Title: Targeting Cx26 Expression by Sustained Release of Cx26 Antisense from Scaffolds Reduces Inflammation and Improves Wound Healing

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Abstract

The gap junction protein Cx26 is expressed at high levels in naturally hyperthickened epidermal layers as well as pathological hyperkeratotic disease states, such as warts, psoriatic plaques and chronic wound edges. The over-expression of Cx26 is also linked with inflammation, breakdown of the skin barrier function and perturbed wound healing. Here we used a collagen scaffold implanted into a rat excisional skin wound. This induced a foreign body type reaction characterised by epidermal thickening with elevated levels of Cx43 and Cx26, increased inflammation and perturbed healing. This was reminiscent of a chronic skin wound. If the same scaffolds were coated with an antisense molecule specifically targeting Cx26, that had a slow sustained release, this prevented the abnormal upregulation of Cx26 protein at the wound edge. Knocking down Cx26 protein levels below those seen in normal wound healing had no adverse effects on the healing process but instead reduced the epidermal thickening and also the inflammatory response, whilst at the same time promoting the healing response. Treatment with Cx43/26 antisense may promote healing of chronic wounds. The Cx26 antisense may also be helpful in treating other skin conditions where Cx26 is overexpressed.

Introduction

Chronic wounds such as venous leg ulcers and diabetic foot ulcers are a growing problem worldwide as the population ages and the prevalence of diabetes and obesity continues to rise[1, 2, 3]. These wounds fail to pass through the normal phases of healing and stall in a highly inflammatory state[4]. The cost to healthcare providers is growing[5], with a 2009 estimate for the USA reported as $25B annually[3] and more recently in 2015 for the UK at £5B a year[6]. These non-healing wounds have a severe effect on the quality of life[7] and the life expectancy of sufferers[8] who in the case of diabetic wounds, can often end up loosing a lower limb, after which they have an average life expectancy of 5 years. Unfortunately there is currently little in the way of effective treatments for these debilitating wounds and new therapeutic solutions are actively being sought[2].

It is now well established that the gap junction protein connexin 43 (Cx43) plays a central role in the wound healing process[9, 10, 11]. Indeed, targeting Cx43 with either antisense or mimetic peptides is able to promote acute wound healing and kick start healing in chronic wounds in man[12-14]. In normal acute wound healing Cx43 is naturally downregulated in wound edge keratinocytes and fibroblasts (but remains high in the proliferative cells behind the healing wound edge). This change takes place within the first 24-48 hours and coincides with the adoption of a migratory phenotype in these wound edge cells[11, 15]. In situations where Cx43 is not downregulated, such as in diabetic and chronic wounds, cell migration is significantly perturbed and wound healing is severely retarded[16, 17]. Cx43 in the cell membrane binds to a number of communicating and adherence junctions to form nexus of junctional proteins, which also links into the cytoskeleton[18]. These communicating and adhering junctions need to be down regulated in order for cell repolarization and normal migration to take place and bring about wound closure [17]. This makes it a promising target for a therapeutic intervention[9]. Targeting Cx43, with antisense and peptide based approaches, in chronic wounds is having promising effects in promoting healing in a number of clinical trials treating venous leg ulcers [19, 20] and diabetic foot ulcers[20].

However, Cx43 is not the only gap junction protein that is upregulated in chronic wounds, which typically have a hyperproliferative epidermis and in which Cx26 & 30 have been shown to be massively elevated[21]. As yet the potential role of Cx26 in wound healing is less clearly defined. It is known that Cx26 and Cx30 are naturally upregulated in the wound edge keratinocytes as Cx43 is downregulated though it is not fully understood how this influences the wound healing process[15]. It is possible that wound edge keratinocytes

form a communication compartment separate to the proliferating keratinocytes further away in the uninjured epidermis. Cx26 and Cx30 can form junctions with each other, but not Cx43, so the two other connexins could establish a unique compartment with properties distinct to the rest of the epidermis[22].

