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Targeting Connexin43 Expression Accelerates the Rate of Wound Repair

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Summary

The repair of tissue damage is a key survival process in all organisms and involves the coordinated activation of several cell types. Cell-cell communication is clearly fundamental to this process, and a great deal is known about extracellular communication within the wound site via cytokines [1, 2]. Here we show that direct cell-cell communication through connexin 43 (Cx43) gap junction channels [3, 4] also plays a major role in the wound healing process. In two different wound healing models, incisional and excisional skin lesions, we show that a single topical application of Cx43 antisense gel brings about a transient downregulation of Cx43 protein levels, and this results in a dramatic increase in the rate of wound closure. Cx43 knockdown reduces inflammation, seen both macroscopically, as a reduction in swelling, redness, and wound gape, and microscopically, as a significant decrease in neutrophil numbers in the tissue around the wound. One long-term consequence of the improved rate of healing is a significant reduction in the extent of granulation tissue deposition and the subsequent formation of a smaller, less distorted, scar. This approach is likely to have widespread therapeutic applications in other injured tissues and opens up new avenues of research into improving the wound healing process.

Results and Discussion

Connexins in Normal and Wounded Skin

In normal skin, several connexins are differentially expressed [5–7], and their importance has recently been highlighted by the discovery that point mutations in several connexins underlie a variety of skin diseases [5]. In the epidermis Cx43 is found localized in the basal and lower spinous cell layers, whereas Cx26, Cx30, and Cx31.1 are expressed in the upper, differentiating epidermal layers [8, 9]. In the dermis, Cx43 is the most ubiquitous connexin, expressed in dermal fibroblasts [10], the vascular system [11, 12], and skin appendages [13]. In the first 24 hr post injury, the level of Cx43 protein decreases within the keratinocytes of the leading edge but later increases in the hyperproliferating epidermis at day 7 [14–16]. In the deep dermis, a few hours after trauma, Cx43 is transiently upregulated in fibroblasts, endothelial cells, and smooth-muscle cells in blood vessels around the wound [16]. At later stages of tissue repair, during tissue remodeling (day 7 onward), upregulation of Cx43 correlates with an increase in granulation tissue maturation, suggesting a potential role during this stage also [10].

Macroscopic Effects of Cx43 Knockdown

Transient knockdown of Cx43 protein in the wound tissues by an antisense oligodeoxynucleotide (AS ODN) to Cx43 [17, 18] gives us the opportunity to test the role of gap junctional communication through Cx43 in the early stages of wound healing. We find that a single topical application of Cx43 AS ODN to a lesion is able to greatly speed and enhance the normal knockdown of Cx43 protein levels in and around the wound within 2 hr (Figure 1) and that recovery only starts after 24 hr. In addition, treatment also prevents the normal upregulation of Cx43 protein in the dermis around the wounded site. This single topical application dramatically improves the rate of healing and the macroscopic wound appearance in two standard wound healing models (Figure 2). Compared to controls, antisense treated wounds appear to be less moist and inflamed (swollen) and have a reduced wound gape within hours of injury (Figure 2). The improved wound appearance with Cx43 AS ODN treatment is most obvious at 1 and 2 days for incisional lesions, when there is almost complete wound closure, with little redness or swelling in the tissues surrounding the lesion as compared to findings for sense controls (Figures 2S and 2T). The macroscopic appearance of wounds in both treatment groups was blindly assessed via a wound rank scoring system that scores the degree of wound closure (wound length and gape) and inflammation (redness and swelling). Cx43 AS ODN-treated wounds consistently had improved wound rank scores compared to those of sense controls, and statistical analysis indicated a significant improvement at 6 hr, 1day, and 2 days (P values 0.028, 6 hr; P 0.001, 1 day; and P 0.012, 2 days).

