Review

Novel therapies for scar reduction and regenerative healing of skin wounds

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Fibrotic scars deposited during skin wound healing can cause disfiguration and loss of dermal function. Scar differentiation involves inputs from multiple cell types in a predictable and overlapping sequence of cellular events that includes inflammation, migration/proliferation and extracellular matrix deposition. Research into the molecular mechanisms underpinning these processes in embryonic and adult wounds has contributed to the development of a growing number of novel therapeutic approaches for improving scar appearance. This review discusses some of these emerging strategies for shifting the balance of healing from scarring to regeneration in the context of non-pathological wounds. Particular focus is given to potential therapies based on transforming growth factor (TGF)-β signaling and recent unexpected findings involving targeting of gap junctional connexins. Lessons learned in promoting scarless healing of cutaneous injuries might provide a basis for regenerative healing in other scenarios, such as spinal cord rupture or myocardial infarction.

Introduction

Interventions that promote the healing of skin wounds have been around for millennia. The phrase 'to lick ones wounds' might be grounded in science showing that mammalian saliva contains anti-bacterial agents and growth factors that aid in wound resolution [1]. Given this history, it is surprising that the modern clinical approach to improving the healed appearance of skin wounds remains largely devoid of drug-based therapies. There are no licensed therapeutics in clinical use that have proven consistent in ameliorating excess deposition of scar tissue – a frequent and undesirable outcome of both surgical and non-surgical injuries to the skin.

The wound-healing progression that results in differentiation of a cutaneous scar involves the orchestration of a complex sequence of interactions between numerous cell types, extracellular matrix components and signaling molecules. There are several excellent reviews detailing the cellular, molecular and signal transduction biology of normal and abnormal wound healing [2–7]. This essay discusses concepts for scar amelioration that have emerged from this work, with a specific focus on the healing of non-pathological wounds in healthy individuals. Recent advances in the development of drug-based therapies for scar reduction are reviewed. Of particular interest are the potential for therapies resulting from manipulation of transforming growth factor (TGF)-β signaling and more recent studies indicating that the targeting of gap junctional connexins might provide an unexpected path to scar-free healing.

Basic aspects of wound healing and scar differentiation

A stereotyped overview of the healing of a skin wound in an adult human is provided in the cartoon shown in Figure 1 [5]. After cessation of bleeding (hemostasis) and clot formation, the process can be divided into three overlapping phases: inflammation, proliferation and scar maturation. Inflammation sets in within minutes of a skin injury; the first inflammatory responders are blood-borne neutrophils – a type of white blood cell, or leukocyte – that transmigrate across endothelia from local blood vessels. Later, monocytes – another leukocyte, and the macrophage progenitor – enter the wound by a similar mechanism. The proliferation phase proceeds typically over the next five to fourteen days and involves the initiation of repair processes for both the epidermal and dermal layers of the skin. Fibroblasts, macrophages and vascular tissues coordinately enter the wound to begin formation of a new dermal composite – the granulation tissue. Fibroblasts and myofibroblasts lay down collagen-rich connective tissues comprising this composite, and myofibroblasts also contribute to wound contraction. Simultaneously, in a process termed reepithelialization, keratinocytes at the wound edge migrate over the granulation tissue to differentiate a new outer layer of epidermis. The healed wound finally enters a maturational phase, during which time granulation tissue continues to be remodeled by its constituent