It is also unclear whether the Cx26 that is expressed in migratory keratinocytes during re-epithelialisation is beneficial to wound healing. There is increasing evidence to suggest that high levels of Cx26 may not be beneficial to wound closure. In streptozotocin (STZ) diabetic rat wounds where healing is perturbed, Cx26 was found to be considerably upregulated in wound edge keratinocytes and for a long distance away from the wound[16]. In an elegant study by Djalilian et al. 2006 where they used involucrin to drive the over expression of Cx26 in keratinocytes, they reported that wound healing was considerably delayed[23]. In addition, in rodent skin wounds containing a collagen scaffold that induced a foreign body reaction and delayed healing, wound edge keratinocytes were found to form a bulb of non-migratory cells, which expressed very high levels of Cx26 (reminiscent of the STZ diabetic rat wounds) [24, 25]. Cx26 up-regulation has also been linked to inflammatory and hyperproliferative skin conditions[26, 27]. Interestingly the buccal mucosa has many more layers of cells than the external epidermis of the skin and appears reminiscent of hyperproliferative skin. Like chronic wound edges it also contains very high levels of connexins Cx26, Cx30 & Cx43. However, lesions to the buccal mucosa heal very rapidly compared to skin lesions. A key reported observation was that buccal mucosa very rapidly down regulates Cx26 within 6 hours of injury, whereas in the slower healing skin epithelium Cx26 becomes slightly elevated in wound edge keratinocytes in the first 24-48 hours after injury[28]. Taken together these observations suggest that high levels of Cx26 may inhibit keratinocyte migration and wound reepithelialisation and that rapid downregulation of Cx26 may be able to improve healing rates.

Here we investigated the effect of a combination of two antisense DNA molecules to target Cx26 in a wound. We induced Cx26 downregulation in a scaffold based wound healing model where we know that Cx26 is greatly upregulated in the wound edge keratinocytes and where healing is perturbed [24]. In this model we have previously found that Cx43 is also upregulated in wound edge keratinocytes and Cx43 antisense treatment improves the healing process[24, 25]. In this study we extended our research theme by comparing the effects of isolated knock down of Cx26 with our previous observations from knocking down

Cx43. We report on the effect of this intervention on the wound healing process and then the effects of a combination of Cx43 and Cx26 antisense treatment.

Materials and Methods

Scaffold fabrication

The collagen electrospinning process has been described in detail previously [25, 29]. Briefly, the polymer solution was spun onto a 9 cm^2 foil sheet and 14ml produced scaffolds with an average thickness of 0.4 mm and 30 ml produced an average thickness of 0.8 mm. Uncoated scaffolds were crosslinked by immersion in a high (15% wt./v.) concentration of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) hydrochloride (99.5% purity, Apollo) in a 1:10 water to acetone solution. Scaffolds were washed for 20 min in standard Phosphate Buffered Saline (PBS) and then sterilised using 70% ethanol for 1 h, followed by washing 3 times with sterile PBS. Scaffold discs were then excised using a 6 mm biopsy punch matching the size of the excisional wounds.

Application of Cx26 and or Cx43 asODN to scaffolds via polymer coatings

Both Cx43 and Cx26 asODN sequences at 300μM concentration were incorporated into polymer coatings using an layer by layer emulsion coating technique. The rat Cx43asODN sequence 5'-GTA ATT GCG GCA GGA GGA ATT GTT TCT GTC-3'. A non-functional sense (sODN) sequence 5'- GAC AGA AAC AAT TCC TCC TGC CGC AAT TAC-3'. In the case of Cx26 asODN, two different novel sequences (termed 'AS3' 5'-TGTATTGGGACAAGGCCAGG-3' and 'AS6' 5'-ATCTCTTCGATGTCCTTAAA-3') that we identified as potentially effective following pilot *in vivo* screening of multiple sequences selected by in-silico and in vitro methodologies[30]. Cross-annealing between the two asODN sequences was ruled out before combining the sequences. Either Polycaprolactone (PCL) or Poly (D, L-lactice-co-glycolide) (PLGA) was dissolved in dimethyl carbonate at 10% wt./v. The asODN sequences were dissolved in water and mixed with the polymer solutions to 300μM final DNA concentration. In the case of Cx26 asODN, both AS3 and AS6 sequences were each dissolved at a final concentration of 300 μM, and for the combined Cx26/Cx43asODN scaffolds the three asODN sequences were each incorporated at a 300μM concentration. The two immiscible layers were gently homogenized for 10 seconds to produce an emulsion. Individual 6 mm diameter collagen scaffolds were dipped in polymer-asODN solutions and transferred to liquid nitrogen and

lyophilised overnight to remove the solvent. This process was repeated either a further 3 times to produce 4 layers of polymer + asODN, or 7 times for ‘double coated’ scaffolds, which had 4 layers of PCL + asODN then 4 layers of PLGA + asODN.

