in the adjacent region was significantly ($P < 0.05$) greater than controls after 2 days (Figs. 3*D* and 4*E*).

On day 5, reepithelialization was well underway in both diabetic and control groups, although the diabetic wounds were significantly retarded (27.4% closed compared with 41% in controls, $P < 0.01$). Cx43 expression increased in the wound edge of control but not diabetic tissue (Figs. 3*C* and 4*C*), such that levels were significantly higher ($P < 0.01$) in control wound edge, a pattern similar to that observed in intact skin. Only low levels of Cx43 expression were observed in keratinocytes in the leading-edge region in both control and diabetic tissue. There were no significant differences in the expression of Cx26 in adjacent, wound edge, or leading edge in control or diabetic tissue after 5 days; the previously elevated Cx26 staining in the diabetic wound-edge and adjacent zones had returned to more normal levels (Figs. 3*D* and 4*F*).

Reepithelialization was complete in both control and diabetic animals by day 10, when high levels of Cx43 could be seen in all of the layers of the proliferative, excessively thickened epidermis (Fig. 5). Examination of the dynamic changes in Cx43 and Cx26 protein expression in the newly regenerated epidermis, as it underwent revision, revealed a marked delay in its return to normality for diabetic wounds. A gradient of maturation could be seen from the edge to the center of the wound (marked 3 and 4 in Figs. 5*Q* and 6*Q*). At day 10, the epidermis was hyperthickened and expressed Cx43 and Cx26 throughout, with higher levels of Cx43 seen in the diabetic epidermis. The nascent epidermis also lacked the laminar structure and restriction of Cx expression of the normal epidermis seen in zone 1 (Figs. 5 and 6). By day 15, wound revision had progressed and the newly formed epidermis had started to thin down and adopt a more laminated appearance. A marked gradient of maturation from the edge to the center of the wound was still evident, and this reflected the gradual restriction of Cx43 expression to the basal layers and Cx26 to the upper layers of the epidermis (Figs. 5 and 6). Throughout the process of maturation, the diabetic skin appeared to be retarded, lagging behind the normal sequence of healing events by several days.

Effects of Cx43-specific antisense treatment on diabetic wounds. Having observed these abnormal patterns of Cx immunostaining, gap junctional communication and morphological response in diabetic wounds, we then assessed the effects of the abnormal increase of Cx43 protein in diabetic wound-edge keratinocytes by preventing the increase with a Cx43-specific antisense gel, applied to the wound at the time of injury (5,7). This effectively inhibited the increase in Cx43 that was seen with control (sense) gel (Fig. 7*A*) and allowed the keratinocytes to adopt a thinner, tongue-like profile (Fig. 7*B*). More importantly, it also improved wound-healing rates, which more than doubled, reaching untreated control levels or exceeding them (Fig. 7*G*). One day after injury, diabetic wounds showed virtually no epidermal regrowth compared with controls (Fig. 7*C* and *D*), but treatment of diabetic skin with the Cx43 antisense ODN induced a regrowth of epidermis to match that of controls (Fig. 7*F*). Healing rates were even higher in antisense-treated wounds in control rats (Fig. 7*E*), as would be expected from our previous findings (5,7).

DISCUSSION

We have demonstrated that the onset of diabetes has a marked effect on the expression levels of Cxs in the skin and that this effect is even more pronounced when the skin is injured. The mechanism whereby diabetes alters the expression of Cxs, with a differential effect on different Cx proteins, is currently unknown. However, hyperglycemia and/or oxidative stress induced by hyperglycemia have been implicated in the development of other complications of diabetes such as neuropathy and angiopathy (3). Both high glucose and oxidative stress in vitro have been shown to alter Cx expression and/or gap junctional communication in a variety of different cell types (22–24), and Cx43 and Cx26 are known to have different promoters (25,26). It should be emphasized that impaired wound healing has been demonstrated in rats as early as 1 week after induction of diabetes, before either neuropathy or angiopathy develops (16,17). Thus, the changes observed here within 2 weeks are likely to be direct consequences of the diabetic condition rather than secondary consequences of long-term diabetes complications.