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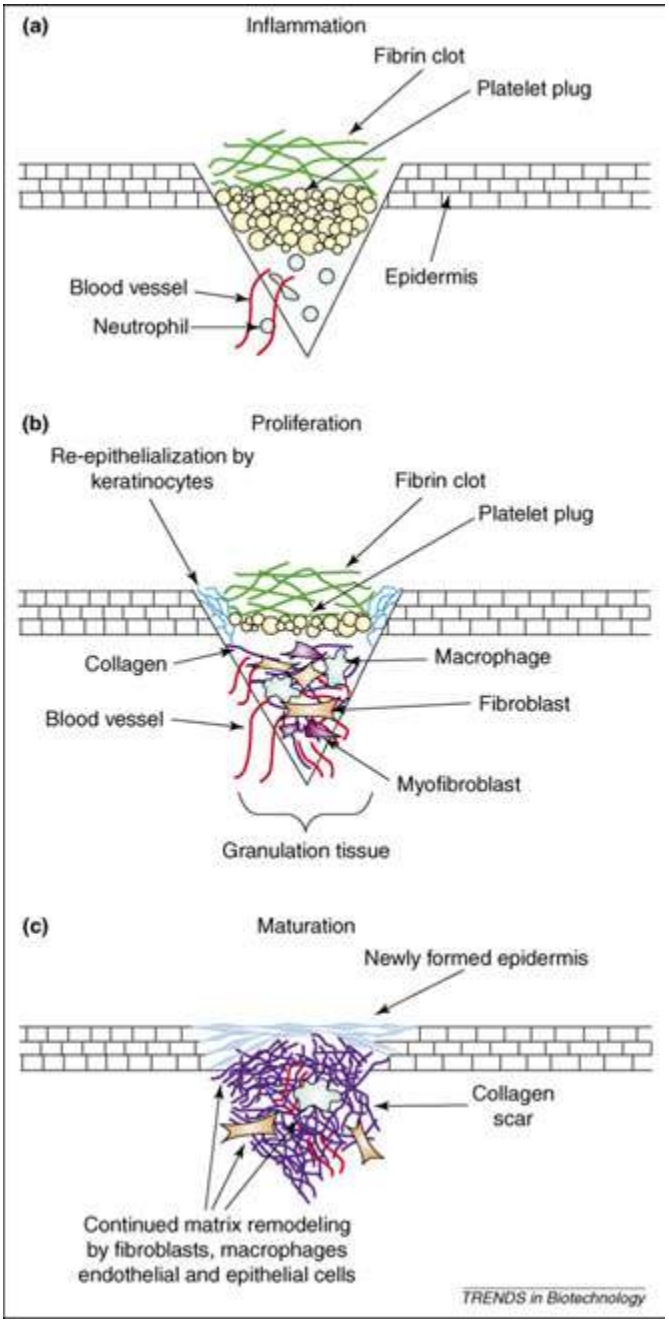

Figure 1. Phases of wound healing. The process of normal wound healing can be divided into a predictable sequence of three overlapping phases: inflammation **(a)**, followed by proliferation **(b)** and, lastly, the ongoing process of scar maturation **(c)** [5]. Inflammation is marked by the infiltration of first neutrophils, then monocytes and macrophages, which function in bacterial destruction and phagocytosis, and tissue debridement. Proliferation involves the coordinated migration of macrophages, vascular endothelia and fibroblasts into the wound bed. During this time, collagen begins to accumulate and wound contraction occurs. Maturation is descriptive of the continuing collagen accumulation and remodeling by the constituent cell types of the healing wound.

cells. During this last phase, synthesis of structural proteins, such as collagen, continues to be elevated for six to twelve months [8], although the scar only ever reaches, at best, 70% of the tensile strength of unwounded skin [5].

The perceived appearance of a scar at the culmination of the healing progression is influenced by several cell- and tissue-specific factors. The organization and quantity of fibrotic tissue deposited is one such factor. The disruption of collagen organization resulting from scarring causes local changes in skin coloration – light scattering by collagen fibers is an important determinant of this color variance [9]. Although abnormal wound healing is not a topic of this review, hypertrophic or keloid scars are pathologies associated with a specific excess of granulation [10]. The distinction with surrounding skin in pathological scars might be further highlighted by disruptions to cutaneous vascular pattern; however, scar vasculature in normally healed wounds has been reported not to differ from that of unwounded skin [11]. The location of a skin injury and patient age also play a role in determining the degree of fibrosis and wound-healing response [12]. Curiously, available evidence suggests that injury-associated changes in the recruitment of melanocytes, the cells responsible for skin pigmentation, do not usually contribute to scar appearance [13]. Epidermally derived appendages within the granulation tissue, including sweat and sebaceous glands, are typically reduced compared to normal cutaneous tissue [3,14]. Sparseness of hair might also heighten scar contrast, although recent evidence from mice indicates that wounding, in association with Wnt signaling, induces hair follicles *de novo* from epidermal progenitor cells [15]. Finally, the regenerated epidermis covering the healed wound might be distinguished by a 'smoothened' appearance [16]. This results from the tendency of the newly formed epidermis to contain lower densities of undulations, termed rete pegs, than adjacent unwounded skin – a change that might also affect the mechanical properties of the tissue [16].