Surgery

Six week old Sprague Dawley rats were used with UK Home Office approval and Singapore Institutional Animal Care and Use Committee (IACUC) approval. Rats were initially anaesthetised using 4% isoflurane, 20% oxygen,10% nitrous oxide, then maintained using 1.5% isoflurane. Buprenorphine (Vetergesic®) 0.03 mg/ml was injected subcutaneously before operation. Their backs were shaved and a layer of Nair® hair removal cream applied and hair was removed using a scraper followed by a moistened gauze pad. Rats were maintained on heated mats and four full-thickness excisional biopsy punch wounds were made using a 6 mm punch (Kai Industries UK). Scaffolds were applied directly to wounds, and covered with sterile Tegaderm™ film dressing (3M). Post-procedure, rats were recovered in a heated chamber and rehoused in individual cages.

Wound Tissue collection

Animals were killed at 1, 3, 5, 10 and 15 days post wounding N=8 per time point Total N=40. Wounds were macroscopically imaged using a Leica MZ8 dissection microscope and then excised and fixed in 4% paraformaldehyde (PFA)-PBS solution for 24h at 4°C. Tissue was transferred to 20% sucrose overnight, washed in PBS and bisected. Each half was frozen in O.C.T. medium (Sakura Finetek, UK) and sectioned using a Leica CM1900 cryostat at 5 μm (for H&E staining) or 10 μm (for immunofluorescence staining).

Haematoxylin and eosin staining

Frozen sections (5 μm) were thawed then immersed in tap water for 5 min before transferring to Harris’ haematoxylin solution for 30 seconds followed by a bath of running tap water for 5 min. Slides were dipped in 1% acid alcohol (1% concentrated hydrochloric acid, 70% ethanol, 29% distilled water) for 3 seconds, to reduce background staining. Then transferred to a container of running tap water for a further 5 min. This was followed by 15 seconds in eosin B solution, followed by 5 seconds in a tap water to rinse away excess stain before immersion in 70% ethanol for 2 min, 100% ethanol for 2 min a second 100% ethanol for 2 min, to dehydrate the tissue. Slides were transferred to xylene for 5 min, then a second xylene for 5 min before mounting in DPX mountant.

Immunofluorescence staining

Sections (10 µm) were immersed in cold acetone for 5 min before blocking with a 0.1 M L-lysine (Sigma UK) PBS solution containing 0.1% Triton-X-100 (Sigma UK). Sections were stained for 1 h using an antibody to Cx43 (1:2000 dilution, C6219, Sigma UK) or Cx26 (1:200 dilution[31]). They were then incubated with a goat anti-rabbit Alexa 488 secondary antibody (A11008, Invitrogen UK) at a 1:400 dilution for 1 h. Sections were counterstained using Hoechst solution (both Hoechst 33528 and Hoechst 33342 dyes at 1:50,000 dilution, Sigma UK).

Imaging

Tissue was imaged using a Leica SP8 confocal microscope with a X40 1.2NA objective. Fluorophores were imaged sequentially. Single optical section 8-bit images were acquired in a distal region of skin and at the wound edge at a resolution of 1024 by 1024 pixels. All settings were kept identical between treatments to allow image analysis comparisons to be made.

Epidermal thickening and re-epithelialisation measurements

Epidermal thickness was measured using ImageJ[24] on H&E stained tissue at the thickest point along the basal to spinous layer axis within 150 µm of nascent epidermis. The length of nascent epidermis was measured on either side of the wound using ImageJ and averaged to give a re-epithelialisation distance (n=8 rats per time point).

Connexin 26 and 43 protein quantification

Cx26 and Cx43 quantified on D1, D3 and D5 wounds in binary images of wound edges using ImageJ as described previously[16, 24]. Images were processed under identical threshold parameters and the number of connexin positive pixels within the terminal 100 µm of the epidermis quantified. Connexin levels were expressed per square micron of epidermis and normalised to distal levels. Cx26, distal levels were extremely low, so small fluctuations between samples could result in large differences between values. Therefore Cx26 distal levels were averaged for the group, and this value was used to calculate changes in wound edge keratinocyte Cx26.

Polymorphonuclear cell quantification

H&E stained D1, D3 and D5 wounds were microscopically assessed at 63 X magnification. At 700 µm from the wound edge in the lower dermis, cells with a polymorphonuclear morphology (PMNs) were counted in three fields of view at this point to quantify inflammatory leukocytes infiltrating uninjured tissue. A total of n=5 wounds were assessed per time point.

Measurement of granulation tissue area

D10 and D15 wounds were H&E stained and imaged using a Zeiss Axio Scan.Z1 slide scanner. A series of brightfield images were captured using a 20 X objective, then stitched together to form a montage, which was imported into ImageJ. The granulation tissue was measured between the wound margins above the muscle and panniculus carnosus layers, using the freehand line tool. A total of n=8 wounds were assessed per time point.

