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Scarless Wound Healing: Finding the Right Cells and Signals

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Abstract

From the moment we are born, every injury to the skin has the potential to form a scar, many of which can impair form and/or function. As such, scar management constitutes a billion-dollar industry. Yet effectively promoting scarless wound healing remains an elusive goal. The complex interactions of wound healing contribute to our inability to recapitulate scarless wound repair as it occurs in nature, such as in fetal skin and oral mucosa. However, many new advances have occurred over recent years, some of which have translated scientific findings from bench to bedside. *In vivo* lineage tracing has helped establish a variety of novel cellular culprits that may act as key drivers of the fibrotic response. These newly characterized cell populations present further targets for therapeutic intervention, some of which have already demonstrated promising results in animal models. Here, we discuss several recent studies that identify exciting approaches to diminishing scar formation (Fig. 1). Particular attention will also be paid to the canonical Wnt/β-catenin signaling pathway, which plays an important role in both embryogenesis and tissue repair. New insights into the differential effects of Wnt signaling on heterogeneous fibroblast and keratinocyte populations within the skin further demonstrate methods by which wound healing can be redirected to a more fetal, scarless phenotype.

Keywords

Wound healing; Fibroblast; Scarless; Wnt; β-catenin

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INTRODUCTION

Human skin has evolved the remarkable ability to repair itself after injury. An unavoidable consequence of the skin's rapid wound healing process is the formation of fibrotic scar tissue. While the presence of a scar for some is merely a cosmetic concern, scars of the face and large burn scars can be severely debilitating. Children are particularly prone to the effects of scarring and suffer from long-term physical (Sheridan, et al., 2000) and psychological (Robert, et al., 1999) harm after developing large scars from surgery or burns. In the United States, $7.5 billion is spent annually on burn care (Finkelstein, et al., 2006) and much of the effort spent treating burn patients is related to the burn scar. Considering non-burn scars, 80 million surgical incisions (CDC, 2010, Cullen, et al., 2009) and 12 million traumatic lacerations (Singer, et al., 1997) occur in the United States annually, resulting in a potential $12 billion market for the treatment of the resulting scars (Sen, et al., 2009).

The ability to cause cutaneous injuries to heal without fibrosis would revolutionize the care of lacerations, incisions, and burns, and would alleviate an enormous amount of suffering. A major advance in the science of scarring took place when it was recognized that the skin of the fetus in mammals heals without scar early in gestation. The oral mucosa demonstrates a similar privileged scarless phenotype following wounding. Unfortunately, wound therapies that result in tissue regeneration in place of physiological reparative healing with scar formation have yet to be realized clinically.

The more recent appreciation for the functional and topographical heterogeneity of cells collectively known as fibroblasts has advanced our ability to more specifically target cells implicated in wound healing, with fewer systemic consequences. While relevant therapeutic interventions are fairly novel, the generalizability of characterizing and targeting specific subsets of cell populations is likely to open many new doors in treating fibrotic disease. In preparation for a proliferation of such studies, we present an overview of our current ability to modulate the wound healing process in favor of a regenerative, scarless phenotype. Special attention will be paid to canonical Wnt/β-catenin signaling, long understood to influence both embryological development and wound healing, as many recent studies have focused on its influence over specific subsets of cells within the dermis and epidermis.

HETEROGENEITY OF SKIN CELL POPULATIONS

Recent advances in stem cell biology have added insight into our understanding of wound healing. The discovery of long-lived epithelial stem cells (ESCs) in the hair follicle bulge region led to the hypothesis that development and repair were dependent on these cells. However, fate-mapping experiments revealed ESCs in the bulge region do not normally contribute to the epidermis, but respond to wounding by generating short-lived transient amplifying cells (TACs) responsible for acute wound repair. (Ito, et al., 2005) Recently, the cellular heterogeneity of the skin interfollicular epidermis (IFE) has been examined using clonal fate analysis and is shown to consist of slow-cycling stem cells and committed progenitor cells. After wounding, only stem cells contribute substantially to the repair and long-term regeneration of the tissue. (Mascre, et al., 2012)

In the dermis, a population of mesenchymal cells, known as dermal papilla (DP) cells are thought to regulate hair follicle growth and development and serve as a reservoir of multipotent stem cells (Driskell, et al., 2011). Another population of cells, known as skin-derived precursors (SKPs), derive from $Sox2^+$ follicle-associated dermal precursors and have been shown to contribute to dermal maintenance, wound healing, and hair follicle morphogenesis. SKPs self-renew and maintain their pluripotency, exhibiting functional properties of an adult dermal stem cell. (Biernaskie, et al., 2009) Recently, Driskell *et al.* have shown that dermal fibroblasts arise from two distinct lineages that form the upper and lower dermis. While the upper papillary dermis contributes to re-epithelization and hair follicle formation, it is the lower dermis that comprises the reticular fibroblasts that synthesize the bulk of the fibrillar extracellular matrix (ECM) and mediates dermal repair after wounding. These fibroblasts are the first to infiltrate the wound bed during healing, and they demonstrate a myofibroblastic phenotype with α-smooth muscle actin (α-SMA) expression. (Driskell, et al., 2013)