When used at low concentrations, unmodified Cx43 AS ODNs are broken down rapidly within the tissues they penetrate, and so their effects are only transient. Our data show that CY3-tagged AS ODNs are rapidly taken up by all cell types at the wound site and extend back in a gradient more than 100 μm from the wound (Figure 1). FRET labeling of ODNs shows that they have

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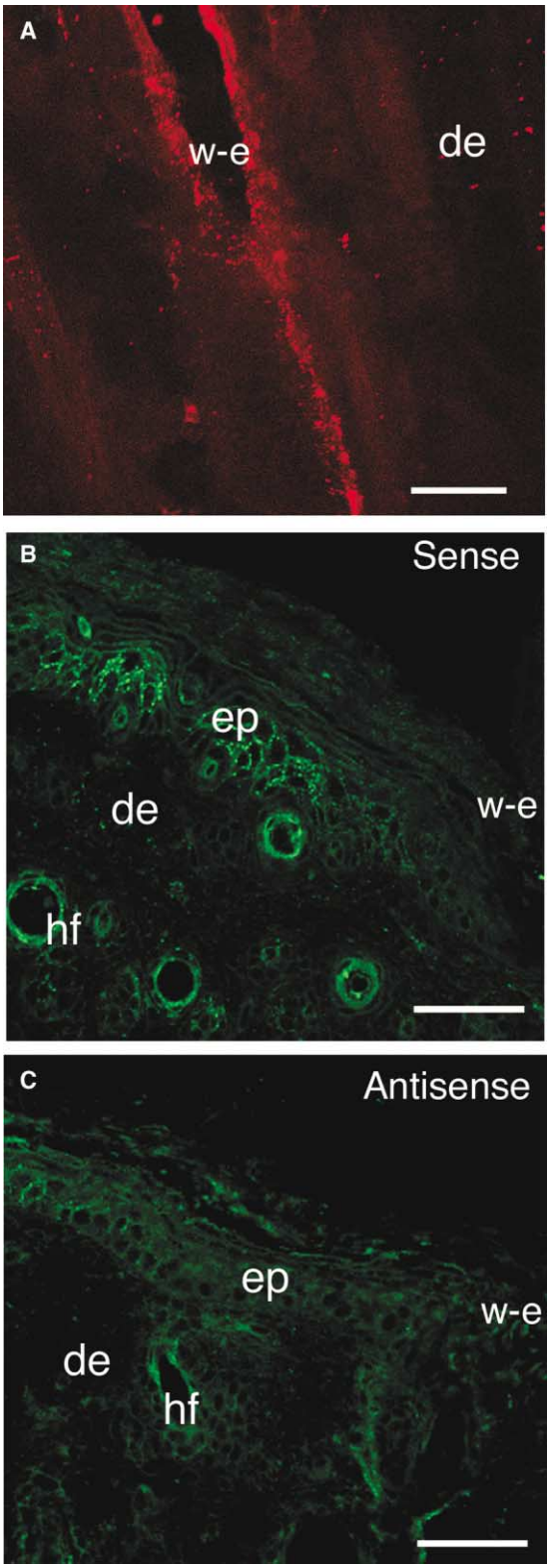


Figure 1. Antisense Penetration and Cx43 Protein Knockdown at the Wound Site and in Adjacent Tissue

(A) CY3-ODN penetration through the dermis at 2 hr in the site of the lesion. Strong labeling can be seen at the edge of the lesion (w-e), where it decreases in a gradient away from the site of application into the dermis (de). (B and C) Cx43 staining of skin 2 hr after wounding. Positive staining appears as small bright puncta in the epidermis (ep), dermis (de), and hair follicles (hf). A decrease in Cx43 protein expression can first be detected in the epidermis of the wound margin (B), but expression is still strong in adjacent epidermis and a little raised in the dermis. Antisense treatment greatly enhances the rate and extent of Cx43 protein knockdown within the epidermis at the wound site and in adjacent tissue and prevents any dermal upregulation of the protein (C). Scale bars represent 50 μm.

a half life of around 30 min inside cells but are constantly replenished with new ODNs as long as the gel remains. The Cx43 knockdown that they produce is likely to affect several diverse cell types involved in the wound healing process, but the consequences of the knockdown extend well beyond the lifetime of the ODNs themselves. Indeed, the beneficial kick-start that they bring to the healing process has visible effects at later stages, after Cx43 protein levels have been restored. By 12 days, the aesthetic appearance of AS-treated scars is improved, and they are generally narrower and flatter against the skin than those of sense controls (Figures 2E and 2J). The short effective life of unmodified AS ODNs may actually be beneficial because it allows Cx43 function to be restored during later stages of re-epithelialization and tissue remodeling.