Scar reduction and the inflammatory phase of wound healing

The initial inflammatory phase (see Figure 1) has received considerable attention from researchers interested in improving the appearance of healed skin injuries. Seminal investigations into the ability of embryonic wounds to heal without apparent scars have been influential in the development of this interest in inflammation [4,14]. Early workers noted that the propensity of embryos for scarless healing correlated inversely with the progressive maturation of the cellular immune response during development [14,17]. Subsequently, the idea that inflammation might be counter-productive to healing also found support from wound healing experiments in knockout and transgenic mouse models [18,19], studies in athymic mice [20], antisense gene knockdown experiments *in vivo* [21,22] and investigations into the relative impermeability of fetal endothelia to neutrophil transmigration [23]. In two examples based on gene targeting in mice, adult Smad3 and PU.1 knockouts are known to be deficient in different aspects of mounting an inflammatory response, but show faster wound closure and less deposition of fibrotic scar after wounding than their wild-type littermates [18,19]. It is noteworthy that the beneficial effect of saliva on skin injuries pointed to in the introduction of this essay has also been proposed to result from a epidermal-growth-factor-mediated reduction in inflammatory neutrophils.

Further evidence that hematopoietic derivatives have key inputs into scarring comes from the wealth of data showing that inflammatory cells emigrating from the blood

provide or activate signals that promote granulation and fibrosis [5,6,24]. These signals include reactive oxygen species, chemokines and cytokines that promote the chemotaxis and collagen deposition of fibroblasts and have the potential to prompt signaling cascades with downstream effects on other cell populations critical to granulation tissue differentiation. However, ongoing work and emerging new data have also raised caveats on the extent to which scarring is causally linked to inflammation. Cox-2 is one of the enzymes responsible for production of prostaglandin, a well-characterized mediator of the inflammatory response [25]. In one report, Cox-2 inhibition was shown to decrease scar deposition after cutaneous injury [26], but work by others suggested that antagonism of Cox-2 had no effect on the macroscopic or microscopic morphology of healed wounds [27]. In a well-conceived study, anti-neutrophil antibodies were used to induce transient neutropenia in mice [28]. Although the targeting of inflammatory neutrophil infiltration was reported to be associated with accelerated wound closure, no difference was found in wound collagen levels between control and neutropenic mice. Furthermore, it appears that inflammatory progenitors are not the only blood-borne cells that contribute to wound-repair mechanisms. There are accumulating data indicating that granulation tissue might in part be derived from circulating fibroblast-like progenitors (fibrocytes), which transmigrate to injury loci from blood vessels in much the same manner as neutrophils and monocytes [2,29].

Thus, pioneering concepts on the role of the cellular immune system in scarring are presently being broadened by new information. Researchers are beginning to entertain the possibility of contributions by other bone-marrow-derived lineages in addition to those that give rise to inflammatory cells. Addressing the questions that have been raised by these ideas and data could eventually lead to new targets for promoting scar reduction.

Scar-reduction strategies based on targeting of the TGF-β signaling pathway

The recognition of differences in inflammatory-cell recruitment to embryonic and adult wounds has been influential in the identification of hematopoietic inputs into scar differentiation – as has just been discussed. The wound environment in the embryo differs in many other important respects from that of late fetal or postnatal stages [4,30]. Recapitulating aspects of the molecular biology of the embryonic wound environment during healing in the adult provides a rationale for several strategies with promise for translation to therapy. Such approaches include examples based on manipulation of hyaluronic acid and fibromodulin – extracellular matrix components that are present at elevated levels in embryonic wounds [31,32]. This being said, by far the most effort in this direction has centered on the potential for altering TGF-βs to prompt embryonic-like healing patterns in mature mammals [4,33].

TGF-β1, TGF-β2 and TGF-β3 are members of the large TGF-β family of secreted signaling molecules [7]. Although there is some variance in the literature, all three TGF-βs appear to show distinct changes in expression during healing of skin wounds in adults (Figure 2) [34]. TGF-βs are secreted by platelets, fibroblasts and macrophages within the injury and are thought to act in various capacities as attractants and/or inhibitors of keratinocyte, fibroblast and inflammatory-cell migration, in upregulation of collagen synthesis and modulation of matrix turnover via inhibitory effects on matrix metalloproteases (MMPs) and MMP inhibitors [6,8]. Additionally, TGF-β1 induces differentiation of myofibroblasts [6] – a cell type critical to wound contraction and marked by active synthesis of granulation tissue constituents, including collagen and fibronectin [35].

