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FETAL WOUND HEALING

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1. ABSTRACT

The developing fetus has the ability to heal wounds by regenerating normal epidermis and dermis with restoration of the extracellular matrix (ECM) architecture, strength, and function. In contrast, adult wounds heal with fibrosis and scar. Scar tissue remains weaker than normal skin with an altered ECM composition. Despite extensive investigation, the mechanism of fetal wound healing remains largely unknown. We do know that early in gestation, fetal skin is developing at a rapid pace and the ECM is a loose network facilitating cellular migration. Wounding in this unique environment triggers a complex cascade of tightly controlled events culminating in a scarless wound phenotype of fine reticular collagen and abundant hyaluronic acid. Comparison between postnatal and fetal wound healing has revealed differences in inflammatory response, cellular mediators, cytokines, growth factors, and ECM modulators. Investigation into cell signaling pathways and transcription factors has demonstrated differences in tyrosine phosphorylation patterns and homeobox gene expression. Further research may reveal novel genes essential to scarless repair that can be manipulated in the adult wound and thus ameliorate scar.

2. INTRODUCTION

Scar and fibrosis are the end result of postnatal tissue injury and disease. They are a major health problem because scarring does not replace lost function at the site of injury, and no proven therapy for scarring exists. Remarkably, fetal full-thickness skin wounds heal with restoration of normal epidermal and dermal architecture and not with scar formation. The biology responsible for

scarless wound healing, a paradigm for ideal tissue repair, is the subject of active investigation.

3. DEVELOPMENT

3.1. Fetal Skin

Fetal skin structure and histology change dramatically with development. The transition from scarless to scarring wound repair occurs in the context of the developing fetal skin. Therefore, investigation of normal skin development may reveal primary regulators of cellular differentiation and proliferation that play a role in fetal wound healing. In early gestation, mutual inductive mechanisms between ectoderm and mesoderm stimulate development of the epidermis and dermis. Epidermal primordial cells derived from ectoderm proliferate at 7 weeks gestation forming a squamous layer of periderm and a basal germinative layer. The periderm cells are keratinized, shed, and eventually replaced by the stratum corneum at 21 weeks. The basal germinative layer becomes the stratum germinativum, a source of new cells for dermal appendages, the intermediate layers found in mature skin, and hair germs. Hair follicles begin development in the 9-12th week. Peripheral follicle cells become the epithelial root sheath and surrounding mesenchymal cells form the dermal root sheath. Mesoderm derived mesenchymal cells produce collagen and elastic connective tissue fibers of the dermis by 11 weeks. Mesenchyme also differentiates to form endothelial-lined blood vessels, which may acquire a muscular coat from myofibroblasts to form arteries and veins. Skin maturation with dermal thickening continues into the postnatal period (1).

3.2. Fetal Extracellular Matrix

The important role of the extracellular matrix in cell adhesion, differentiation, and proliferation has only recently been discovered (2). In the past, the ECM was regarded as inert scaffolding. We now know it is a dynamic layer of collagen, proteoglycans, and glycosaminoglycans which facilitates cellular migration in the fetus and serves as a reservoir for growth factors. The fetal ECM undergoes a series of changes before reaching the adult phenotype. Fetal ECM differs from adult ECM in collagen composition, hyaluronic acid (HA) content, and proteoglycan ECM modulators. This may have implications in scarless repair.

3.2.1. Collagen

Collagen is the dominant structural protein in all human connective tissue. Although several types of collagen exist, Type I predominates and is the principal component of both adult and fetal ECM (2). Its strength is derived from a triple helix configuration of polypeptide chains, which are cross-linked and stabilized by lysyl oxidase. Fetal skin has a higher ratio of type III to type I collagen than adult skin (3). With maturation, the relative amount of type III collagen in fetal skin diminishes, although the adult phenotype is not seen until the postnatal period (4).

3.2.2. Hyaluronic acid

Hyaluronic acid (HA) is a negatively charged, unsulfated glycosaminoglycan of the ECM found in soluble form or complexed with proteoglycans. Increased HA content in the ECM is noted during rapid cellular migration and angiogenesis (5). The net negative charges of HA trap and impede water molecules, which allows resistance to deformation and facilitates cellular movement (2). Fetal skin contains more HA than adult skin (6). HA stimulates collagen synthesis by fibroblasts *in vitro* (7).