Effects of Cx43 Knockdown on Neutrophils

Having established the general effectiveness of our Cx43 AS ODNs in promoting wound healing, we next sought to study its effects on specific cell types. The first inflammatory cells to enter a wound are the neutrophils. Their primary role is in the phagocytosis of cells and debris around the wound as a first line of defense against any microbes that might cause infection [1, 19]. In addition, they also release pro-inflammatory cytokines, which may be key in coordinating wound repair [19]. We studied the effects of Cx43 AS ODNs on the inflammatory response by immunostaining tissue for neutrophils. In AS-treated wounds, their number was dramatically reduced and their distribution was greatly restricted in comparison with controls (Figure 3). Neutrophil numbers on days 1 and 2 were reduced by 20% in the immediate vicinity of the wound clot and were half those of controls in adjacent dermal zones ($P < 0.028$) (Figure 3).

Cx43 is normally transiently upregulated in the smooth-muscle cells and endothelial cells of blood vessels around the wound site within 6 hr after lesion injury [11, 16, 20]. This upregulation is prevented in blood vessels of the deep dermis after treatment with Cx43 AS ODN. The normal increase in gap-junctional expression has been suggested to enhance vasodilation, which in turn is permissive to leukocyte diapedesis and migration into the wound site [21]. Indeed, perturbation of gap-junctional communication in renal blood vessels with connexin mimetic peptides prevents renal vasodilation [22]. Cx43 gap junctions have been described between endothelial cells and leukocytes in vivo during the inflammatory response, and it has been suggested that this communication is required for transmigration across the endothelium [23]. Cx43 AS ODN treatment may therefore