In 1991, Whitby and Ferguson reported that TGF-β was undetectable in mouse embryonic wounds [17]. Later analyses by the same group confirmed TGF-β1 was decreased in fetal skin injuries as compared to those in adult skin but, furthermore, they reported that TGF-β3 was significantly elevated in the fetal epidermis [36]. Treatments of wounds to recapitulate this embryonic profile of TGF-βs, either via combinatorial administration of antibodies neutralizing endogenous TGF-β1 and TGF-β2 or by treatment with exogenous TGF-β3, were subsequently found to prompt reductions in scarring of adult

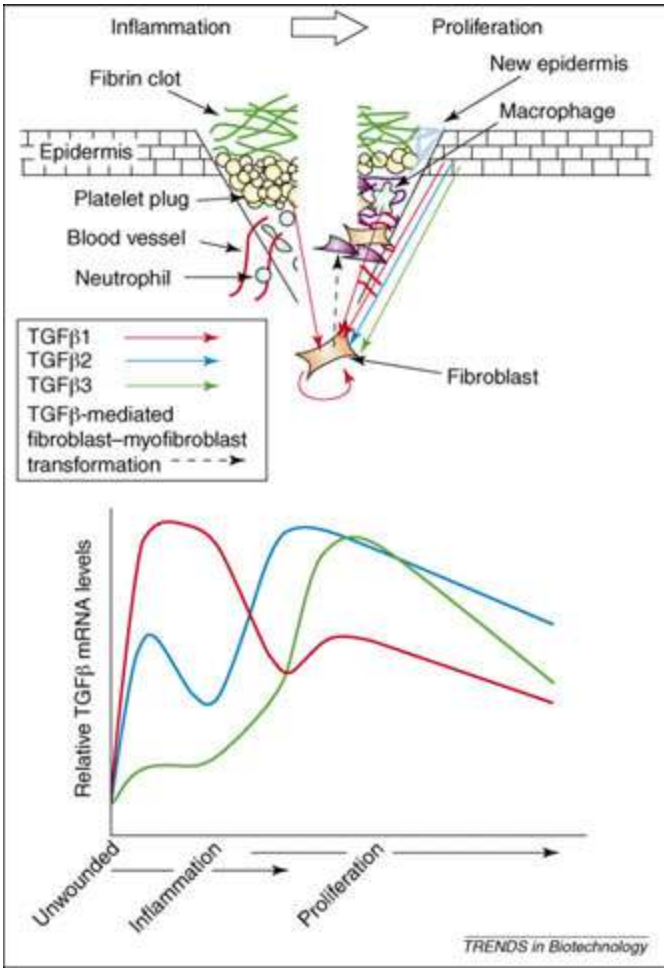

Figure 2. TGF-β signaling during wound repair. TGF-β signaling influences many events during cutaneous healing: keratinocyte, fibroblast and inflammatory cell migration, collagen synthesis and matrix turnover and myofibroblast differentiation and wound contraction [6,34,35]. The graph shows relative variance in TGF-β1 (red), TGF-β2 (blue) and TGF-β3 (green) mRNA levels over the course of healing of a skin wound [34].

wounds [33]. Interestingly, antibody neutralization of either TGF-β1 or TGF-β2 alone had marginal or no affect on scar tissue differentiation, although later studies by others have reported that solely knocking down TGF-β1 expression level using antisense RNA was efficacious in reducing fibrotic tissue deposition after skin injury [37]. In a related finding, suppression of endogenous TGF-β1 has been proposed to account for the fibrosis-reducing effect of overexpression of hepatocyte growth factor mediated by a viral gene vector in cutaneous wounds [38].

It should be noted that the body of data on TGF-βs has not been unanimous in emphasis on the effects of manipulation of these growth factors on wound healing. Experiments in the late 1980s in a model based on outgrowth of cells from explants of pig skin led to the conclusion that TGF-βs stimulated keratinocyte migration *in vitro* [39]. In apparent agreement with this, a contemporaneous study in rats *in vivo* reported that exogenous TGF-β1 prompted accelerated healing, increased collagen deposition and improved tensile strength of skin wounds [40]. Subsequent work by the same group in an avascular rabbit ear model confirmed that treatment with TGF-β1 promoted granulation tissue formation, although it inhibited wound reepithelialization [41]. Other researchers came to a similar conclusion that TGF-β1 inhibited reepithelialization based on experiments using human keratinocytes in an organotypic model designed to mimic properties of a living epidermis *in vitro* [42]. These discrepancies are understandable given that TGF-β1 has since been shown to activate chemotaxis in cell types other than epidermal cells that are involved in wound healing, including monocytes, lymphocytes, neutrophils and fibroblasts [7], and the effect of exogenous TGF-β1 seems to depend on the local matrix and cellular context.