Statistical analyses

Connexin quantification, PMN count and wound edge measurements were analysed using GraphPad Prism 5.0 software. Having confirmed normality we used a one-way Analysis of Variance ANOVA tests followed by Tukey's post-hoc tests to determine statistical significance[24]. Data is presented as mean and SEM. $P<0.05$ was taken as significant.

Results

Macroscopic appearance of wounds

In this study we had seven different variations of wound treatments to compare: No treatment, Uncoated Scaffold, PCL/PGLA Coated Scaffold, Cx26 Antisense Coated Scaffold, Cx43 Antisense Coated Scaffold, Cx43 Sense Coated Scaffold and Cx26/Cx43 Antisense Coated Scaffold.

In order to assess the efficacy of the Cx26 asODN sequences on Cx26 knockdown *in vivo*, collagen scaffolds that had been coated with the combination of two Cx26 asODN sequences were applied to 6 mm diameter full thickness wounds on rats. In addition, some scaffolds were coated with both the Cx26 asODN sequences as well as the Cx43 asODN sequence resulting in a combined Cx26/43 asODN coated scaffold. Rats were culled at D1, 3 and 5, after which the wounds were macroscopically assessed (Figure 1) and compared to the control groups: namely, no treatment, uncoated scaffold and PCL/PGLA

coated scaffold . Images of control wounds are reproduced from Gilmartin et al 2016[25] with permission from Wiley-VCH (at the request of reviewers). These shared control group from this previously reported study were used for welfare and ethical reasons to avoid unnecessary animal usage by repeating redundant control groups. The current Cx26 and Cx26/43 experiments were undertaken at the same time as the shared controls.

At D1, wounds across all treatments appeared macroscopically similar to the initial 6 mm wound. At D3, uncoated scaffold wounds had changed very little in size and the coated scaffold and sense coated control scaffold were both only slightly smaller. The Cx26 and Cx26/43 asODN coated scaffold treated wounds had all reduced in size to a level comparable to that of untreated wounds. By D5, the uncoated scaffold wounds were still the largest. The coated scaffold and sense coated scaffold had reduced some more but were still much larger than untreated wounds. Wounds treated with the Cx26 and Cx26/43 asODN coated scaffolds had considerably reduced in size, to a similar or greater extent to that of untreated wounds. Interestingly the combined Cx26/43 asODN scaffold treated wounds did not, on average, appear to be more reduced in size relative to the Cx26-only or Cx43-only scaffold treatments.

Day 1

Cx26 asODN coated scaffolds significantly improve wound re-epithelialisation

Measurements of wound re-epithelialisation at D1, wounds containing scaffold only, coated scaffold and sense scaffold were all retarded in their re-epithelialization compared to untreated wounds. Whereas wounds treated with the Cx26 asODN only scaffold had an average re-epithelialised a distance of 404 μm a 48% greater than the 274 μm of untreated wounds ($P < 0.05$) (Figure 2). This was similar to the average distance for Cx43 asODN scaffold treated wounds (407 μm)[25]. The combined Cx26/43 asODN coated scaffolds resulted in a moderately higher re-epithelialisation distance (494 μm) 22% more than Cx43 asODN only scaffolds. All three types of antisense containing scaffolds re-epithelialized significantly further than all control wounds without antisense treatment.

Cx26 asODN prevents epithelial wound edge Cx26 upregulation

In untreated wounds there is normally a significant increase in the levels of Cx26 in the wound edge epithelium and this was seen here with an increase of around 2000%

compared to distal levels of Cx26. Uncoated scaffolds raised this level of Cx26 to 3000%, as did coated (2500%) and sense coated scaffolds (2000%). However, Cx26 upregulation in both Cx26 asODN and Cx26/43ODN scaffold treated wounds was significantly reduced (141% and 226% upregulation respectively) compared to the wounds without antisense (Figure 2). Interestingly, the application of Cx26 asODN coated scaffolds did not result in a reduced level of wound edge Cx43 expression at D1 and was very similar (at 122% upregulation) to that of the control scaffold wounds (Figure 2).

Day 3

The presence of a scaffold (without antisense) continued to retard re-epithelialization on D3. However, Cx26 asODN only coated scaffolds had re-epithelialised on average 791 μm (Figure 3), which was significantly more than the three different control scaffold treatments, which had on average re-epithelialised only 51% as much. This re-epithelialization was very similar to Cx43 asODN only treated wounds[25]. Wounds treated with the combined Cx26 and Cx43 asODN had re-epithelialised still further with an average of 972 μm. This was 12% further than those treated with the Cx43 asODN scaffold, and 19% further than those treated with the Cx26 asODN only scaffold.