3.2.3. Proteoglycan ECM Modulators

Proteoglycan ECM modulators decorin, fibromodulin, lysyl oxidase, and matrix metalloproteinases (MMPs) serve a role in collagen synthesis, maturation, and degradation. Decorin production increases by 72% during the transition period in fetal rat skin and continues to increase into the postnatal period achieving a level 300x that of early gestational fibroblasts (8). Likewise, increased expression of enzymes lysyl oxidase and MMP occurs with development (9-10). Fibromodulin, another modulator of collagen fibrillogenesis in the decorin family of proteoglycans, has decreased production with maturation (11). Fibromodulin binds and inactivates transforming growth factor beta (TGF-??? a key cytokine implicated in adult wound healing and scar (12).

4. SCARLESS FETAL WOUND REPAIR SPECIFICITY

4.1. Scarless Fetal Wound Phenotype

Phenotypic differences in collagen deposition and cross-linking patterns, hyaluronic acid content, and differential expression of proteoglycan ECM modulators and adhesion proteins distinguish fetal wounds from adult wounds (8-14).

4.1.1. Collagen Content

In scarless fetal wounds, collagen is rapidly deposited in a fine reticular pattern indistinguishable from uninjured skin. In contrast, adult scarring wounds have disorganized type I collagen bundles with more collagen cross-linking (13-15) (Figures 1-4). Type I collagen and the molecular chaperone heat shock protein 47 (HSP 47) both increase expression in adult rat wounds, as shown by reverse transcriptase-polymerase chain reaction (RT-PCR). In contrast, fetal wounds showed no difference in collagen I production or HSP 47 expression (16). Lovvorn et. al implanted PVA sponges in fetal sheep wounds and noted increased collagen cross-linking with advancing gestational age that paralleled the transition from scarless to scar-forming repair (17). Although Type I collagen cross-linking is essential for adult wound healing and strength, its rigidity may impede the movement of cellular mediators required for rapid cellular regeneration in the fetus.

4.1.2. Hyaluronic acid

The hyaluronic acid (HA) content of scarless fetal wounds increases more rapidly, is more sustained, and is overall greater than that of adult wounds (6). Fetal wounds have greater HA stimulating activity and fewer pro-inflammatory cytokines, such as IL-1 and TNF-alpha, that down-regulate HA expression (18).

4.1.3. ECM Adhesion Proteins

Scarless fetal wounds are characterized by a more rapid upregulation of ECM adhesion proteins and

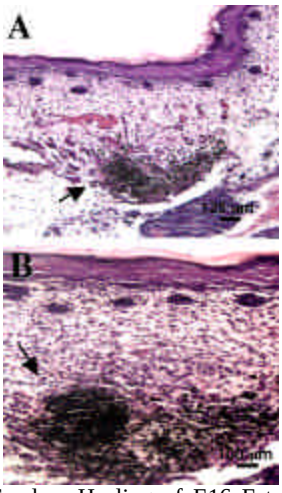

Figure 1. Scarless Healing of E16 Fetal Wounds (H&E Stain). Black arrows indicate India ink tattoo in the wound base made at the time of wounding in order to localize scarless location. A. E16 healed wound at 72 hours (150X). B. E16 wound at 72 hours (200X). The epidermal appendage (developing hair follicles) pattern shows numerous appendages directly in the healed wound. No inflammatory infiltrate is present.

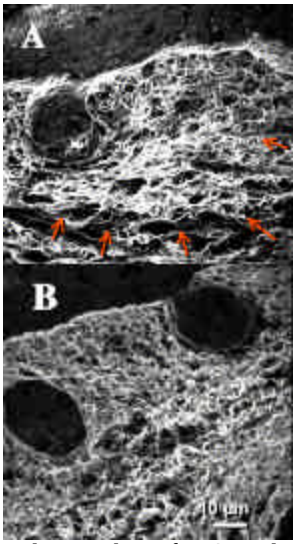

Figure 2. Scarless Healing of E16 Fetal Wounds (Confocal Microscopy). Collagen fibers are stained with sirius red and appear white. A. Healed E16 wound at 72 hours (1000X). The wound collagen fibers are arranged in a wispy reticular pattern (red arrows). The fibers are thin and closely approximate each other with little interfiber space. B. Non-wounded E19 skin at the same magnification as A (1000X). The dermal collagen fiber pattern is identical to A.