The effect of hyperglycemia in inhibiting Cx43 expression has been described in a number of cell types in in vitro models, where it has been suggested that the inhibition is due to increased protein kinase C phosphorylation (27,28). Phosphorylation of Cx43 may lead to its removal from the membrane (29), but this explanation cannot account for our observation of reduced levels of Cx26, which does not have any phosphorylation site on its COOH-terminus. Clearly, the effect of diabetes also is not a uniform downregulation of Cxs, as fibroblasts respond by elevating Cx43. Again, the mechanism of action remains to be determined. High levels of Cx43 in diabetic fibroblasts are likely to have a detrimental effect on their migration to form granulation tissue in the wound-healing process because actively downregulating Cx43 in fibroblasts enhances their migration and proliferation (30).

A significant finding in our study was the abnormal upregulation of Cx43 at the epidermal wound edge in diabetes. This has the potential to affect the process of wound closure in various ways. The formation of communication compartments within the regenerating epidermis has been proposed to play a role in wound healing (1,9,31). Compartmentalization could be effectively brought about in normal conditions by expression of Cx26 and removal of Cx43 in leading-edge cells, as these Cxs do not form junctions with one another. Thus, the delay in wound healing in diabetes could reflect the additional time required for Cx43 expression to downregulate to a point where such a compartmentalization can occur. Alternatively, as the C-tail of Cx43 is known to interact with cytoskeletal components or with P120ctn/Rho GTPase, downregulation of Cx43 could be necessary for changing the motility of keratinocytes at the wound edge, enabling them to migrate and close the wound (32). In this regard, it was notable that, 1 day after wounding, when their Cx43 expression was high, diabetic keratinocytes formed a