Studies of transgenic and gene knockout mice have provided further nuance in the evolving understanding of the role of TGF-β in wound healing and scar differentiation. A transgenic mouse overexpressing TGF-β1 in plasma under an albumin promoter was unexpectedly found to display faster wound healing and less scarring than controls [43]. Smad3 is a key cytoplasmic mediator of the TGF-β signal transduction pathway [18,44]. Although incisional skin wounds on mice in which the Smad3 gene has been genetically deleted heal more quickly and with reduced scarring compared to genetically normal littermates [18], excisional wounds on the ear of Smad3 gene nulls become enlarged compared to controls [44]. In another example indicating that inhibition of TGF-β1 signaling might not always result in beneficial outcomes, wound healing is significantly delayed in immunodeficient SCID1 (severe combined immunodeficiency 1)-knockout mice that also lack the TGF-β1 gene [45]. SCID1-knockouts that possess a normal TGF-β1 genotype demonstrate no such delay in healing. The results from gene-targeted mice are as yet not fully understood. However, these data do suggest that future therapeutic strategies based on manipulation of TGF-β signaling might have to take into account several variables, including acute versus chronic exposure to treatment, body location and patient immune status, if the outcomes of such therapies are to be optimized.

Potential scar-reduction therapies based on targeting TFG-βs are presently in the biotech pipeline, and some have completed clinical phase I and II safety and efficacy trials. Products include those being developed by Renovo Ltd – a company spun off from the Ferguson laboratory at the University of Manchester. A recombinant TGF-β3 (Juvista) polypeptide is Renovo's lead drug candidate, and formulations of this product have completed phase II efficacy trials in the UK. According to the company website (http://www.renovo.com/), human wounds treated with TGF-β3 showed significant improvement in scar appearance, as evaluated by a panel composed of surgeons and lay persons, with over 70% of individuals in the trial exhibiting a response to the therapy. Other therapies under development by Renovo include formulations of mannose-6-phosphate (M6P, marketed as Juvidex™) and other proprietary compositions based on estradiol (marketed as Zesteem™). M6P is thought to act by inhibiting activation of TGF-β1 and TGF-β2 and has been reported to improve healing and reduce scarring in M6P/IGF-2 (insulin-like growth factor 2)-knockout mice [3]. Estradiol has been suggested to mediate beneficial effects on wound healing by downregulating TGF-β1 levels at the wound locus [46].

Scar-reduction strategies based on targeting of gap junctions and connexins

Polypeptide growth factors such as TGF-βs are secreted ligands that bind external cell-surface receptors. Secreted ligands can affect cellular signaling over relatively large tissue fields. Direct cytoplasmic couplings between cells mediated by gap junctions provide another avenue for the spread of biological information within tissues [47]. Communication between any two adjacent cells involves a localized dialog, but gap junctions can also underpin the formation of coupled cellular networks that provide for the regulated and long-range propagation of signaling. The conduction of action potential during the heart beat is perhaps the most widely known and tangible example of this aspect of gap junction function [48]. Gap junctions are also recognized as playing key roles in morphogenesis and cellular differentiation, proliferation and migration during embryonic development [47].

Gap junctional couplings between cells are formed by aggregates of intercellular channels composed of proteins encoded by the connexin multigene family [47,48]. Intercellular communication mediated by gap junctions is now recognized as an important aspect of cutaneous injury response (Figure 3). Although the details of specific mechanisms remain to be characterized, connexins appear to have functions that include coordinating inflammatory response, propagation of injury signals between cells, wound closure and scar-tissue formation after injury [21,22,49–56]. Several different connexin gene family members are expressed in skin, including connexin 26 (Cx26), Cx30, Cx31.1 and Cx43 [21,52,57–59]. Of these isoforms, the potential of targeting Cx43 to enhance the outcome of the healing progression has been studied most extensively [21,22,58]. Cx43 is expressed in both the epidermal and dermal cutaneous layers [21,52,57–59]. In the first day or two after injury to skin in rodent models, Cx43 expression and cell communication has been reported to decrease transiently in epidermal cells at the wound edge