differential expression of cell surface receptors (integrins). The adhesion protein, fibronectin, mediates cellular attachment to the ECM and attracts fibroblasts, keritinocytes, and endothelial cells to the site of injury (5). Early gestation fetal rabbit wounds express fibronectin 4 hours after wounding while its expression is not seen until 12 hours in the adult (19). Whitby *et. al* found no difference in the onset of fibronectin expression, but noted more sustained expression in the adult (14). Tenascin blocks fibronectin-mediated cellular attachment. In upper lip wounds of mice, tenascin appears more rapidly in the fetus (1h) compared to the adult (24h) and precedes cellular migration (14). This suggests a role for tenascin in the rapid closure of fetal wounds. Collagen integrin receptors in fetal fibroblasts are differentially expressed with increasing gestational age. Fetal fibroblasts have increased alpha 2 integrin subunit expression and decreased alpha 1 and 3 integrin subunit expression compared to the adult. This correlates with the low capacity of fetal fibroblasts to contract a collagen gel and may have implications in the differences seen between fetal and adult wound contraction (20).

4.1.4. ECM Proteoglycan Modulators

Regulators of collagen organization and degradation influence the ECM architecture. Decorin, a modulator of collagen fibrillogenesis, shows no change in fetal wounds but is up-regulated in adult wounds (8). Fibromodulin facilitated cellular migration is down-regulated in adult wounds and unchanged in the fetal wound. (11,21). This may prove useful as a marker of wound phenotype--if exogenous factors decrease scarring, they may decrease decorin and increase fibromodulin. Lysyl oxidase expression is greater during adult wound repair and has been implicated in fibrotic diseases (9). Matrix metalloproteinases (MMPs) and tissue-derived inhibitors (TIMPs) function in ECM turnover. Levels of MMP1 and MMP9 are increased in scarless wounds while scarring wounds have down-regulation of MMP2 and contain greater TIMP expression. Overall, scarless wounds have a higher ratio of MMP to TIMP expression, likely favoring remodeling and less accumulation of collagen (10).

4.2. Scarless Repair Is Intrinsic to Fetal Skin

The capacity for scarless repair was initially attributed to the sterile intrauterine environment. Amniotic fluid is rich in hyaluronic acid and growth factors but devoid of bacteria and inflammatory stimulators, thus it was thought to be permissive for scarless repair. However, early studies demonstrated that the intrauterine environment is neither essential nor sufficient for scarless repair. Fetal marsupials develop outside the uterus in a maternal pouch and heal cutaneous wounds without scar (22). Adult sheep skin transplanted onto the backs of fetal sheep bathed in the amniotic fluid of the intrauterine environment healed incisional wounds with scar while adjacent incisional wounds in fetal skin healed scarlessly (23).

Fetal scarless repair is also organ-specific. At time points early in gestation where fetal skin heals without scar, fetal stomach, intestine, and diaphragm heal with scar formation (24-25). This suggests certain subpopulations of cells in skin modulate the local wound healing response. Further evidence implicates the fetal fibroblast as the effector cell responsible for scarless repair. Human fetal skin from 15-22 weeks gestation was transplanted subcutaneously and cutaneously onto the backs of athymic

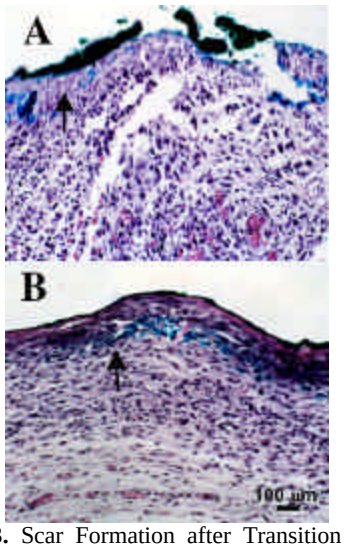

Figure 3. Scar Formation after Transition Point in E18 Fetal Wounds (H&E Stain). Green vital dye tattoo made at the time of wounding is visible at the wound sites (black arrows). A. E18 wound at 24 hours. The wound has not epithelialized (200X). B. E18 wound at 72 hours. No epidermal appendages are present, consistent with adult-type repair and scar formation (200X). The wound has epithelialized.