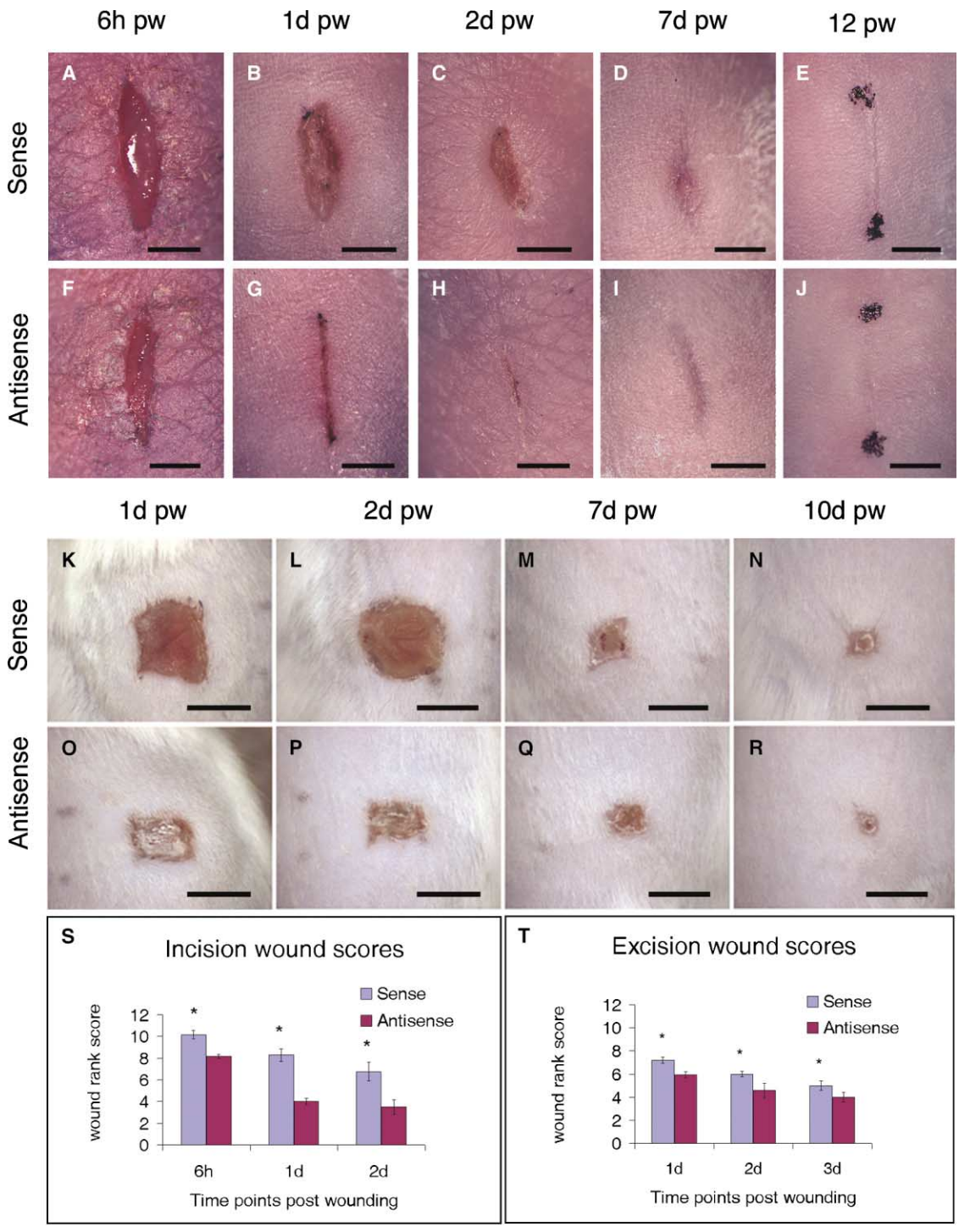


Figure 2. Macroscopic Appearance of the Healing Process in Pairs of Treated and Untreated Incisional and Excisional Wounds

(A–J) Macroscopic images of pairs of control (A–E) and antisense-treated (F–J) full-thickness, incisional lesions on the backs of neonatal mice at 6 hr (A and F), 1 day (B and G), 2 days (C and H), 7 days (D and I), and 10 days (E and J) after wounding.

(K–R) Macroscopic images of pairs of control and antisense-treated full-thickness, excisional lesions on the backs of adult mice at 1 day (K and O), 2 days (L and P), 7 days (M and Q), and 10 days (N and R) after wounding.

(S and T) Histograms showing the wound rank scores of Cx43 antisense-treated wounds and control wounds in the neonatal incisional model (S) and the adult excisional model (T). Each bar represents the mean wound rank score ± SEM from eight animals. A significant difference was found between Cx43 antisense-treated wounds and control wounds when these were analyzed with the Wilcoxon Signed Rank test. An asterisk indicates *P* values, as follows: 0.028, 6 hr, P 0.001, 1day and P 0.012, 2 days). Scale bars in (A)–(J) represent 1 mm, and those in (K)–(R) represent 0.5 mm

reduce the influx of neutrophils into the wound site and adjacent skin by targeting communication between neutrophils and endothelial cells. Alternatively, the reduced neutrophil numbers in AS ODN-treated wounds may reflect a decreased propagation of injury signals into adjacent healthy blood vessels. This would confine neutrophil exudation to the wound site and thus explain the reduced inflammation around the injury site and the limited spread of tissue damage because neutrophils are known to cause substantial tissue damage as they release reactive free-oxygen radicals [19].