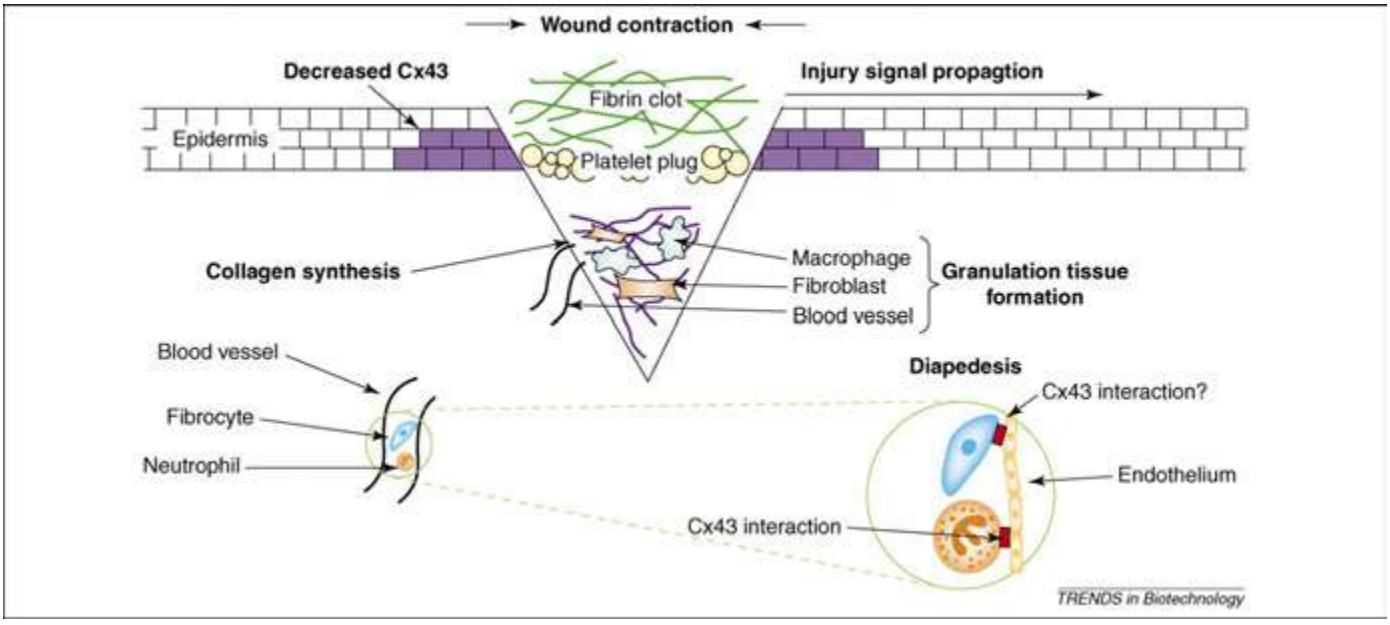

Figure 3. Gap junction function in wound healing. Gap junctions are involved in many processes in healing (indicated by bold type), including wound contraction by myofibroblasts [54], keratinocyte response [52], propagation of injury signals [53] and neutrophil diapedesis [51]. Fibrocytes enter the wound from circulation in a similar fashion to neutrophils, although it remains to be determined if gap junctions are involved [2,29].

[52,57]. The effects of this decrease on intercellular coupling might be further enhanced by increases in phosphorylation of Cx43 at serine368, a modification that has been shown to be associated with decreased gap junctional communication levels [60].

Application of Cx43 antisense oligonucleotides to skin wounds on mice was reported by Green, Becker and colleagues to reduce infiltration of inflammatory cells, accelerate healing and reduce the overall area of granulation tissue formation after skin wounding in mice [21]. A presumed aspect of the mechanism was to further diminish the injury-induced reduction in endogenous Cx43 function within epidermis adjacent to the wound. Subsequent work demonstrated benefits of Cx43 antisense to reduced scarring after cutaneous thermal injury [61] and also in the enhanced reepithelialization of wounds in a rodent diabetic model [62]. Other recent work on the effects of Cx43 antisense from the same group has provided unexpected new information: it was reported that Cx43 antisense treatment significantly increased levels of TGF-β1 mRNA [22]. In addition, enhanced expression of collagen and evidence of increased rates of granulation-tissue formation were observed.