Cx26 levels increased by about 4000% in untreated wounds, which was similar to coated and sense coated scaffold but the levels in uncoated scaffold were even higher (6000%). Cx26 asODN coated scaffolds were able to prevent the elevation in Cx26 expression at the wound edge to only a 48% rise, while for the combined Cx26/43 asODN scaffold there was a 50% rise (Figure 3). For both of these types of scaffold the rise was significantly lower than all wounds without antisense[25]. Interestingly, the Cx43 asODN coated scaffold wound edges expressed significantly lower levels of Cx26 than the no antisense control treatments. At this stage untreated wounds had a downregulation of Cx43 in wound edge keratinocytes whereas scaffolds without antisense caused an increase in Cx43 of between 200-300%. This increase was very similar to that seen with the Cx26 only scaffold whereas scaffolds that contained Cx43 asODN had a decrease in Cx43 of around 90%.

Day 5

On D5 re-epithelialization of untreated wounds (882 μm) and wounds treated with the control scaffolds without antisense was very similar[25] (Figure 4). Cx26 asODN coated scaffold wounds had re-epithelialised a distance of 1325 μm, which was significantly further than any of the control groups but very similar to that of Cx43 asODN treatment (1345 μm). However, the combined Cx26 and Cx43 asODN scaffold again produced in the greatest increase in re-epithelialisation, at 1695 μm, which was 26% more than Cx43 asODN only scaffolds and 28% greater than the Cx26 asODN coated scaffolds. Across all wounds at D5, those with a scaffold that contained Cx43 asODN (either Cx43 asODN only scaffolds or both Cx43 and Cx26 asODN) had on 5 occasions fully re-epithelialised. Only 1 wound treated with the Cx26 asODN only scaffold had fully re-epithelialised and only one of the untreated control wounds had fully re-epithelialised.

At D5, wound edge keratinocyte Cx26 levels were considerably reduced compared to the levels at D3 across all treatments (Figure 4). Untreated wounds were only showing 199% increase, with uncoated scaffolds, control coated scaffolds and Cx43 sODN scaffolds 417-431% increase. However, Cx26 asODN scaffolds resulted in significantly lower levels of Cx26 expression at the wound edge compared to these control treatments and had increased Cx26 levels to only 68% above distal levels. Combined Cx26/43 asODN scaffolds resulted in a slightly greater repression of Cx26 asODN (87% above distal levels). Cx43 asODN only scaffolds also reduced the elevation in Cx26 levels but not to the same degree. In contrast to D1 and D3, at D5 the Cx26 asODN only scaffold resulted in a significant reduction in Cx43 levels relative to the untreated control scaffold and the coated control scaffold treatments (Figure 4). While control scaffolds without antisense all resulted in an upregulation of Cx43 at the wound edge epidermis, Cx26 asODN scaffolds resulted in 62% of distal levels Cx43, similar to the 57% of distal levels observed in untreated wounds.

Epidermal thickening

Thickening of the nascent epidermis following Cx26 asODN scaffold application was also measured, since Cx26 expression is commonly linked to epithelial thickening disorders[26, 27]. Uncoated scaffolds induced an epidermal thickening to about 100 μm which was significantly thicker than untreated controls at around 50 μm on D1 and this difference was maintained at all time points. Coated scaffolds had lower epithelial thickening than the uncoated scaffold and were not significantly different from untreated controls at all time points. Cx26 asODN coated scaffolds had significantly lower epithelial thickening values

than the scaffolds without antisense at most time points. The thickening was a similar level to that of Cx43 asODN coated scaffolds at each time points and was always less than untreated controls (Figure 5). The combined Cx26+43 asODN scaffold was significantly thinner than untreated wounds at D3 (P <0.05) but did not appear to have a synergistic effect in reducing epithelial thickening compared to individual connexin antisense treatments.

The effect of Cx26 asODN coated scaffolds on the inflammatory cell response

The number of cells with a rounded morphology with the characteristic multi-lobbed nuclei of polymorphonuclear leukocytes (PMNs) were identified and counted in a region of dermis distal to the wound[25]. This was performed in order to determine whether treated scaffolds induced a less extensive inflammatory cell response in the surrounding tissues (Figure 6).