Effects of Cx43 Knockdown on Re-Epithelialization and Granulation Tissue

To assess granulation tissue deposition and rates directly, we stained tissue sections with haematoxylin and eosin (H&E) or an antibody to fibronectin. Quantification of incisional lesion re-epithelialization rates showed that AS ODN-treated wounds closed significantly more rapidly than sense-treated controls at all time points measured. At 1, 2, and 4 days after wounding, re-epithelialization rates in AS-treated lesions were raised by 47%, 55%, and 27%, respectively (P values of 0.017, 1 day, P 0.012, 2 days, and P 0.028, 4 days) (Figures 4A–4F). Even before re-epithelialization begins, the reduced swelling and gape of the treated wound has decreased the distance that the migratory keratinocytes will have to travel to achieve closure. In addition, the Cx43 AS ODN treatment also speeds the normal downregulation of Cx43 protein within the keratinocytes at the leading edge [24, 16], and in this way may speed up their transformation to the migratory phenotype and thus contribute to the enhanced re-epithelialization rate seen in treated wounds.

Accelerating wound closure and reducing the tissue damage caused by neutrophils impacts downstream events such as granulation tissue deposition. Measurements of granulation tissue area in H&E-stained sections showed that, compared to controls, AS ODN-treated wounds had significantly ($P < 0.046$ 7 days and 12 days) smaller areas of granulation tissue at 7 days (Figures 4I–4K) and 12 days (data not shown) after lesioning. The nature of the granulation tissue was very similar in both groups, as assessed by fibronectin staining for extracellular matrix distribution and VEGF staining for blood vessel density.

Conclusions

The complex process of wound healing involves the coordinated activity of a variety of cell types and mechanisms. By transiently disrupting one signaling pathway, through transient knockdown of a specific type of gap junction channel, Cx43, we have been able to damp down the inflammatory response at two different kinds of wound site while enhancing the rate of re-epithelialization. Positively influencing early events in the wound healing process has long-term effects on the continuing cascade of events that completes it. Transient Cx43 downregulation targets the early inflammatory re-