The ground-breaking finding that Cx43 antisense can improve the healed appearance of wounds [21] and yet increase TGF-β1 expression and promote granulation-tissue formation [22] is interesting in light of the conclusions by others [33,63,64]. Evidently, the questions raised here on potential interplay between TGF-β signaling and Cx43 function will require further work. There is, nonetheless, other evidence supporting the concept that Cx43 deficiency has beneficial effects on cutaneous injury response from studies showing that Cx43-knockout mice display enhanced healing of skin wounds [58]. Further work supporting a role for connexins and/or gap junctions in scar formation comes from Ehrlich *et al.*, who showed that synthesis of type I collagen protein was decreased in the presence of chemical uncouplers of intercellular communication although, oddly, collagen type I mRNA levels were unaffected by these treatments [50]. Conversely, and consistent with the observed effect of inhibition, lithium chloride enhancement of gap junctional coupling between fibroblasts in skin wounds appears to advance the maturation of granulation tissue [54]. Also worth mentioning in this context are mimetic peptides, which are thought to interact directly with the extracellular loop domains of connexin molecules and thereby inhibit the function of channels assembled from these proteins [65]. In cutting-edge studies using live cell imaging techniques, Martin and co-workers recently demonstrated that connexin mimetic peptides designed to target Cx43 enhanced wound-closure rates in keratinocyte monocultures and in an organotypic 'living skin equivalent' model [66].

Work currently underway in the Gourdie laboratory has provided data for a further Cx43-related approach to modulating wound healing [49,56]. In 2005, we reported the synthesis of a membrane permeant peptide comprised of an antennapedia internalization sequence linked to the last nine amino acids of the carboxyl-terminus of Cx43 – RPRPDDLEI [67] (Figure 4). This short sequence was rationally designed to inhibit interaction of the actin-binding protein ZO-1 (zonula occludens-1) with Cx43. It was demonstrated that the inhibitory peptide (which we now refer to as alpha-connexin carboxyl-terminal peptide 1, or ACT1 peptide) reduced levels of ZO-1 localization at the edge of gap junctional plaques comprised of Cx43, resulting in effects on the size of the plaque [67]. Based on these data, we concluded that ACT1 peptide influenced plaque organization by inhibiting the rate of accretion of channels to the gap junction.

Subsequently, we have found that ACT1 peptide provides benefits in the healing of skin wounds [49,56]. The peptide was designed to bind the PDZ2 domain of ZO-1 [67]. However, the extreme carboxyl terminus of Cx43, in