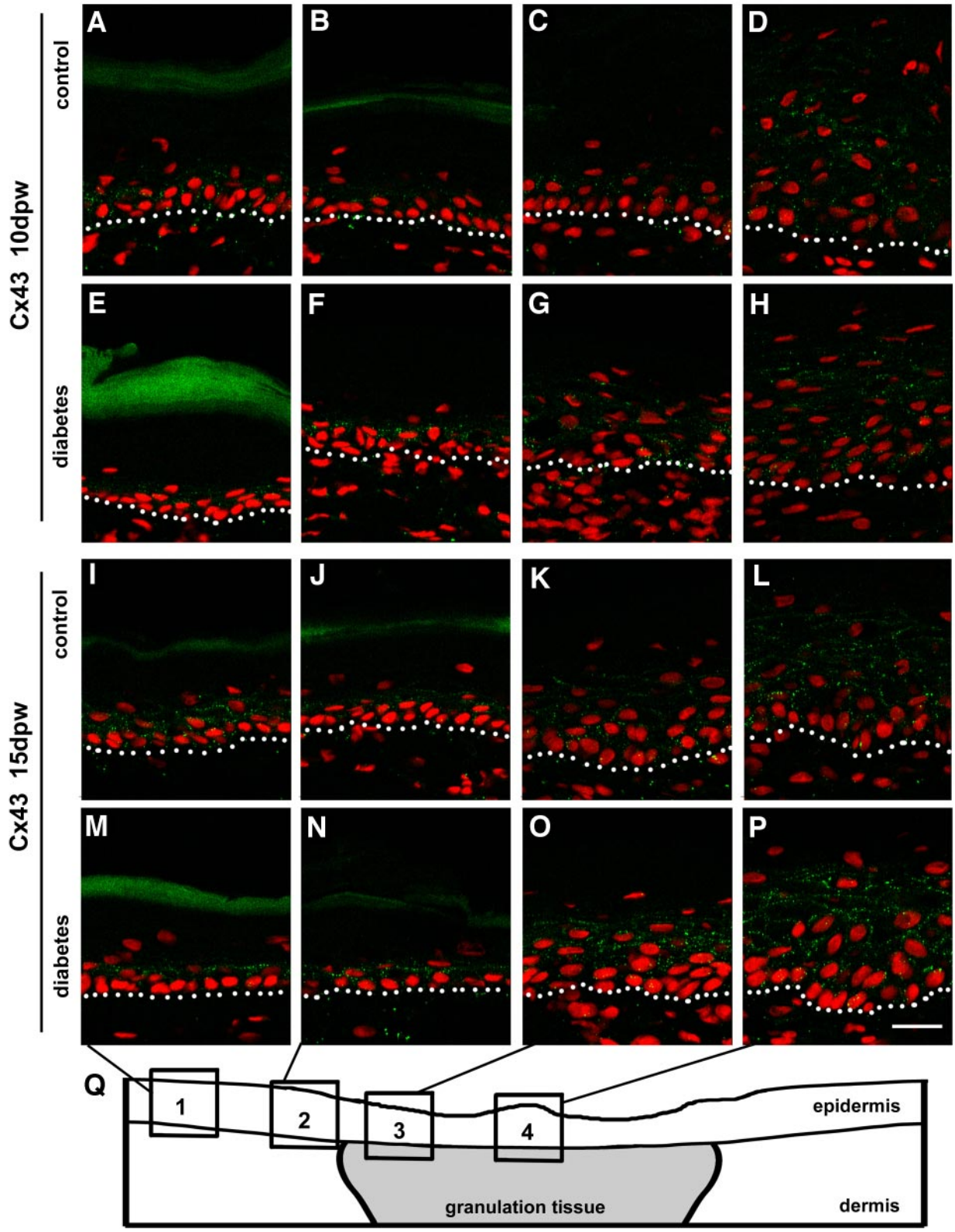

FIG. 5. Cx43 staining (green puncta) and nuclear staining (red) in control and diabetic skin at days 10 and 15 after injury, showing the dynamic changes in expression during maturation of the nascent epidermis. The numbered zones represent *1*) uninjured epidermis away from the wound, *2*) uninjured epidermis at the edge of the wound, *3*) nascent epidermis at the edge of the wound, and *4*) nascent epidermis in the center of the wound, as diagrammed in *Q*. The dotted white line shows the border between the epidermis and the dermis. Cx43 can be seen to be in all layers of the newly formed, hyperthickened epidermis, returning to a more basal expression as the skin thins down during tissue remodeling. This change can be seen both in time and as a gradient of maturation from the edge to the center of the wound. A green band of autofluorescent keratin can be seen over the intact skin (*E* and *M*), but this has not yet formed in the nascent epidermis. Scale bar = 25 μm. (Please see http://dx.doi.org/10.2337/db07-0613 for a high-quality digital representation of this figure.)