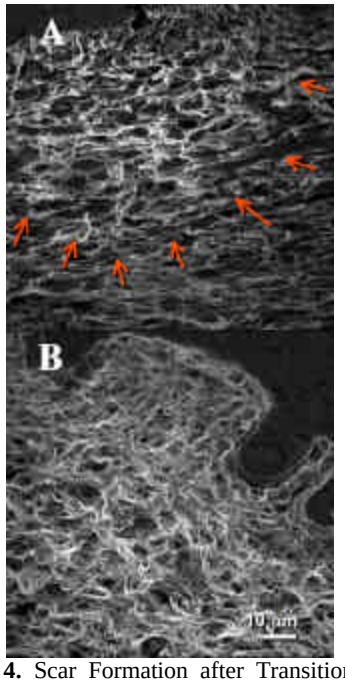

Figure 4. Scar Formation after Transition Point in E18 Wounds (Confocal Microscopy). Collagen fibers are stained with sirius red and appear white. A. Healed E18 wound at 72 hours (1000X). The wound dermal collagen pattern (red arrows) is different from the surrounding non-wounded dermis. The wound collagen fibers are less densely compacted. The fibers are thicker and have greater interfiber space. No epidermal appendages are present. B. Non-wounded E21 skin (1000X). When compared to wound collagen fibers (A), non-wound dermal collagen fibers are thinner and have less interfiber space.

adult mice. In this adult system, wounds created in the subcutaneous fetal grafts healed scarlessly with human collagen from fetal fibroblasts. Conversely, wounds made in the gestationally equivalent cutaneous fetal grafts healed with scar composed of mouse collagen from adult fibroblasts (26).

4.3. Scarless Repair Depends on Gestational Age and Wound Size

There is a developmentally regulated threshold for scarless healing based on gestational age and the extent of injury. The ontogenetic transition of rat skin has been defined in an organ culture system and confirmed *in vivo* with confocal microscopic analysis (15,27). This transition point lies between days 16.5 and 18.5 of gestation (Term = 21.5 d). In a human fetal skin model, the transition point occurs after 24 weeks of gestation (26). Wound size modulates the transition point. In fetal lambs, increasing wound size increased the frequency of scarring at a gestational age when smaller wounds healed scarlessly (28). In nonhuman primates, the transition from scarless to scarring repair has been shown to proceed through an intermediate wound phenotype. Fetal monkey lip incisional wounds heal with restoration of normal epidermal appendage and dermal collagen architecture in midgestation. At the start of the third trimester, these wounds do not restore epidermal appendage (hair follicle and sebaceous gland) architecture, but still heal with a normal collagen dermal pattern. Thus, a "transition wound" phenotype occurs. By the mid-third trimester, the wounds heal with a typical scar pattern - no appendages and collagen scar (29).

5. MECHANISMS OF SCARLESS REPAIR

Fetal wounds heal rapidly with a paucity of inflammatory cells. This key observation has stimulated interest in the role of cellular inflammatory mediators, cytokines, and growth factors in fetal wound healing. We know that in the postnatal animal, disruption of tissue integrity stimulates platelet activation, cytokine production, and chemotaxis of macrophages and neutrophils (30). However, scarless wounds are characterized by a relative lack of inflammation (5). Furthermore, introduction of inflammation into normally scarless wounds produces dose-dependent increases in wound macrophages, neutrophils, collagen deposition, and scarring (31). This suggests an important role of inflammation in scar formation.

5.1. Cellular Inflammatory Mediators

5.1.1. Platelets

The absence of an acute inflammatory infiltrate in scarless wounds may be partly explained by decreased fetal platelet degranulation and aggregation. Although there is no difference in size, organization, or granule content by transition electron microscopy in fetal compared to adult platelets, fetal platelets produce less platelet-derived growth factor (PDGF), TGF-β1, and TGF-β2 than their adult counterparts (32). Fetal platelet exposure to collagen *in vitro* does stimulate growth factor release; however, the

platelets still do not aggregate (33). Olutoye, *et al.* further investigated the aggregatory capabilities of adult and fetal porcine platelets after exposure to collagen and ADP. The fetal platelets responded suboptimally to collagen and showed an age-dependent aggregatory response to ADP exposure corresponding with the transition period for cutaneous scarless to scar-forming wounds (34). Additionally, HA suppresses aggregation and release of PDGF from fetal platelets in a dose-dependent fashion, having the greatest effect in the HA-rich fetal environment (35).