The appreciation for the functional heterogeneity of dermal fibroblasts has led to advances in our understanding of their responses to signaling pathways during the wound healing process. The distinct lineages of dermal fibroblasts demonstrate differential responses to epidermal Wnt/β-catenin paracrine signaling. Specifically, epidermal Hedgehog (Hh) promotes papillary fibroblast proliferation whereas transforming growth factor-β (TGF-β) meditates ECM remodeling the reticular dermis. Pharmacological inhibition of either TGF-β or Hh was shown to specifically target these fibroblast lineages. (Lichtenberger, et al., 2016) While the authors did not directly study wound healing and scar formation, their findings did corroborate the findings of Rinkevich *et al.* as they relate to a fibroblast lineage originating from Engrailed-1 (En1)-expressing progenitors. Both the reticular fibroblasts of Lichtenberger, which co-express Sca1 and CD26, and the $CD26^+$ fibroblasts derived from $En1^+$ embryonic cells, were found to comprise 70–80% of all dermal fibroblasts. It is these cells that are responsible for the bulk of connective tissue deposition in the dorsal dermis leading to scar formation. (Lichtenberger, et al., 2016, Rinkevich, et al., 2015) Overall, these studies have improved our understanding of stem cell biology and wound healing, giving hope for improving aberrant wound repair.

SCARLESS WOUND HEALING AS IT OCCURS IN NATURE

Fetal scarless wound healing

Interestingly, wounds in the early, but not late, mammalian gestational period heal without the formation of scar in a process resembling regeneration (Gurtner, et al., 2008). Although, the exact mechanism is yet unknown, there are many characteristics of the fetal skin that differentiate it from adult skin, both at the cellular and tissue levels (Fig. 2). One notable feature is the decreased tensile strength in early fetal skin. Based on this observation, a study by Wong *et al.* has elucidated focal adhesion kinase (FAK) as the pathway that ties mechanotransduction with fibrosis. FAK, through extracellular-related kinase (ERK), triggers the secretion of monocyte chemoattractant protein-1 (MCP-1), which has been associated with a number of human fibrotic disorders (Wynn, 2008). By knocking out or inhibiting various components of the inflammatory FAK-ERK-MCP-1 pathway, scar

formation was attenuated. (Wong, et al., 2012) This work has translated to the development of a stress-shielding device that has been shown in two randomized controlled clinical trials to reduce scar formation (Lim, et al., 2014, Longaker, et al., 2014). Early gestational cells may also modify their embryonic niche in a way that promotes regenerative healing, while the cells of late gestation may be restricted to reparative healing. As an example, human keratinocytes from mid-gestation have the ability to modulate fibroblast gene expression, causing increased proliferation and migration relative to co-culture with late-gestation fibroblasts (Wang, et al., 2015). Moreover, proteome analysis of cultured fibroblasts revealed different patterns of protein expression when comparing adult and fetal fibroblasts, further supporting scar formation during wound healing as a cell-intrinsic property (Ho, et al., 2014).

Oral mucosa

In adult mammals, healing of the oral mucosa provides the closest example of regenerative healing as it demonstrates very minimal scarring. Inflammation at the wound bed is known to contribute to the formation of a scar. Both oral mucosa and fetal skin may be considered as “protected environments” for wound healing. Human fetal skin was observed to heal without scar when transplanted subcutaneously into immunodeficient nude mice, while cutaneous transplantation of age-matched fetal skin resulted in scar formation (Lorenz, et al., 1992). Similarly, in the oral cavity salivary mucins form hydrophilic viscoelastic gels, which in turn act as barriers protecting the underlying mucosa from mechanical damage and direct interaction with pathogens and other harmful substances (Amerongen and Veerman, 2002). However, the role of this “protected environment” was determined to be necessary but insufficient to achieve scarless wound healing, given that adult skin transplanted to the fetus heals with a scar following incisional wounding (Longaker, et al., 1994).