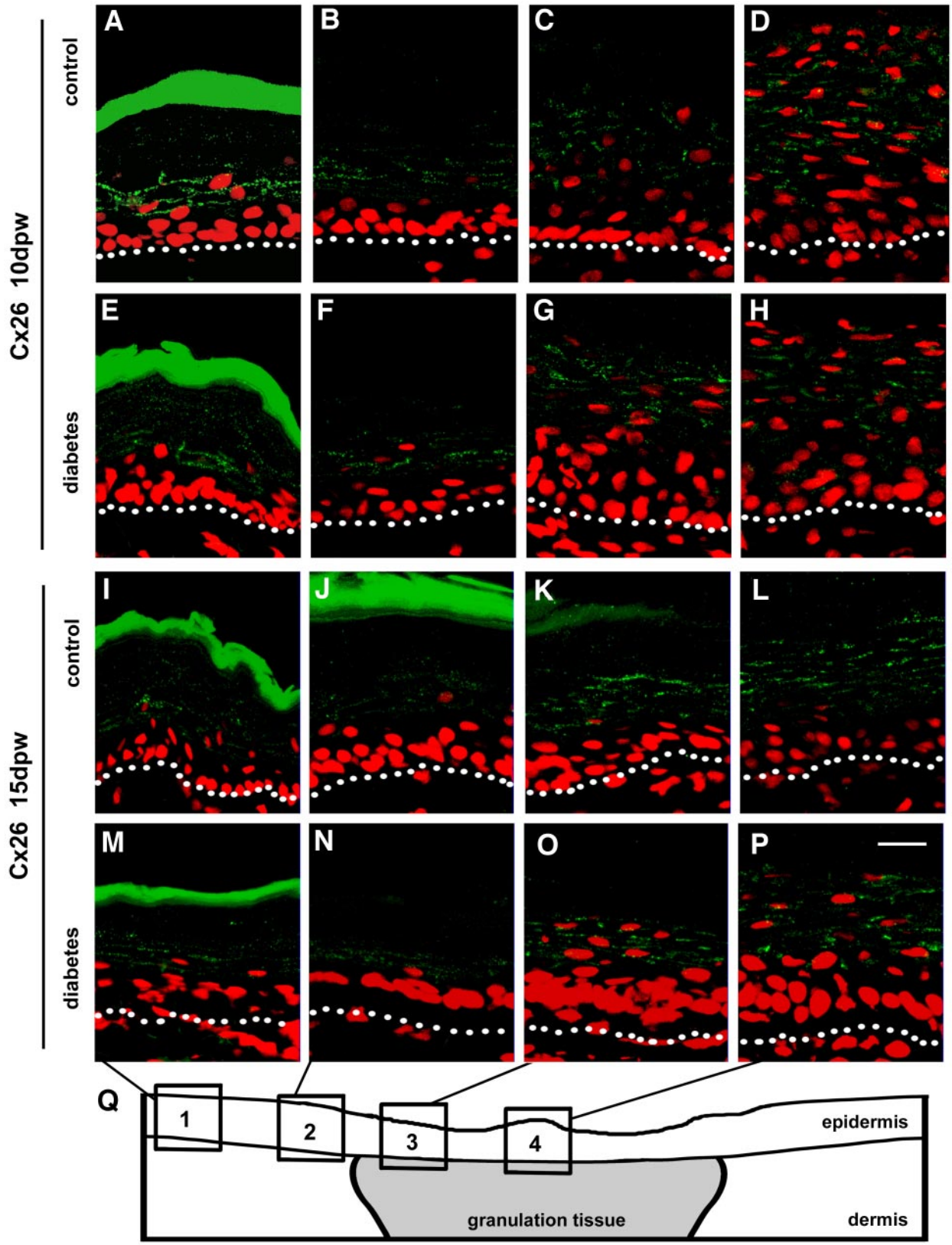

FIG. 6. Cx26 staining (green puncta) and nuclear staining (red) in control and diabetic skin at days 10 and 15 after injury showing the dynamic changes in expression during maturation of the nascent epidermis. The numbered zones represent *1*) uninjured epidermis away from the wound, *2*) uninjured epidermis at the edge of the wound, *3*) nascent epidermis at the edge of the wound, and *4*) nascent epidermis in the center of the wound, as diagrammed in *Q*. The dotted white line shows the border between the epidermis and the dermis. Cx26 can be seen to be in all layers of the newly formed, hyperthickened epidermis at day 10, returning to a more superficial expression in the upper spinous layers as the skin thins down during tissue remodeling. This change can be seen both in time and as a gradient of maturation from the edge to the center of the wound. A green band of autofluorescent keratin can be seen over the intact skin. A small amount of keratin can be seen forming at the edge of the wound in control rats at day 15 but not in diabetic rats. Scale bar = 25 μm. (Please see http://dx.doi.org/10.2337/db07-0613 for a high-quality digital representation of this figure.)