At D1 the uncoated scaffold produced the highest level of PMNs around three times that of untreated wounds. Coating the control scaffolds reduced the number of PMNs but these were again higher than untreated wounds. All of the antisense coated scaffolds resulted in lower PMN counts than untreated wounds but these were only significant for the scaffolds without antisense. Cx26 asODN treated tissue was not significantly different to the Cx43 asODN coated scaffolds or the combined Cx26 + Cx43 asODN scaffolds.

On D3, the number of PMNs in each treatment had a very similar profile to that seen on D1. At D5 however, PMN numbers were greatly reduced in all treatments. The PMN numbers were still greatest in scaffolds that did not contain antisense. All three types of scaffold containing antisense had very similar PMN numbers, which were significantly lower than the scaffolds without antisense.

Granulation tissue area measurements at D10 and 15.

The granulation tissue area at D10 in untreated wounds was 3.6 ± 0.2 mm^2, which was not significantly different from any of the scaffolds that did not contain antisense. However, all three of the different antisense containing scaffolds had similar and significantly smaller granulation tissue areas than untreated wounds and scaffolds without antisense (Figure 7).

By D15 the granulation tissue in all groups had shrunk to approximately half the size of D10. The differences in granulation tissue area were maintained and all three containing antisense were significantly smaller than untreated controls and scaffolds without

antisense. Scaffolds that contained Cx43 antisense had slightly smaller granulation tissue areas that Cx26 antisense alone but this difference was not significant.

Discussion

It has been reported that placing solid collagen scaffolds into a wound bed can induce a foreign body type reaction in the cells of the wound edge, which can inhibit the healing process[24]. It has been proposed that this may also be a useful model to study perturbed wound healing in rats[24]. As part of this foreign body inflammatory response reaction there is an influx of neutrophils and the epidermal wound edge forms a thickened bulb of non-migratory cells that express high levels of Cx43 and Cx26[24]. This has a very similar appearance to the epidermal wound edge from a STZ diabetic rat, which also has elevated Cx43 and Cx26 and shows perturbed healing[16]. It is also consistent, although not as extreme, as the hyper-thickening of the epidermis and over expression of connexins reported in a wide range of human chronic wounds[21, 32]. In these chronic wounds, Cx43 upregulation is a notable common characteristic [16, 21] however, less well appreciated is the fact that Cx26 is also frequently upregulated many fold beyond the normal levels encountered during acute wound healing[16, 21, 32]. It is not yet clear what effects this upregulation may bring about with respect to wound healing.

Our previous studies have shown that scaffolds coated in a specific Cx43asODNs are able to reduce the inflammation in the wound around the scaffold[24]. At the same time this reduces the levels of Cx43 and indirectly that of Cx26, which in turn is associated with improved healing. Previously expression levels of Cx43 and Cx26 have been shown to be linked as when Cx43 was knocked down in keratinocytes with a specific Cx43 shRNA, the levels of Cx26 protein was also found to be reduced[33]. Application of Cx26 asODN scaffolds to wounds knocked Cx26 protein levels down below those levels seen in a normal untreated wound and did not appear to inhibit wound closure at a macroscopic level. These wounds were similar in size to untreated wounds and to those with Cx43 asODN coated scaffolds. This supports the hypothesis that the elevation in Cx26 protein seen in the migratory wound edge keratinocytes is not required for normal wound healing and may potentially even slow it down. Uncoated scaffolds inhibited wound closure and caused an elevation of Cx26 protein in the epidermal wound edge. Combined Cx26/43 asODN coated scaffold treated wounds did not seem to enhance the reduction in wound

size beyond the effect of each of the individual antisense treatments. Unlike Cx43, Cx26 knockdown in wounds has not previously been linked to a faster reduction in wound area, and it still remains unclear how Cx26 knockdown could mechanistically affect wound closure.

Hyperthickening

There is a considerable amount of evidence correlating high levels of Cx26 with hyperthickened epidermis. For instance, following tape stripping of skin Cx26 is found to become elevated in the epidermis, which precedes an increase in cell proliferation and epidermal thickening[27]. Cx26 is also found at high levels in hyperproliferative vaginal and buccal epithelia[27, 28]. Similarly Cx26 has been reported to be highly expressed in hyperproliferative viral warts where it can become colocalized with Cx43[34], which may be a marker of the diseased state as normally Cx43 and Cx26 do not interact[22]. Another example of high levels of Cx26 and hyperthickend skin is in psoriatic plaques though the role of Cx26 in these plaques is yet to be fully determined[26, 35]. Skin hyperthickening can also be induced in humans by topical application of retinoic acid, which results in a 2.5 fold increase in epidermal thickness and a turn on of Cx26[36]. Evidence to suggest that it is in fact the Cx26 overexpression that drives the thickening comes from a study of genetically modified mice, where involucrin was used to drive the overexpression of Cx26 in keratinocytes, which resulted in keratinocyte hyperproliferation, epidermal thickening and slowed wound healing[23]. Indeed the levels of Cx43 and Cx26 in keratinocytes has been shown to be linked to their differentiation as when they are reduced, premature differentiation takes place[33]. This suggests that targeting the expression of Cx26 when it is overexpressed, as reported in chronic wounds[21], may have therapeutic benefits in reducing hyperthickening and promoting healing. This current animal model used in this study is consistent with illustrating this potential therapeutic effect.