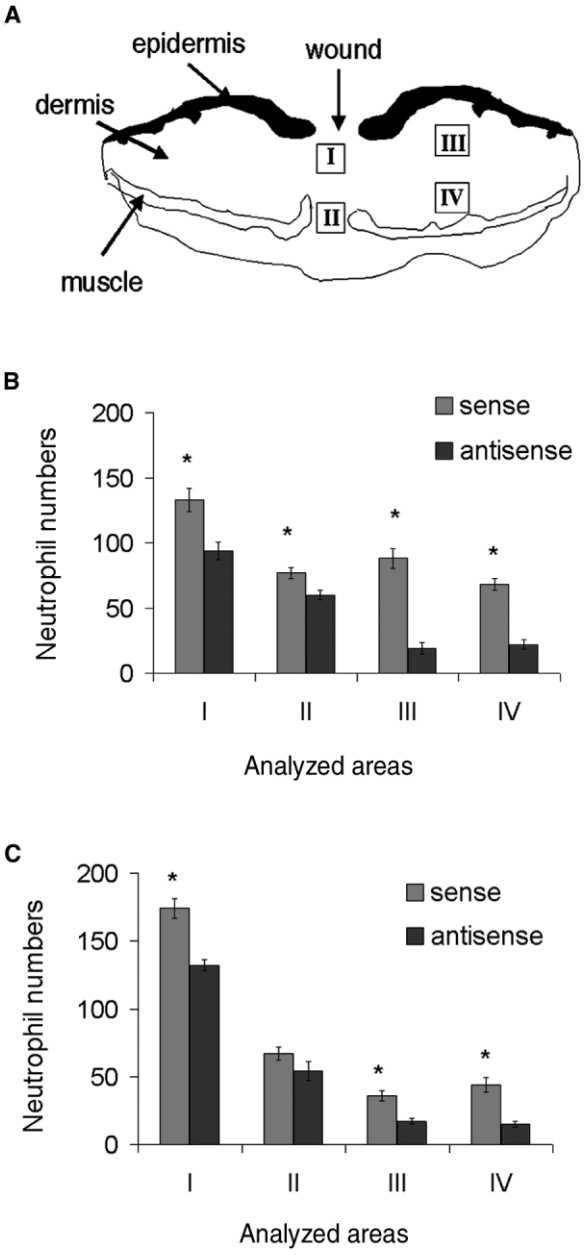
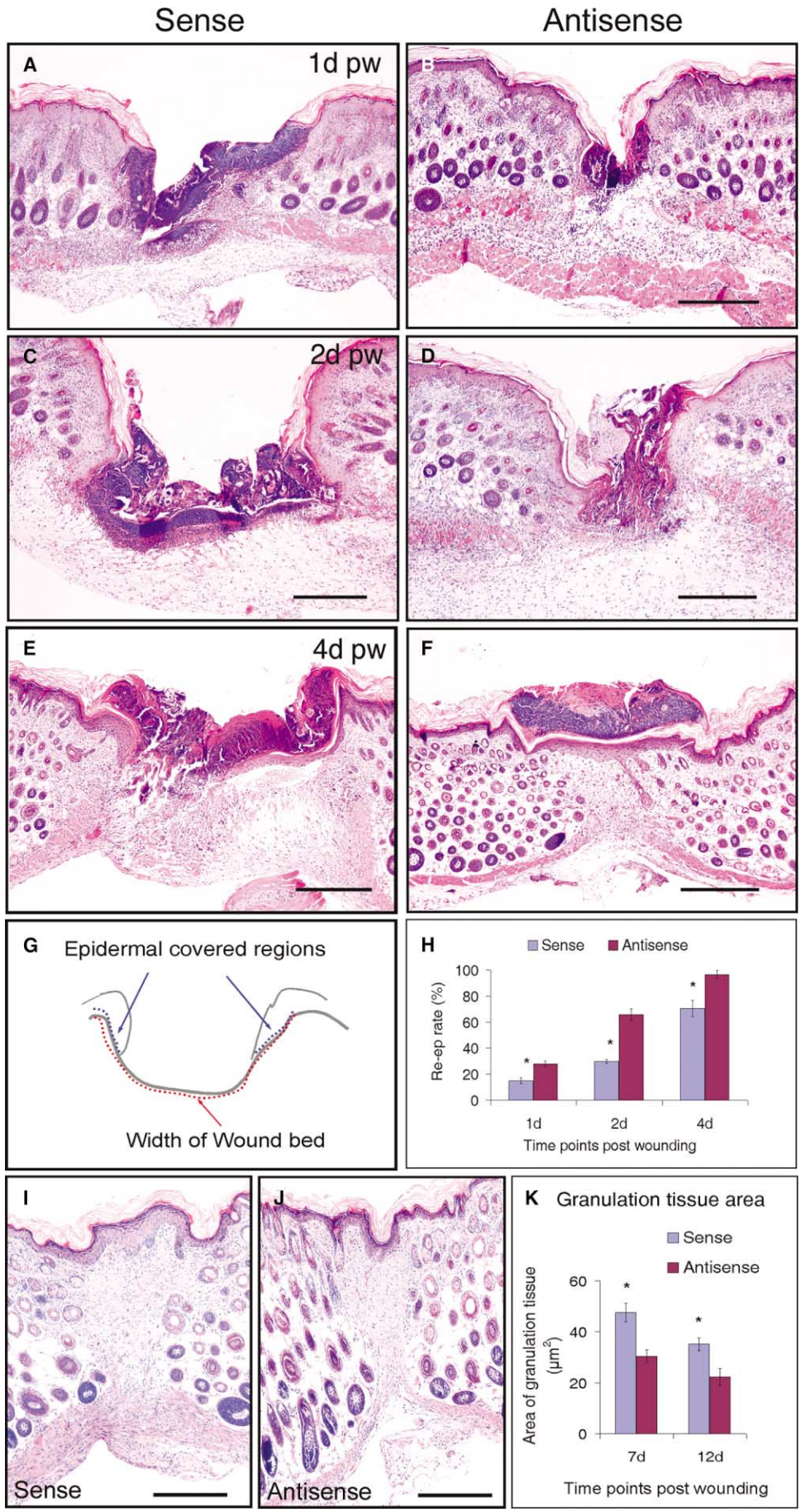