Analysis of healthy human oral mucosa revealed significantly lower numbers of neutrophils and macrophages, compared to healthy skin (Glim, et al., 2015). However, the authors were unable to study these differences in acute wounds. The attenuated inflammatory environment of the oral mucosa likens it to the fetal wound healing environment and may partially explain the similarities in reduced scar formation. ECM composition prior to wounding has also been hypothesized as a contributor to privileged scarless healing. Relative to skin, the oral mucosa exhibits increased fibronectin, its splice variant ED-A, and chondroitin sulfate in the former. Pro-fibrotic myofibroblast α-SMA expression relies on cell surface ED-A fibronectin interaction in addition to TGF-β1 (Tomasek, et al., 2002). Conversely, the presence of elastin was more pronounced in the skin. Additionally, the rate of proliferation was higher in oral keratinocytes, with keratinocytes located in the dermis demonstrating increased differentiation. (Glim, et al., 2014) The unique characteristics of the oral mucosa relative to the cutaneous skin contribute to its ability to heal with minimal scarring. However, significant advances are required to establish this privileged healing in cutaneous wounds.

RECENT ADVANCES IN SCARLESS WOUND HEALING RESEARCH

Administration of therapeutics with known anti-inflammatory activity

Fetal scarless wound healing is associated with significantly reduced inflammation, whereas pathological scarring often involves an unregulated inflammatory response (Larson, et al., 2010). Therefore, significant effort has been devoted to modulating the inflammatory processes that may negatively impact wound healing. Utilization of physiological anti-inflammatory molecules offers the potential to direct wound healing down regenerative pathways. For example, systemic administration of the neuropeptide α-melanocyte-stimulating hormone (α-MSH) led to a more regenerative healing process characterized by less macroscopic scar formation as well as organization of collagen fibers more closely mimicking unwounded skin (de Souza, et al., 2015). While this effect required administration of α-MSH prior to wounding, prophylactic administration of anti-fibrotic agents is not without clinical utility, particularly in cases such as scar revision surgery.

Fibroblast growth factor 9 (Fgf9) also provides a means for modulating the inflammatory environment, for example by promoting monocyte to M2 macrophage differentiation in cardiac ischemic injury (Singla, et al., 2015). Importantly in cutaneous injury, Fgf9 from dermal γδ T cells was shown by Gay *et al.* to facilitate follicle neogenesis. In humans, wound healing with scar formation occurs without regeneration of hair follicles. Given that Fgf9 overexpression potentiates increased follicle neogenesis in mice, these findings may translate to therapies promoting regenerative healing. (Gay, et al., 2013)

In a study by Morris *et al.* the authors aimed to better understand how wound size and inflammation impact fetal ability to undergo regenerative healing rather than scar formation. Larger (8 mm) wounds created in fetal lambs were found to heal with scar formation while 2 mm wounds healed without scar. These findings also correlated with different gene expression profiles and increased inflammation in large fetal wounds. Administration of lentiviral IL-10 resulted in restoration of normal skin architecture and attenuation of pro-inflammatory gene expression. (Morris, et al., 2014) However, use of viral vectors is greatly limited in clinical use due to the risks of oncogenesis. Low concentrations of recombinant human interkleukin-10 injected intradermally in humans prior to incision showed improved macroscopic scar appearance at 12 months (Kieran, et al., 2013).

Findings of upregulated stromal cell-derived factor 1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR-4) after burn injury and subsequent hypertrophic scar formation have also led to novel therapeutic targets as CXCR-4 antagonism inhibits inflammatory cell recruitment and fibroblast activation. In this case, Ding *et al.* transplanted split-thickness human skin grafts into full-thickness wounds in nude mice. Serial injection of the CXCR-4 antagonist CTCE-9908 resulted in grafts that remained flat, with only limited contraction, contrary to findings in controls demonstrating more hypertrophic scars. Results of associated gene and protein expression studies, coupled with analysis of tissue architecture and cell recruitment, collectively suggest that CXCR-4 antagonism exhibits a potent anti-fibrotic and pro-regenerative phenotype. (Ding, et al., 2014) Limiting the fibrotic response following skin grafting has the potential to reduce functional deficits due to contractures, particularly when grafts are placed over mobile joints. Furthermore, such therapies may preclude the need to

harvest full-thickness skin grafts, which demonstrate less contraction relative to split-thickness skin grafts.

Cellular targets for scarless wound healing

Advances in developmental biology, by lineage tracing and transplantation assays, have allowed researchers to identify populations of cells, contributing to scar formation. Dulauroy *et al.* utilized transient expression of a disintegrin and metalloprotease 12 (ADAM12) to distinguish a pro-inflammatory subset of perivascular cells that are activated upon acute injury in muscle and dermis. Ablating these cells, or knocking down ADAM12, was sufficient to decrease fibrosis and scarring. (Dulauroy, et al., 2012) Recent studies by Rinkevich *et al.* have further elucidated the heterogenic nature of dermal fibroblast populations. Of particular interest, fibroblasts originating from En1-lineages have been identified as the main culprits in cutaneous scarring that occurs beyond early gestation. These cells act as the major contributor of connective tissue in scar formation. Concomitant expression of CD26 (also known as dipeptidyl peptidase-4, DPP4) surface marker allowed selective abrogation of the En1-fibroblast lineage with diprotin A. Treatment with this small molecule reduced cutaneous scarring secondary to full-thickness excisional wounding without compromising the integrity of the healed tissue. (Rinkevich, et al., 2015) *In vivo* experiments have demonstrated the ability of DPP4 inhibitors to curb the fibrogenic phenotypes of keloid-derived fibroblasts as well as normal fibroblasts. The underlying mechanisms associated with the observed decrease in collagen production and TGF-β1 expression involve the pro-fibrotic pp38 and pERK1/2 pathways. (Thielitz, et al., 2008)