5.1.2. Neutrophils

Neutrophils neutralize and engulf bacteria. Cytokines TGF-β1 and PDGF recruit neutrophils to the site of injury. In turn, neutrophils release self-stimulating cytokines and chemoattractants for fibroblasts and macrophages (30). Fewer neutrophils are present in the fetal wound, and an age dependent defect in the ability of fetal neutrophils to phagocytose pathogenic bacteria has been demonstrated in fetal sheep (36).

5.1.3. Fibroblasts

Synthesis and remodeling of the ECM by fibroblasts is essential for wound healing. Adult and fetal fibroblasts are recruited to the site of injury by soluble chemoattractants released by macrophages and neutrophils (2). Fetal wounds characteristically have less inflammatory cells and cytokine expression yet heal more rapidly than adult wounds. This may be partly explained by intrinsic differences between adult and fetal fibroblasts.

Fetal and adult fibroblasts display differences in synthetic function of collagen, HA, and other ECM components. *In vitro*, fetal fibroblasts synthesize more type III and IV collagen than their adult counterparts, correlating with an increase in prolyl hydrolase activity, the rate-limiting step in collagen synthesis (37-38). Collagen synthesis is delayed in the adult wound while fibroblasts proliferate. In contrast, fetal fibroblasts simultaneously proliferate and synthesize collagen (2). Increases in cell density diminish HA production in the adult but has no effect on fetal HA synthesis (39).

Fetal fibroblasts have a greater ability to migrate into collagen gels than adult fibroblasts. A migration stimulation factor secreted by fetal fibroblasts is purported to be responsible for this enhanced migratory ability (40). Fetal fibroblasts have more surface receptors for hyaluronic acid, which also serves to enhance fibroblast migration (39). Additionally, TGF-β, which inhibits migration of confluent fibroblasts *in vitro*, is decreased in the fetal wound (41).

Differences in contractile fibroblasts, termed "myofibroblasts", have also been reported. Myofibroblasts, detected by the presence of alpha smooth muscle actin, appear in the adult wound one week after wounding. The content of myofibroblasts is greatest during the 2nd-3rd week and then decreases with time (2). Wounds made early in gestation have virtually no myofibroblasts. In contrast, scarring fetal and postnatal wounds have progressively more active myofibroblasts, which correlates with contraction and degree of scarring (42).

5.2. Cytokines

5.2.1. Transforming Growth Factor - Beta (TGF-b)

The transforming growth factors were linked to wound healing shortly after their discovery more than twenty years ago. TGF-β is chemotactic for fibroblasts, keritinocytes, and inflammatory cells and stimulates collagen I production by fibroblasts (43). Isoforms TGF-β1 and TGF-β2 are thought to be pro-fibrotic and to promote scar formation because their expression is increased in adult wounds and their exogenous administration to adult wounds increases collagen, protein, and inflammatory cell accumulation (43). Expression is modified by decorin, fibromodulin, hypoxia, hypoxia-inducible factor-1 (HIF), and other ECM proteoglycan modulators (12,44). In turn, TGF-β modulates MMP expression (43).

Evidence implicating TGF-β1 as a pro-scarring cytokine is well established. Immunohistochemical analysis reveals no change in TGF-β1 and -β2 expression in fetal rabbit wounds but increased expression in adult wounds (45). Scarless wounds in fetal mice have less TGF-β1 staining than neonatal or adult wounds (46). Insertion of PVA sponges containing TGF-β1 into rabbit wounds causes normally scarless wounds to heal with scar (46). Treatment of adult rat wounds with neutralizing antibodies to TGF-β1 and TGF-β2 reduces scar formation (45,47).

Furthermore, the relative proportion of TGF-β isoforms, and not the absolute amount of any one isoform, may determine the wound phenotype. In scarless fetal wounds, TGF-β3 expression is increased while TGF-β1 expression is unchanged. Conversely, TGF-β1 expression is increased and TGF-β3 decreased in scarring fetal wounds (48-49). Treatment of adult rat wounds with exogenous TGF-β3 reduces scar formation (50). This suggests the ratio of TGF-β3 to TGF-β1 may determine whether tissue regenerates or forms scar.