Figure 3. Reduced Neutrophil Infiltration in Cx43 Antisense-Treated Wounds

(A) A diagrammatic representation of a section through a 1 day incisional lesion indicates areas I–IV where counts of neutrophils were made. Site I lies beneath the fibrin clot, site II is in the deep dermal region of the wound site, and sites III and IV are corresponding regions in the skin adjacent to the lesion.

(B and C) Histograms showing the comparison of neutrophil numbers in defined areas I–IV in treated and untreated incisional lesions at 1 day (B) and 2 days (C) after wounding. The data represent the mean ± SEM values of six animals. The statistical significance of the data was analyzed with the Wilcoxon Signed Rank test. A significant difference (*) was found on days 1 and 2 at the wound site ($P < 0.046$) and in adjacent dermal tissue ($P < 0.028$). Scale bars represent 25 μm.


Sense
Antisense
A
1d pw
B
C
2d pw
D
E
4d pw
F
G
Epidermal covered regions
Width of Wound bed
H
Sense
Antisense
Re-ep rate (%)
100
80
60
40
20
0
1d
2d
4d
Time points post wounding
I
Sense
J
Antisense
K
Granulation tissue area
Sense
Antisense
Area of granulation tissue (μm²)
60
40
20
0
7d
12d
Time points post wounding

sponse, prevents its subsequent amplification, and blocks the exuberant influx of reactive leuckocytes that leads to scarring [25]. Concurrently, the same Cx43 knockdown also appears to kick-start the early stages of epidermal closure by bringing forward the natural downregulation of Cx43 in the cells of the wound leading edge. This study suggests that Cx43 AS ODN treatment may be an effective and safe wound healing therapy that may have widespread applications in tissue repair.

Supplemental Data

Supplemental Experimental Procedures are available online at http://www.current-biology.com/cgi/content/full/13/19/1697/DC1/.

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Figure 4. Cx43 Antisense Treatment Accelerates Wound Re-Epithelialization and Reduces the Area of Granulation-Tissue Deposition

(A–F) Representative examples of H&E-stained sections through incisional lesions treated with sense (A, C, and E) or Cx43 antisense (B, D, and F) ODNs at 1 day (A and B), 2 days (C and D), and 4 days (E and F) after wounding.

(G) Diagram illustrating the method of measurement of re-epithelialization in histological sections taken from the center of the lesion. The red dotted line indicates the length of the wound bed, and the blue dotted line from the leading edge of the epidermis to the end of thickened epidermis, indicates the re-epithelialized region. Measurements were taken from a minimum of eight animals per time point.

(H) Histogram showing the re-epithelialization rates in Cx43 sense- and antisense-treated incisional lesions at 1 day, 2 days, and 4 days after wounding. A statistically significant improvement in the rate of re-epithelialization was shown via the Wilcoxon Signed Rank test (an asterisk indicates P values of <0.017 at 1 day, < 0.012 at 2 days, and <0.028 at 4 days).

(I and J) Examples of H&E-stained sections through the center of a 7 day incisional wound treated with Cx43 antisense (I) and sense (J) ODNs show the reduced granulation tissue deposition in antisense-treated lesions. Measurements of granulation tissue area were made in a minimum of six animals per time point.

(K) Histogram showing the comparison of granulation tissue areas in Cx43 sense- and antisense-treated wounds at 7 days and 12 days after wounding. A Wilcoxon Signed Rank test showed that, compared to controls, the antisense-treated incisional lesions had significantly smaller areas of granulation tissue deposition at both 7 days and 12 days (an asterisk indicates $P < 0.046$ at 7 and 12 days).

Scale bars (A–F, I, and J) represent 100 μm.

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