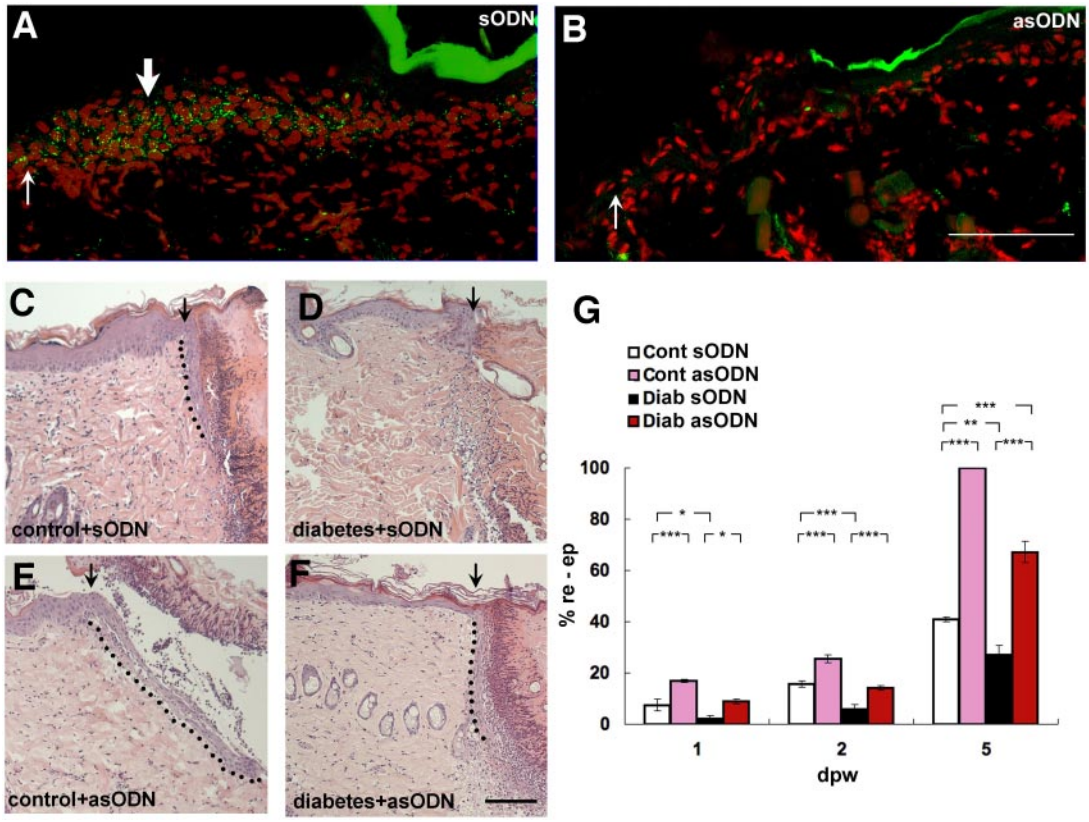

FIG. 7. Effects of Cx43 sense and antisense ODN treatment on Cx43 expression 1 day after wounding and on reepithelialization rates at 1, 2, and 5 days. *A*: Elevated levels of Cx43 staining (green puncta, thick arrow) can be seen at 1 day postwounding in the swollen epidermal bulb (red nuclei) at the edge of a diabetic wound treated with Cx43 sense ODN. However, this elevation in Cx43 protein can be prevented by treatment with Cx43 antisense ODN (*B*), which also results in a thinner layer of keratinocytes that appear more like those of control animals rather than a bulb of cells. Thin arrows mark the edge of the wound. *C–F*: H&E staining of sections through the edge of the wound at 1 day after wounding in diabetic (*D* and *F*) and control (*C* and *E*) rats treated with Cx43 sense (*C* and *D*) or antisense (*E* and *F*) ODNs. Antisense treatment results in double the rate of reepithelialization for both control and diabetic rats. Importantly, it brings the rate of diabetic wound healing back to that of sense-treated (or untreated) control levels. The significantly increased rate of reepithelialization, following antisense treatment of both control and diabetic skin, was maintained 1, 2, and 5 days after wounding (*G*). Scale bar = 50 μm. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. (Please see http://dx.doi.org/10.2337/db07-0613 for a high-quality digital representation of this figure.)

thickened bulb at the wound edge rather than flattening out and starting to crawl forward across the wound.

Persistence of Cx43 at the wound edge has been reported in chronic diabetic ulcers in humans (10). Thus, our observation that the same Cx is abnormally upregulated in diabetic rats is likely to have implications for the delayed wound healing that occurs in diabetic patients. Clearly, the normal, prompt downregulation of Cx43 after wounding is critical for normal healing, because we have shown here that knocking down Cx43 expression by a single application of the Cx43-specific antisense gel at the time of wounding enables the entire process, from initial migration to complete wound closure, to occur at or above a normal rate, despite the continued presence of the diabetic condition. Thus, antisense treatment has considerable potential as a novel approach for the treatment of chronic wounds in diabetic patients.

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