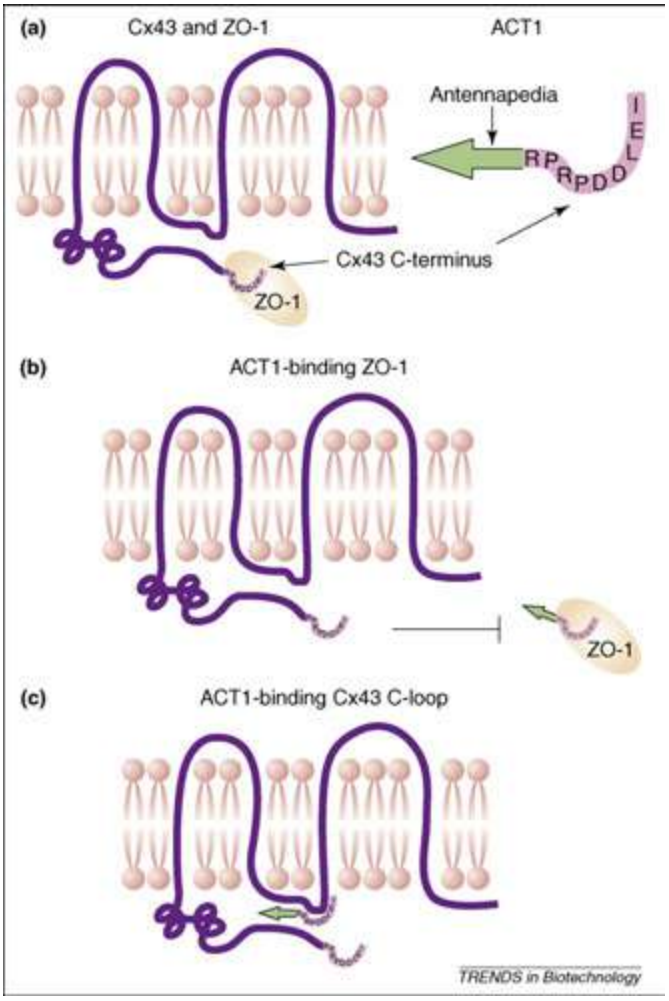

Figure 4. Cx43, ZO-1 and ACT1 peptide Interactions. **(a)** The C-terminal PDZ2-binding domain of Cx43 (DLEI) interacts with ZO-1. ACT1 peptide comprises an N-terminal antennapedia sequence (green arrow) coupled to the carboxyl-terminal-most nine amino acids of Cx43 (red), including the PDZ-binding sequence. **(b)** Once ACT1 enters the cytoplasm via its antennapedia membrane permeabilization sequence, it competitively inhibits Cx43 binding with endogenous ZO-1. **(c)** Hypothetical interaction of ACT1 with the cytoplasmic loop domain of Cx43.

addition to binding ZO-1 [68,69], is involved in interactions with other proteins, including CCN3 – a matricellular protein implicated in glioma and tissue-injury response [70,71], 14-3-3 proteins [72], SH3-mediated interactions [73] and various protein kinases [74]. The carboxy-terminal-most amino acids of Cx43 have also recently been reported to be involved in interactions with the cytoplasmic loop domain of Cx43 itself [75]. ACT1 peptide does not alter Cx43 expression [67], suggesting that loss of Cx43 gene function is not likely to be an aspect of mechanism of ACT peptides, unlike the presumed mechanism of Cx43 antisense [21] and gene knockdown [58]. Finally, with respect to mode of action, it is noteworthy that a recent study reported that short, ACT-like sequences are generated by peptidase cleavage of the Cx43 C-terminus as part of the natural injury response after myocardial infarction [76]. This raises the intriguing possibility that Cx43 might release ligand-like peptides similar in structure to exogenously administered ACT peptide used in our current work.

As is the case with TGF-β targeting, progress in technology transfer is also underway for connexin-related scar-reduction therapies. A Cx43 antisense-based gene therapy (Nexagon^{TM}) provides a platform technology for CoDa Therapeutics Inc. (http://www.codatherapeutics.com), a San Diego-based biotechnology company with links to University College London, UK, and the University of Auckland, New Zealand. According to CoDa's website, the company has obtained venture capital to pursue clinical testing of their lead product. FirstString Research Inc. (http://www.firststringresearch.com), a spin-off of the Medical University of South Carolina in Charleston, South Carolina, is pursuing clinical development of ACT technology based on short bioengineered peptides derived from the Cx43 C-terminus. FirstString anticipates beginning phase 1 safety trials on ACT peptides early in 2008.

Other mechanistically based scar-reduction strategies

The strategies with potential for reducing scarring that this essay has mainly focused on have stemmed from research on TGF-βs and connexins. Inevitably, several drugs and biologics with promise for promoting the regenerative healing of skin wounds fall outside of this scope. Approaches based on hyaluronic acid, hepatocyte growth factor and fibromodulin have already been mentioned in brief [31,32,38]. Other therapeutic candidates found in the literature include angiotensin peptides [77] and inhibitors of MMP [78], pro-collagen C-proteinase [79] and dipeptidyl peptidase IV [80] enzyme functions. Adenoviral overexpression of the transcription factor p21 has also been reported to promote decreased cutaneous scarring [81].

Concluding remarks

It is an unfortunate fact that deposition of fibrotic scar, as opposed to restoration of normal cutaneous histology, is the endpoint that dominates wound resolution in humans. An effective approach to improving the appearance of healed skin wounds would obviously be of the utmost significance to cosmetic surgeons, but could also be important to those interested in enhancing the healing of other injured tissues and organs. Spinal cord ruptures, scarification of hand tendons after extremity injury, corneal scarring and myocardial infarction are but a few examples where reduction in fibrosis and regeneration of previously existing tissue architecture could be of enormous clinical benefit. Reducing fibrosis in and around medical implants (e.g. stents), engrafted stem cells and tissue-engineered devices (e.g. skin substitutes, scaffold-containing stem cells) is a further area where such progress might eventually prove extremely helpful to clinicians. Indeed, as Anthony Atala (Wake Forest University, NC) has pointed out [82], 'In tissue engineering, everything goes back to scar formation.' It is therefore encouraging that the prospects for novel approaches to shifting the balance from scarring to regenerative healing of skin wounds seem stronger than ever.

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