Cytoskeletal interactions

As part of the wound healing response leading edge keratinocytes need to down regulate their junctional complexes, freeing them up to allow the cells to become migratory, whilst remaining on in the proliferating cells behind[15]. It is well known that Cx43 has multiple junctional and cytoskeletal binding partners and this may be one of the reasons why it naturally downregulates as cells become migratory [18, 37]. However, it appears that despite having a very short C-terminal tail Cx26 is able to directly interact with the coiled-coil

domain of the tight junction protein occludin[38]. In the wound healing response occludin needs to relocate to the leading edge to repolarize the microtubule organizing centre (MTOC) and organize the cytoskeleton for migration to take place [39]. Cx26 is normally upregulated in the leading edge keratinocytes, however, very high levels of Cx26 potentially binding to occludin may impair its ability to relocate to the leading edge and hence inhibit migration.

Neutrophil response

Polymorphonuclear leukocytes (PMNs) could be easily identified in the dermis from the histological morphology of their multi-lobbed nuclei. Similar to Cx43 asODN, Cx26 asODN coated scaffolds had a significant effect on reducing the numbers of inflammatory PMNs spreading into the uninjured dermis. The link between Cx26 and inflammation has been shown to be associated with increased hemichannel activity when Cx26 is overexpressed in keratinocytes, resulting in an increase in release of ATP, which in turn increased the inflammatory response[23]. The levels of Cx43 and Cx26 play an important role in the differentiation of the skin[33] and this elevated Cx26 level produced a psoriasis like effect damaging the skins natural barrier. Indeed extracellular ATP has been shown to be proinflammatory resulting in activation of the inflammasome and causing tissue damage[40]. Irritant chemicals have been shown to induce rapid release of ATP from keratinocytes, which promotes skin inflammation[41]. In this instance, in the control treatment of uncoated collagen scaffolds, we consider it likely that it may generate an irritation causing the elevation of Cx26, ATP release and promotion of the inflammatory response recruiting leukocytes into the tissues around the wound. The reduced inflammatory response seen with coating the collagen scaffold may reflect better biocompatibility of PCL/PGLA coating material in the wound compared to the uncoated collagen scaffold.

Conclusions

Elevated levels of Cx26 in the skin are associated with hyperproliferation of the epidermis, elevated inflammation, breakdown of the epidermal barrier and impaired wound healing. It appears that Cx26 antisense treatment can have a beneficial effect on the wound healing process, perhaps by reducing proinflammatory ATP release within the skin. In addition it may act by freeing up the Cx26 interaction with occludin allowing the leading edge keratinocytes to more easily polarise and become migratory. In addition, uncoated collagen scaffolds used in this context may provide us with a new rodent wound model for

exploring impaired healing and recreating aspects of human chronic wound biology. Aside from chronic wound healing applications Cx26 antisense treatments offer therapeutic opportunities in other disease states such as psoriasis, which has reported elevated Cx26. In these setting it is predicted to reduce both inflammation and hyperthickened plaque formation as well as improve the skin barrier function.

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Disclosures:

DLB is a founding scientist of CoDaTherapeutics Inc and holds shares in the company. ARJP, MM & SO were employed by CoDaTherapeutics at the time of the study.

Figure legends:

Figure 1. Effect of scaffold application on macroscopic wound appearance. Full-thickness rat wounds were treated with scaffolds coated with Cx26 asODN or a combination of both Cx26 and Cx43 asODN (denoted Cx26/43 asODN). Animals (n = 8) were culled at days 1, 3, or 5 (D1, D3, D5) after wounding and the wounds were macroscopically imaged. Scalebar = 1 mm. *Control data reproduced with permission* (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

Figure 2. D1 full-thickness wound healing following application of scaffolds coated with Cx26 asODN or a combination of both Cx26 and Cx43 asODN. Wound re-epithelialisation distances outlined by yellow dotted lines were measured for each type of scaffold applied and plotted as a graph (left column). Relative changes in Cx26 and Cx43 expression were also quantified in keratinocytes at the wound edge (WE) outlined by white dotted lines in confocal images of the corresponding immunostained sections (middle, right columns).