Myofibroblasts are key players in the normal wound healing response, contributing to wound contraction and ECM production (Gurtner, et al., 2008). Cells demonstrating persistent myofibroblast-like phenotypes are key contributors to fibrosis, associated with excess ECM deposition and disinhibited contraction make them ideal targets to reduce scar formation (Powell, et al., 1999). However, normally as the scar enters the maturation phase, these cells largely undergo apoptosis (Desmouliere, et al., 1995). Reversal of the myofibroblast phenotype may also contribute to decreased numbers of these cells (Desai, et al., 2014, Maltseva, et al., 2001). Additionally, myofibroblasts (or myofibroblast-like cells) have also been determined to be a functionally heterogeneous cell population, with numerous potential precursors, including fibroblasts, mesenchymal stem cells (MSCs), smooth muscle cells, endothelial cells, and fibrocytes (Hinz, et al., 2007). Adipocytes have also been found to appear simultaneously during wound healing. While it is unclear if fibroblasts and adipocytes share a common progenitor, Schmidt *et al.* have demonstrated that dermal adipocytes, via intercellular communication, are necessary for fibroblast recruitment in wound healing (Schmidt and Horsley, 2013). Thus, experimental interventions have been targeted at a variety of cell populations, both directly and indirectly responsible for scar formation.

Studies conducted by Desai *et al.* have demonstrated both the ability to direct differentiation of myofibroblast precursors (adipose-derived mesenchymal stem cells, ASCs) away from a myofibroblastic phenotype, as well as reverse differentiate cells towards a more fibroblast-like phenotype. Specifically, they showed that myofibroblastic cells treated with basic

fibroblast growth factor (bFGF) led to diminished expression of α-SMA, collagen I, and fibronectin, as well as a loss of focal adhesions and stress fibers. These cells were also more migratory, associated with tenascin-C and vimentin upregulation, in-line with a more fibroblast-like phenotype. Their findings suggest that the myofibroblast differentiation process is not terminal, elucidating bFGF as a phenotypic reversing agent. (Desai, et al., 2014)

Extracorporeal shockwaves (ESW), more commonly used in urological or orthopedic interventions, may also have a role in reducing scar formation. ESW treatment caused myofibroblast precursors to differentiate into more fibroblast-like cells with lower contractility and higher migration potential, simultaneously reducing α-SMA and type I collagen expression. (Rinella, et al., 2016)

Stem cells and scarless wound healing

Stem cell-based wound therapies are generally directed at treating recalcitrant wounds, while their potential for scarless wound healing has been less well established *in vivo*. Stem cells have certainly demonstrated an ability to modulate the wound environment to improve healing, in part by reducing inflammation (Corcione, et al., 2006, Di Nicola, et al., 2002, Loots, et al., 1998). However, whether this translates to significant decreases in scarring is unclear as a result of sometimes conflicting findings (Doi, et al., 2016, Sabapathy, et al., 2014). Furthermore, practical barriers associated with stem cell-based therapies may restrict their utility in pursuit of scarless wound healing (Nuschke, 2014). Despite the potential limitations of direct stem cell-based scarless wound therapies, one recent study by Li *et al.* demonstrated the potential benefits of conditioned media from umbilical cord (UC)-MSC cultures. Dermal fibroblasts under the paracrine influence of these cells exhibited characteristics reminiscent of fetal fibroblasts, such as low capacity to form myofibroblasts, a decreased TGF-β1/TGF-β3 ratio, and increased expression of enzymes involved in ECM remodeling (matrix metalloproteinases, MMPs). Wounds treated with the UC-MSC conditioned media also healed faster with decreased collagen accumulation. (Li, et al., 2015) Additionally, *in vitro* studies have shown that human amniotic fluid-derived MSC conditioned media has the potential to inhibit the pro-fibrotic actions of TGF-β1 and even reverse the myofibroblast phenotype to a fibroblast-like state. Conditioned media from ASCs produced similar results, but to a lesser extent. (Mia and Bank, 2015) Taken together, these findings suggest that stem cells may be able to promote a scarless phenotype without the requirement of direct inoculation to the wound bed, drastically reducing the risk of resultant oncogenesis (Fig. 3).

Wnt and regeneration

Analysis of gene expression in fetal mouse keratinocytes and fibroblasts at embryonic day (E)16 and E18 time points, straddling the transition from scarless to scar forming repair, has revealed numerous differentially regulated pathways, such as canonical Wnt/β-catenin signaling; many others have yet to be examined under the lens of wound healing (Hu, et al., 2014). These pathways create numerous novel targets to modulate the wound healing process towards a more scarless phenotype (Fig. 4).

Wnt signaling is a key component of embryological development but is also involved in all phases of the wound healing response. Of the various subsets of Wnt signaling pathways, the canonical Wnt pathway is most relevant to tissue regeneration and repair, and will be the focus of this section. In this pathway, Wnt ligand binding at the cell surface leads to cytoplasmic β-catenin accumulation, which would otherwise be degraded by the APC/Axin/GSK3 complex. β-catenin subsequently translocates to the nucleus to exert its effects as a transcriptional co-activator. (Houschyar, et al., 2015) This section will focus on the influence of Wnt signaling in wound healing as it pertains to scar formation. For more in-depth review of the role of Wnt signaling in tissue renewal and regeneration, the reader is directed to the following review article (Clevers, et al., 2014).

Multiple studies have elucidated a link between TGF-β and the canonical Wnt/β-catenin pathway in fibrosis. For example, analysis of pathological scars (hypertrophic scars and keloids) uncovered upregulated Wnt signaling secondary to TGF-β (Sato, 2006). Even in normal wound healing with scar formation, β-catenin levels have been shown to double during the proliferative phase (Cheon, et al., 2002). Another study found that human keloids display Wnt-3a overexpression, inducing fibroblasts of endothelial origin to transition to mesenchymal cells, which in turn lead to collagen accumulation (Lee, et al., 2015b). While Wnt-3a downregulates CD31 expression, it is unclear if these cells are directly related to the aforementioned $ADAM12^{+}$ lineage, which are $CD31^{-}$ (Dulauroy, et al., 2012). Future studies may translate our understanding of these endothelial or perivascular progenitors to eliminate fibrosis in wound healing.

While TGF-β signaling is central to the pro-fibrotic fibroblast phenotype, several studies have shown that the Wnt pathway is an important mediator. For example, Cheon *et al.* demonstrated that TGF-β1 is unable to promote hyperplastic wounds in transgenic mice lacking β-catenin (Cheon, et al., 2006). Akmetshina *et al.* found that TGF-β downregulates the Dickkopf-1 (Dkk-1) Wnt-antagonist, thereby upregulating the canonical Wnt signaling pathway. This claim was further substantiated by enhanced Wnt and decreased Dkk-1 expression in fibrotic human tissue samples. It thus follows that overexpression of endogenous Dkk-1 in a transgenic mouse line prevented bleomycin-induced fibrosis. (Akhmetshina, et al., 2012) However, topical application of Dkk-1 in a full-thickness excisional model inhibited wound healing (Shi, et al., 2015). Therefore, harnessing Dkk-1 for therapeutic intervention will require further insight into its mechanism of action, particularly as it relates to fibrotic etiology and means of administration.

Modulation of the Wnt/β-catenin pathway is not restricted to the use of endogenous mediators. Bastakoty *et al.* demonstrated the potential therapeutic nature of small molecules promote a regenerative wound healing phenotype. Topical application of Wnt pathway inhibitors (XAV-939, a tankyrase inhibitor, and pyrvinium, a casein kinase activator) led to formation of rete pegs and dermal papillae following wound closure. These structures are found in intact skin rather than a mature scar. Scar formation was also inhibited, as demonstrated by fewer myofibroblasts expressing α-SMA and reduced type I collagen synthesis. Wnt activation with lithium chloride (LiCl) in turn reduced regenerative repair. (Bastakoty, et al., 2015) However, in a full thickness excisional wound model in rats, topical LiCl application demonstrated reduction in gross scar appearance without impacting time to

wound closure (Shi, et al., 2015). As in the case with therapeutic Dkk-1 administration, more studies are needed to produce the desired regenerative healing phenotype *in vivo*.

A recent study by Lee *et al.* demonstrates the balance that must be struck in regulating pathways of wound healing, such as Wnt, in the pursuit of improved wound healing. Here the authors demonstrated that CXXC5, a zinc finger family protein, inhibits β-catenin and blocks wound healing in part by decreasing contraction, migration, and collagen production in dermal fibroblasts/myofibroblast-like features of dermal fibroblasts. The authors subsequently developed synthetic peptides to block interactions between CXXC5 and Dishevelled (Dvl), a mediator of Wnt signaling, which together lead to β-catenin degradation. While topical treatment of cutaneous wounds in mice resulted in faster healing, which could be further accelerated with positive Wnt regulators, this therapy may result in more scar formation. However, the authors did not quantify final scar size across treatment and control groups. (Lee, et al., 2015a)

Not only do cells exhibit different genetic expression profiles as they transition into the adult scar-forming phenotype, developmental stage and tissue of origin dramatically impact how fibroblasts respond to Wnt signaling (Lam and Gottardi, 2011). For example, Carre *et al.* elucidated differences between mouse embryonic (E16.5) fibroblasts and postnatal (P1) fibroblasts following stimulation with Wnt-3a. Wnt-3a increased postnatal, but not fetal, fibroblast proliferation, with concomitant collagen type I synthesis. Furthermore, recombinant Wnt-3a produced similar findings to administration of pro-fibrotic TGf-β1 and also shifted postnatal fibroblast expression of TGF-β isoforms towards that of scarring wounds, with increased TGF-β1 and decreased TGF-β3. Finally, based on gene expression analyses, endogenous Wnt signaling was found to be elevated, but to a substantially lesser degree in fetal cutaneous wound repair compared with adult skin. (Carre, et al., 2010)

Despite these findings, by inducing sustained epidermal β-catenin signaling, Collins *et al.* observed increased fibroblast proliferation and enhanced remodeling of the dermal ECM at levels akin to neonatal dermis. The connective tissue composition also more closely resembled developing skin. (Collins, et al., 2011) Furthermore, the activation of epidermal Wnt/β-catenin signaling can cause expansion of the upper dermal fibroblast lineage, which in turn allows hair follicle growth not traditionally observed in physiological wound healing (Driskell, et al., 2013, Gurtner, et al., 2008). This observation has led some to conclude that the upper dermal fibroblasts could be utilized as a means for resolving scar formation and promoting scarless wound healing, given their more abated fibrillary collagen production relative to the lower dermis lineage (Driskell and Watt, 2015).

Many Wnt studies in skin have focused on its role in hair follicle neogenesis and its involvement in cutaneous homeostasis and regeneration. While researchers such as Bastakoty *et al.* specifically targeted the dermis with topical application of Wnt modulators/inhibitors, many other studies into the skin regenerative phenotype have focused on follicular neogenesis, relying on epithelial or follicle-specific paracrine Wnt signaling (Bastakoty, et al., 2015, Ito, et al., 2007). Following full-thickness wound epithelialization, Ito *et al.* noted that formation of *de novo* hair follicles both requires Wnt signaling and is promoted by Wnt overexpression. Given that mammalian hair follicles are believed to form

only during embryonic development, the authors postulated that wounding could induce an embryonic phenotype in the skin, which in turn can be regulated by Wnt proteins. (Ito, et al., 2007) Specific models used to study the impact of Wnt in scar formation may differ in part due to the transient mode of action and the different means by which this pathway is manipulated experimentally. De la Roche *et al.* noted that long-term Wnt activation limits the efficacy of small molecule Wnt inhibitors (de la Roche, et al., 2014). The differential outcomes of Wnt stimulation based on target tissues, partly related to the mode of Wnt pathway modulation, is likely to account for some of the aforementioned discrepancies relating to upregulation of the canonical pathway either promoting or inhibiting scarless wound healing.

MicroRNA (miRNA)

MicroRNA (miRNA) gene therapies are another more recent avenue for potential therapeutic intervention. These molecules exert an inhibitory role on mRNA transcription within eukaryotic cells, effectively silencing genes at a post-transcriptional level (Sen and Ghatak, 2015). Comparison of genome-wide miRNA expression between mid- (E16) and late-gestational (E19) mouse skin discovered global repression of these molecules at the earlier time point, which is known to heal without scar formation (Cheng, et al., 2010). In human fetal keratinocytes, Zhao *et al.* established the miR-34 family as potential candidates for scarless wound healing. Expression of these miRNAs was significantly lower in late- as compared to mid-gestational keratinocytes. This family may also heavily suppress genes involved throughout the TGF-β pathway. (Zhao, et al., 2015) Numerous tools have been developed to modulate cell and tissue miRNA levels, several of which have been directed towards regenerative wound healing. For example, miR-145, has been found at three times normal levels in hypertrophic scars and pro-fibrotic TGF-β1-induced myofibroblasts. Using a commercial inhibitor of miR-145, Gras *et al.* were able to show a significant decrease in type I collagen expression, TGF-β1 secretion, and contractility in skin myofibroblasts. (Gras, et al., 2015) Additionally, miRNAs can be combined with biomimetic scaffolds to enhance wound healing, furthering their clinical potential (Monaghan, et al., 2014). Future *in vivo* studies will hopefully enumerate the clinical potential for other miRNA-based therapies.

CONCLUSION

Aberrant wound healing, scarring, and fibrosis results in a tremendous burden on public health. Thankfully in this regard, recent advances in stem cell and developmental biology have elucidated novel pathways and cell types involved in scarring and fibrosis. However, to accelerate generalizability and translation, future studies aimed at assessing therapeutic potential for scarless wound healing would benefit from a more standardized model. It is not uncommon for decreased scarring to be interpreted from histological data showing less collagen staining. While collagen accumulation is a fundamental component of scar formation, clinically speaking, it is the macroscopic cutaneous scarring that most negatively impacts form and function following wound healing. Therefore, studies would benefit from a macroscopic assessment of scar size in wound healing models. Additionally, translation of research findings may be aided through utilization of a humanized wound model, relying

either on porcine tissue or splinted murine skin, as loose-skinned animals heal primarily by contraction whereas humans heal by granulation tissue formation (Galiano, et al., 2004). Increased standardization and rigor in defining what truly promotes scarless wound healing *in vivo* will also improve generalizability and translatability of findings.

While stem cell-based therapies may provide a more complex mélange of factors to promote scarless wound healing, there exist many barriers to their utility. By definition stem cells are multipotent or pluripotent and thus carry a potential risk of tumor formation. These cells also exhibit difficulty with survival and engraftment following administration. However, the latter may be less of an issue given their largely paracrine mechanisms of action and therapeutic benefits of stem cell conditioned media. (Cerqueira, et al., 2015) Conversely, molecular therapies offer increased specificity in their targets, allowing for more predictable effects. However, individual agents may be inadequate to successfully augment the complex wound healing process to the more privileged regenerative phenotype. Combinations of endogenous and exogenous wound healing modulators may likely prove to be the most practical means of successfully and reproducibly achieving scarless wound repair in patients. As our understanding of regenerative over reparative healing increases, translation of an improved understanding of scarring from fundamental science can result in novel clinical therapeutics.

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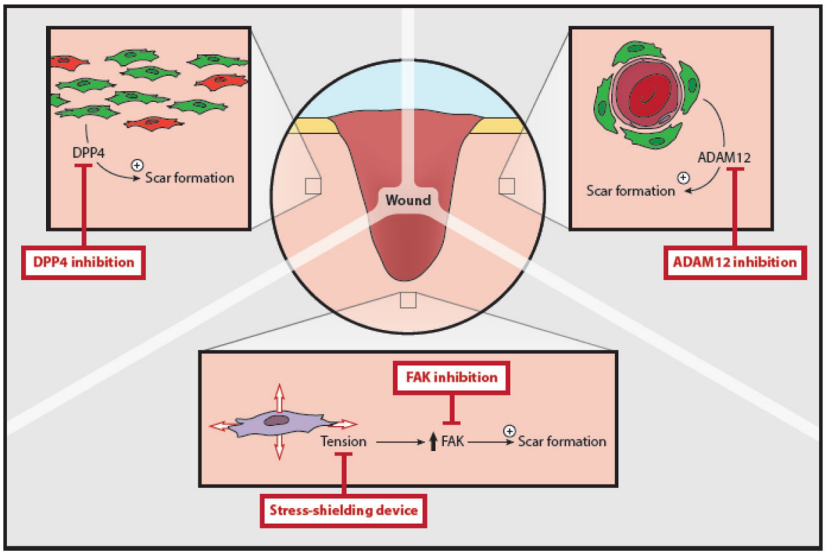

Figure 1. Recent approaches to reducing scar formation

Schematic showing novel scientific approaches for decreasing scar formation, including targeting pro-fibrotic cell populations based on surface molecule expression. Modulation of cellular mechanotransduction pathways are another means to reduce scar formation, both at the molecular level, or macroscopically with dressings designed to offload tension at cutaneous wound sites. ADAM12, a disintegrin and metalloprotease 12; DPP4, dipeptidyl peptidase-4; FAK, focal adhesion kinase.

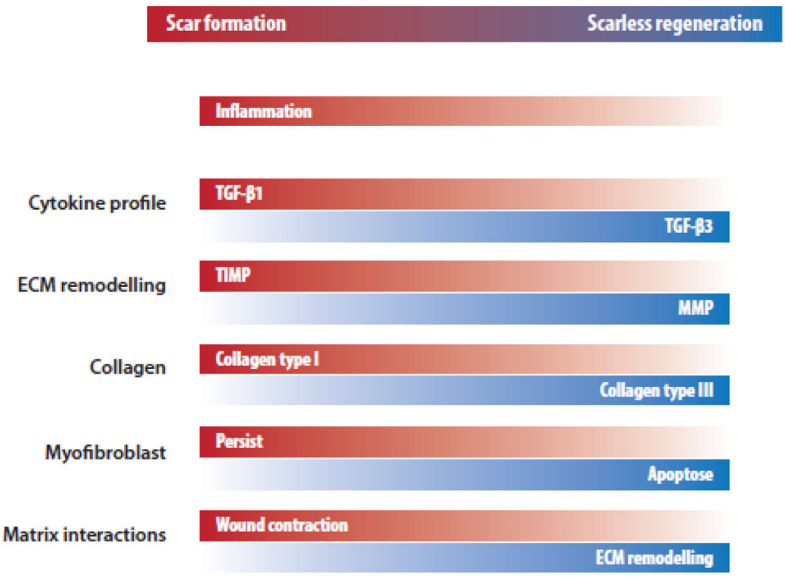

Figure 2. Features of reparative scar formation and scarless regeneration in wound healing
ECM, extracellular matrix; MMP, matrix metalloproteinase; TGF-β, transforming growth factor beta; TIMP, tissue inhibitor of metalloproteinase.

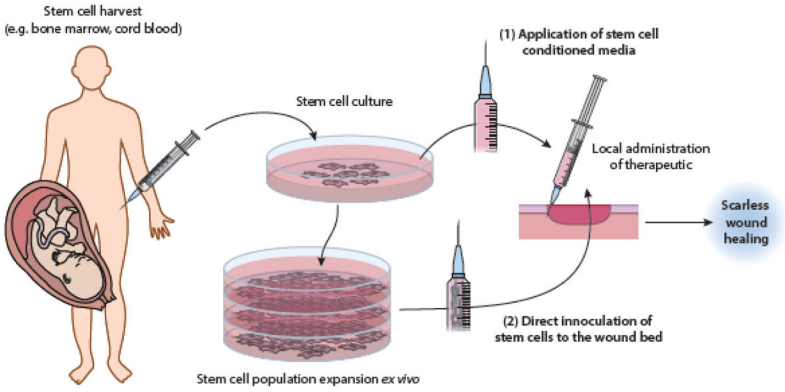

Figure 3. Stem cell-based therapies to promote scarless wound healing

Schematic showing general principles of two cell-based therapeutic methodologies, (1) application of stem cell conditioned media and (2) direct application of stem cells to the wound bed. The poor survivability of mesenchymal stem cells (MSCs) transplanted to the wound bed has prompted development of other novel therapies that take advantage of the paracrine mechanisms of action of these cells. Application of conditioned media from umbilical cord blood-derived MSC (UCB-MSC) culture is one such example (Doi, et al., 2016).

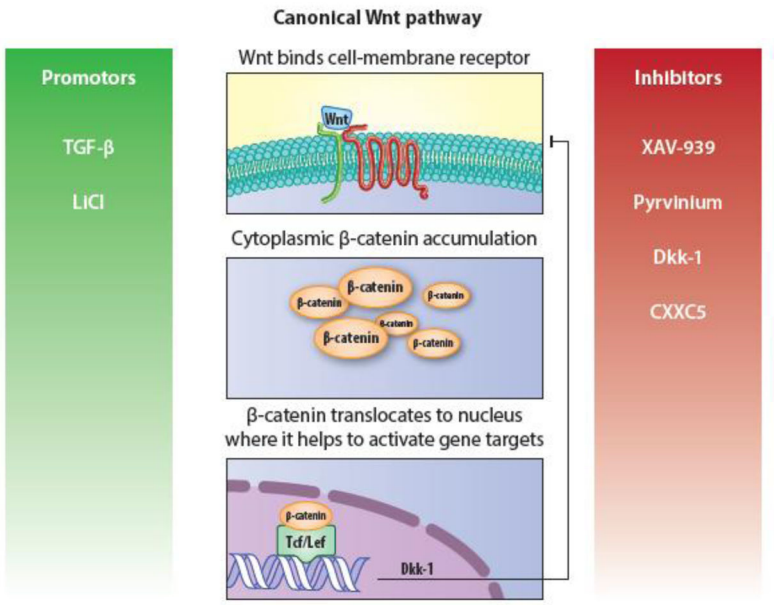

Figure 4. Brief overview of canonical Wnt signaling and its associated targets

Wnt binding results in cytoplasmic accumulation of β-catenin, which is degraded in the absence of ligand binding. Subsequently, β-catenin translocates to the nucleus where it interacts with T cell-specific transcription factor/lymphoid enhancer-binding factor (Tcf/Lef) in order to modulate gene expression. CXXC5 (CXXC Finger Protein 5); Dkk-1, Dickkopf-1; LiCl, lithium chloride; TGF-β, transforming growth factor beta.