5.2.2. Other Growth Factors

Platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) are additional pro-fibrotic cytokines. PDGF, a potent mitogen and chemoattractant for fibroblasts, has prolonged expression during scar formation but disappears by 24 hours in fetal wounds (51). Treatment of rabbit fetal wounds with PDGF induces a marked increase in acute inflammation, fibroblast recruitment, and collagen deposition (52). The FGF family of cytokines, including keritinocyte growth factors 1 and 2, has greater expression with increasing gestational age in fetal skin and during adult wounding (53). In contrast, a mitogen for endothelial cells, vascular endothelial growth factor (VEGF), increases two-fold in scarless wounds while its expression remains unchanged in scarring fetal wounds (54). Thus, an increased stimulus for angiogenesis and vascular permeability may assist in the rapid healing of fetal wounds.

5.2.3. Interleukins

Interleukins are cytokines important in chemotaxis and activation of inflammatory cell mediators. IL-6 stimulates monocyte chemotaxis and macrophage

activation while IL-8 attracts neutrophils and stimulates neovascularization (55). Wounding stimulates a rapid increase in IL-6 and IL-8, which persists at 72 hours in the adult but disappears by twelve hours in the fetus (55,57). PDGF induces adult fibroblast production of IL-6 (55). In turn, the addition of IL-6 to fetal wounds produces scar in normally scarless wounds. Both IL-6 and IL-8 expression are significantly lower in early fetal fibroblasts at baseline and with PDGF stimulation compared to in adult fibroblasts (55-57). IL-10 has an anti-inflammatory function through decreased production of IL-6 and IL-8. Wounds in fetal skin grafts harvested from early gestation IL-10 knock-out mice and grafted onto syngeneic adult mice heal with significant inflammation and scar (58). In an initial study, adult mouse wounds were treated with an IL-10 over-expression adenoviral vector. Inflammation was reduced and scarless healing occurred (59). This will likely have potential therapeutic implications for human adult wounds.

5.3. Molecular Control of Scarless Repair

Efforts toward defining the scarless fibroblast phenotype have examined cellular signaling via receptor tyrosine kinase phosphorylation patterns and adapter protein Shc expression. Shc couples receptor tyrosine kinase to mitogen-activated protein kinase (MAPK) (60). It serves as a key intermediate for discoid domain receptor (DDR) signaling and may contribute to hypoxia-induced HIF protein stabilization and endothelial migration (61-62). Although TGF-β signaling is mediated through intrinsic serine/threonine kinase receptors, tyrosine kinase receptor signaling controls fundamental reaction sequences leading to gene activation (63). Different receptor tyrosine kinase (RTK) phosphorylation patterns are observed between fetal and adult rat fibroblasts with increased amounts of epidermal growth factor receptor, DDR, and Shc proteins in fetal fibroblasts suggesting that RTK signaling may play a role in scarless repair (63).

Ultimately, the mechanistic differences between scarless and scarring repair may be regulated at the gene expression level. Homeobox genes are transcription factors that are implicated in the patterning and cell type specificiation events during development. These genes determine the direction taken by major developmental pathways involving activity of hundreds of genes. Their role in skin embryogenesis and wound healing is being investigated. Human homeobox genes MSX-1, MSX-2, and MOX-1 are differentially expressed during skin development (64). Additionally, human fetal scarless repair is associated with decreased expression of HOXB13 and increased PRX-2 expression (65). Given that scarless repair is inherent to developing skin, it seems likely that coordinated control of groups of genes by transcription factors, such as homeobox genes, has a crucial function during the repair process.

6. PERSPECTIVE

Experimental data obtained in the past decade has greatly increased our knowledge of fetal wound healing, but the precise mechanism of this complex event remains unknown. Fetal wound repair is a tightly regulated process involving various cellular mediators and cytokines. *In vivo* up- or down-regulation of these repair elements interrupts the orderly sequence of regeneration resulting in scar formation. In turn, manipulation of the post-natal scarring wound may allow skin to regenerate. Identification of more key wound repair regulators will be facilitated with microarray analysis of scarless healing. This will likely enable further manipulation of repair mechanisms in the adult wound in order to eliminate scar formation.

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