Scalebar = 100 μm. Data plotted as mean + SEM. 8 rat wounds per condition assessed. One-way ANOVA with posthoc Tukey's multiple comparisons test conducted to establish significance (*p<0.05, **p<0.01, ***p<0.001 Significant difference from No treatment, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Uncoated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Coated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Cx43 sODN, □p<0.05 Significant difference from Cx26 asODN). Control data reproduced with permission (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

Figure 3. D3 full-thickness wound healing following application of scaffolds coated with Cx26 asODN or a combination of both Cx26 and Cx43 asODN. Wound re-epithelialisation distances outlined by yellow dotted lines were measured for each type of scaffold applied and plotted as a graph (left column). Relative changes in Cx26 and Cx43 expression were also quantified in keratinocytes at the wound edge (WE) outlined by white dotted lines in confocal images of the corresponding immunostained sections (middle, right columns). Scalebar = 100 μm. Data plotted as mean + SEM. 8 rat wounds per condition assessed. One-way ANOVA with posthoc Tukey's multiple comparisons test conducted to establish significance (*p<0.05, **p<0.01, ***p<0.001 Significant difference from No treatment, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Uncoated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Coated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Cx43 sODN, □p<0.05 Significant difference from Cx26 asODN). Control data reproduced with permission (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

Figure 4. D5 full-thickness wound healing following application of scaffolds coated with Cx26 asODN or a combination of both Cx26 and Cx43 asODN. Wound re-epithelialisation distances outlined by yellow dotted lines were measured for each type of scaffold applied and plotted as a graph (left column). Relative changes in Cx26 and Cx43 expression were also quantified in keratinocytes at the wound edge (WE) outlined by white dotted lines in confocal images of the corresponding immunostained sections (middle, right columns). Scalebar = 100 μm. Data plotted as mean + SEM. 8 rat wounds per condition assessed. One-way ANOVA with posthoc Tukey's multiple comparisons test conducted to establish significance (*p<0.05, **p<0.01, ***p<0.001 Significant difference from No treatment,

□p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Uncoated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Coated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Cx43 sODN). *Control data reproduced with permission* (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

Figure 5. Thickness of the nascent epidermis following scaffold application. Full-thickness wounds treated with different types of scaffolds were measured for epithelial thickness within the end 150 μm of the nascent tip of the epidermis. Measurements recorded across time points of D1, D3 and D5 were plotted as means + SEM of n = 8 samples. One-way ANOVA with posthoc Tukey's multiple comparisons test conducted to establish significance (*p<0.05 Significant difference from No treatment, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Uncoated Scaffold, □p<0.05, □□p<0.01 Significant difference from Coated Scaffold, □p<0.05, □□p<0.01 Significant difference from Cx43 sODN). *Control data reproduced with permission* (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

Figure 6. Effect of scaffold application on the number of polymorphonuclear cells (PMNs) infiltrating the unwounded dermis. Full-thickness wounds treated with different types of scaffolds were assessed for polymorphonuclear cell invasion into the lower dermis 700 μm away from the wound edge. The images shown are typical of unwounded dermis distal to D3 wounds. Scale bar = 50 μm. Quantification of individual PMNs for each treatment across time points of D1, D3 and D5 were plotted as means + SEM of n = 5 samples. One-way ANOVA with posthoc Tukey's multiple comparisons test conducted to establish significance (□p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Uncoated Scaffold, □p<0.05 Significant difference from Coated Scaffold, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Cx43 sODN). *Control data reproduced with permission* (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

Figure 7. Effect of scaffold application on the late stages of wound healing. Full-thickness wounds treated with different types of scaffolds were measured for granulation tissue or wound area at days 10 or 15 (D10, D15) after wounding. Typical H&E stained images illustrating the quantified granulation tissue area of the individual wounds bounded by white dotted lines. Eight rat wounds per condition assessed. Data plotted as means + SEM. One-way ANOVA with posthoc Tukey's multiple comparisons test conducted to establish significance (*p<0.05, **p<0.01, Significant difference from No treatment, □p<0.05, □□p<0.01, □□□p<0.001 Significant difference from Uncoated Scaffold, □p<0.05, □□p<0.01 Significant difference from Coated Scaffold, □p<0.05, □□p<0.01 difference from Cx43 sODN). *Control data reproduced with permission* (2016) Advanced Healthcare Materials 5, 1786-99. Wiley-VCH.

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Figures: