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Embryonic Wound Healing: A Primer for Engineering Novel Therapies for Tissue Repair

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Abstract

Scar is the default tissue repair used by the body in response to most injuries—a response that occurs in wounds ranging in seriousness from minor skin cuts to complete severance of the spinal cord. By contrast, before the third trimester of pregnancy embryonic mammals tend to heal without scarring due to a variety of mechanisms and factors that are uniquely in operation during development in utero. The goal of tissue engineering is to develop safe and clinically effective biological substitutes that restore, maintain, or improve tissue function in patients. This review provides a comparative overview of wound healing during development and maturation and seeks to provide a perspective on just how much the embryo may be able to teach us in the engineering of new therapies for tissue repair.

Keywords

tissue engineering; embryonic wound healing

INTRODUCTION

Youngsters should learn from their elders—the old chestnut goes—but sometimes the table turns. The tissue repair mechanisms of immature mammals tend to be superior to those of adults. Indeed, during the earliest stages of development the embryo is capable of scar-free and sometimes regenerative healing—tricks that once learned and translated to the adult could have major impacts on clinical medicine. The embryo may be one of the most important textbooks for the growing field of *Tissue Engineering*, as this discipline moves toward the development of clinically useful biological substitutes that restore, maintain, or improve tissue function (Lysaght et al., 2008). Most adult injuries, from skin wounds to myocardial infarctions and spinal cord injuries, result in some level of scarring. Unfortunately, the implementation of tissue-engineered solutions can themselves lead to further scarring (Vistnes et al., 1978; Grill and Mortimer, 2000). With this in mind, engineers, researchers, and clinicians stand to be taught much from the embryonic paradigm of scar-free healing.

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Scar is a physical stand-in for the functional tissue it replaces following injury. Once formed, fibrotic scar is an effective mechanical repair, but in excess, it can inhibit optimal function of a tissue or organ. Scar tissue in obligate organs such as the heart can be especially problematic. Cardiologists often admit that dealing with the clinical fallout from fibrotic tissue is almost the “whole game” in their area of medical practice. In one example, scar tissue forms after a myocardial infarction prompted by cell death caused by ischemia (Eltzschig and Eckle, 2011; van Nieuwenhoven and Turner, 2012). Due to the active contractile function of the heart, this injury must be healed rapidly while the heart continues to function, most often resulting in scarring. Cardiac scar tissue is unable to contract and is thought not to conduct electrical signals effectively, potentially leading to arrhythmias and heart failure (Palatinus et al., 2010). The clinical implications of scarring also encompass sclerosis of the liver, lungs, kidneys, and other organs, making the excess deposition of fibrotic tissue one of the most wide spread “pathologies.”

Scarring does not occur to the same degree in all organs. This variability is likely due to differences in the intrinsic properties of the cell types involved, the extracellular matrix (ECM) and cytokine signaling within the tissue (Rhett et al., 2008; Dobaczewski et al., 2010; Mahdavian et al., 2011). Mechanical activity also plays a role in scarring, (Aarabi et al., 2007) as evidenced by the tendency of scrapes on knees and elbows to heal more slowly than areas of skin subject to less stress and strain. Generally, tissues with cell populations that turnover more rapidly (e.g., intestines and liver) demonstrate less scarring in response to injury, while tissues with lower mitogenic activity, like the heart, tend to scar more readily (Bertalanffy, 1967; Laflamme and Murry, 2011). This being said, cell populations within mature organs such as the heart, including fibroblasts, vascular cells, and immune cells undergo constant renewal and regenerate quite effectively in response to injury (Baudino et al., 2006). For those interested in reading further on adult wound healing, the topic is reviewed (Gurtner et al., 2008).

In contrast to wound repair in the adult, the tissues of the mammalian embryo tend to heal scarlessly before the third trimester of pregnancy (Gurtner et al., 2008). The factors contributing to this are heavily studied but complex. One consideration likely relates to the relative immaturity of the embryonic immune system, which does not start to take on fully adult characteristics until late in gestation (Ygberg and Nilsson, 2012). Another aspect centers on signaling in the wound environment. Numerous signaling factors and pathways have been identified as showing distinct patterns of activity during development, as well as demonstrating responses to injury that are different from those occurring in mature tissues (Whitby and Ferguson, 1991; Buchanan et al., 2009; Leung et al., 2012). In one salient example, Fergusson and coworkers showed that transforming growth factor (TGF)- β isoform expression was altered in embryonic wound healing; observing that TGF- β_1 was downregulated while TGF- β_3 was upregulated. The distinctive composition, structure, and function of ECM in the embryo is a further consideration that should be taken into account (Schultz and Wsocki, 2009; Laflamme and Murry, 2011; Namazi et al., 2011; Sonnemann and Bement, 2011). A broad range of factors are thus likely to contribute to scarless healing in the embryo, each of which may provide lessons in the development of potential wound healing therapies.

DIFFERENCES IN THE MORPHOLOGIC SEQUENCE OF ADULT AND EMBRYONIC WOUND CLOSURE

The skin is an excellent experimental model for wound healing owing to its accessibility, the low morbidity, and mortality associated with modest cutaneous injuries and the large amount of data already accumulated over many years of research. Normal healing of adult skin wounds follows a stereotypic sequence over time. This sequence goes from the acute hemostasis phase in which bleeding is rapidly halted, to the inflammatory phase (0–3 days), granulation (2–10 days), wherein scar progenitor tissue is differentiated and the final, relatively protracted scar remodeling phase (10 + days; Clark, 1996; Gurtner et al., 2008; Namazi et al., 2011; Sonnemann and Bement, 2011). During the first week, the primary consideration for successful healing is wound closure and the re-establishment of the dermal barrier. Failure to achieve timely closure via re-epithelialization and myofibroblast-mediated contracture is a characteristic of pathologic, slow, or nonhealing wounds, such as diabetic foot ulcers.

Martin and Lewis carried out a classic study some 20 years ago that demonstrated closure of skin wounds in embryos occurred in a fundamentally different manner from that in the adult. Upon injury of chick and mouse embryos by removal of a limb bud, an actin cable was assembled by epidermal cells around the injury border (Martin and Lewis, 1992; McCluskey et al., 1993). This cable then closed the wound in a “purse-string”-like manner. Because the cable assembled rapidly within minutes, its formation was thought to be dependent on redeploying existing actin, myosin and intercellular junctional molecules (Martin and Lewis, 1992). Interestingly, the embryonic wound closure mechanism closely parallels that observed to mediate neuropore closure during morphogenesis of the (unwounded) *Drosophila* embryo. In this model, Rho GTPase activity was shown to be essential for the assembly of this structure (Wood et al., 2002). In *Rho1* mutant *drosophila*, a cable fails to assemble but, after a lag phase of several hours, epithelial cells compensate for the absence of the actin “purse-string” by tugging on immediate epithelial neighbors using filopodia and lamellae that are assembled in place of the cable (Brock et al., 1996). Filopodia also seem to be responsible for the cell-to-cell matching needed for the final “zippering” shut of epithelial wounds (Jacinto et al., 2000; Wood et al., 2002).

CELLULAR DIFFERENCES ADULT AND EMBRYONIC WOUND HEALING

Platelets

The differences between embryonic and adult wound healing extend to the most acute phases of the injury response. Platelets are responsible for quickly walling off the injury and promoting hemostasis. When platelets are activated, adhesive proteins are discharged; some of which act as aggregation factors (e.g. fibrinogen) while others promote adhesion to the surrounding ECM (e.g. von Willebrand factor; Redd et al., 2004). Together these activities seal the wound by creating a fibrin clot, which is especially important in the prevention of infection of exposed surfaces of wounded skin not covered by an intact epidermis. Once aggregated in the wound, platelets degranulate secreting an array of chemotactic factors for inflammatory cells (Redd et al., 2004). Fetal platelets show decreased aggregation and

degranulation compared to adult counterparts (Olutoye et al., 1996). Despite poor functional interaction with collagen, fetal platelets secrete platelet derived growth factor (PDGF), TGF- β_1 , and TGF- β_2 when exposed to this ECM protein (Clark, 1996; Olutoye et al., 1997a). It is thought that the increased interaction with hyaluronic acid (HA), which is more prevalent in embryonic wounds, is also inhibitory to platelet aggregation (Olutoye et al., 1997b).

Immune Cells

The formation of the fibrin clot initiates recruitment of the first cellular responders of the adult immune system—neutrophils, followed by macrophages. The embryo is capable of producing a cellular immune response but it differs from that of the adult. The embryonic wound heals faster and, prior to the later stages of fetal development, with less immune presence [for more detail see (Cowin et al., 1998)]. In addition to the less differentiated state of immune cells, the lower quantity of neutrophils and macrophages may be contributed to by differences in the composition of the embryonic ECM. For example, higher molecular weight forms of HA are found in abundance and seem to inhibit the infiltration of leukocytic immune progenitors (Chen and Abatangelo, 1999; Savanti et al., 2000). The complement system also contributes to the initial injury response in mature tissues and plays an important role in inflammatory and fibrotic diseases (Schepp-Berglind et al., 2012). However, the complement system is thought not to be a significant mediator of embryonic wound healing (Wahl et al., 1974).

For skin wounds in the adult, and at later developmental stages, neutrophils begin arriving within minutes of an insult. In mature tissues, these cells are responsible for destroying contaminating bacteria by degranulation and phagocytosis. Barring a substantial bacterial infiltration, neutrophil activity decreases after the first few days. These cells will become senescent and later be phagocytosed by macrophages (Clark, 1996). Depletion studies have shown that the removal of neutrophils and macrophages does not seem to hinder but actually accelerates adult wound closure (Simpson and Ross, 1972; Dovi et al., 2003, 2004; Martin et al., 2003). By reducing the cellular immune response in this manner, immune cell-depleted mice mimic aspects of the embryonic wound environment, wherein lower densities of neutrophils and macrophages are present—with downstream effects on the levels of cytokines normally induced by these immune cells (Redd et al., 2004).

From 48 to 96 hr postinjury, macrophages become the predominant population in healing adult wounds. The progenitors of these cells, monocytes, are recruited from the circulation in response to signaling molecules including TGF β s, interferon- γ , tissue necrosis factor (TNF)- α (Parham, 2009, pp 49–53). Monocytes can also home to gradients of soluble fragments of collagen, elastin, and thrombin (Clark, 1996). Macrophages phagocytose debris, including remnants of apoptosed neutrophils, dead bacteria, and damaged ECM in the wound. In addition, these cells secrete chemoattractants and activators of fibroblasts and fibroblast progenitors in the wound, such as PDGF and TNF- α (Satish and Kathju, 2010; Koh and DiPietro, 2011). Depending on the wound environment, macrophages are putatively activated in a pro or anti-inflammatory manner—classified as either type 1 or type 2, respectively (Gordon, 2003). These phenotypes are transient and thought dependent upon the microenvironment that the macrophage encounters (Stout et al., 2005). Of particular

importance during remodeling, macrophages also produce a variety of proteases [e.g., matrix metalloproteinases (MMPs)] that assist in break-down and remodeling of the granulation tissue (Clark, 1996).

The activation state of macrophages in embryonic tissues in response to wound healing is less well understood. Indeed, incisional wounds in embryos show significantly reduced macrophage densities. On the other hand, burn cauterization of embryonic skin induces a more adult like-response by macrophages (Hopkinson-Woolley et al., 1994). This difference between embryonic incisional wounds and burn injuries may be due to the fact that cauterization produces increased tissue necrosis and thus may be more stimulatory to resident macrophages. Macrophages have been generally accepted as helpful for wound repair (Browder et al., 1988; Danon et al., 1989). However, the wound healing response of PU.1 null mouse embryos or adults is not delayed, despite the inability of this mutant to raise a cellular immune response, including that part of the response mediated by macrophages (Martin et al., 2003).

Fibroblasts and Myofibroblasts

As the inflammatory phase resolves, fibroblasts enter the wound and initiate formation of granulation tissue (Clark, 1996). In the adult, granulation tissue is the progenitor of the mature scar. However, in the embryonic skin, while collagenous granulation-like tissue is formed in response to injury, it is remodeled, and typically does not mature into scar tissue (Namazi et al., 2011). Interestingly, a similar process in which granulation tissue forms, but is eventually replaced by regenerated muscle has been observed following injury of the giant zebrafish heart (Lafontant et al., 2012). Upon exposure to TGF- β (Desmoulière et al., 1993) and the ECM of adult wounds, (Serini et al., 1998) fibroblasts take on a contractile phenotype, becoming myofibroblasts. These mechanically active cells have some characteristics in common smooth muscle cells and express α -smooth muscle actin (Chen et al., 2007; Hinz, 2010; Hinz et al., 2012). The myofibroblast is critical for wound closure, contributing to wound contraction by creating tension via interaction with the ECM, in an irreversible process that is regulated by a Rho/Rho kinase-mediated inhibition of myosin phosphatase (Kato et al., 2001). Other markers that differentiate myofibroblasts from fibroblasts include specific myosin heavy chain isoforms, as well as desmin and vimentin (Clark, 1996).

It was originally thought that resident fibroblasts had no potential to undergo transformation to myofibroblasts in embryonic wounds (Estes et al., 1994; McCluskey and Martin, 1995). However, when exposed to large doses of TGF- β_1 in vitro, it was determined that fetal fibroblasts were capable of differentiating into myofibroblasts. This transition was nonetheless found to be ephemeral and resulted in less collagen production than was observed in myofibroblasts from adult tissues (Rolfe et al., 2007). Interestingly, when embryonic fibroblasts are transplanted into adult wounds, these injuries have been reported to heal with reticular collagen patterns indistinguishable from healthy skin (Lorenz et al., 1995).

Adult myofibroblasts secrete high levels of collagen type I. By contrast, embryonic fibroblasts synthesize less collagen I and more collagen types III and IV. Developing

fibroblasts are also capable of simultaneously synthesizing collagens and undergoing proliferation, whereas adult fibroblasts proliferate as a prelude to collagen synthesis. Fetal fibroblasts also have more receptors for HA, but lower levels of TGF- β receptors (Alaish et al., 1994; Namazi et al., 2011). This could make these cells less responsive to TGF- β and more sensitive to HA, a characteristic that may explain the reduced densities of myofibroblasts observed in embryonic wounds in vivo.

Fibrocytes

Fibroblastic cell types are thought to be either derived from cells resident in tissue proximal to the wound or recruited from progenitors in the circulation (Baudino et al., 2006). The lineage relationships between these populations of fibroblastic populations remain a topic of interest and debate. While little is known about fibrocytes in embryonic wound healing, their contribution to adult wound healing should not be ignored. Circulating fibrocytes make up 0.1–0.5% of cells in the peripheral blood, excluding erythrocytes, and are phenotypically distinct from other white blood cells. The differentiation of fibrocytes is sensitive to TGF- β and could require T-cell interaction, much like the activation of dendritic cells (Chesney et al., 1997). Fibrocytes can also play a role in antigen presentation, expressing both Major histocompatibility complexes-I and II, as well as the ability to migrate to lymph tissues (Chesney et al., 1997; Abe et al., 2001). Fibrocytes also display potential for differentiating into adipocytes and chondrocytes, and appear to aid in diverse processes during adult wound repair that include angiogenesis and matrix degradation (Chesney et al., 1997).

Stem Cells

The embryo is in essence a conglomeration of stem cells—albeit that these cells exhibit varying degrees of differentiation potential (Nagy et al., 1990; Bishop et al., 2002; Rippon and Bishop, 2004). Embryonic wound repair will thus involve the direct incorporation of progenitor cells into the healed tissue (Lumelsky et al., 2001; Wichterle et al., 2002). Populations of stem cells are maintained in adult tissues to facilitate cell turnover and tissue regeneration (Young and Black, 2004). The extent to which stem cells are involved in wound healing in the adult is a question under active investigation (Herdrich et al., 2008; Metcalfe and Ferguson, 2008; Kuroda et al., 2011; Jackson et al., 2012). While some stem cells contribute directly to the repopulation of injured tissues (e.g., skeletal muscle satellite cells), others may provide paracrine cues that facilitate repair (Németh et al., 2009). Mesenchymal stem cells from adult bone marrow can produce a variety of factors which counteract inflammatory cytokines and induce angiogenesis (Wu et al., 2007). For instance, adipose-derived stem cells have been shown to act directly on inflammatory macrophages by secreting interleukin (IL)-10 (González et al., 2009).

“Small stem cells” (or “dot cells”) are a rare subpopulation of nonhematopoietic bone marrow stem cells, 2–4 μm in diameter, which maintain embryonic like characteristics in adults and embryos beyond the blastocyst stage of development. “Small stem cells” are greater than 95% Sca-1⁺/Lin[−]/CD45[−] and possess the potential to give rise to tissues from all three germ layers (Zuba-Surma et al., 2009; Ratajczak et al., 2010). Kong reported that the transplantation of bone marrow-derived “dot cells” prompted skin wounds to heal with less scarring. Transplantation of “dot cells” moreover reduced the amount of α -smooth

muscle actin and collagen expressed in the healed tissue, as well as inducing reticular patterns of ECM, similar to those found in nonwounded skin. “Small stem cells” efficiently homed to injury sites, via a CD184/SDF-1 signaling mechanism, and differentiated into dermal fibroblast-like cells that produced reduced levels of collagen (Kong et al., 2008). Bone marrow derived cells have been reported to enhance wound healing (Kuroda et al., 2011). It has been suggested that “small stem cells” may be responsible for the beneficial effects of treatments based on bone marrow derived cells (Kong et al., 2008).

EXTRA CELLULAR MATRIX

Collagen

Collagen is the most abundant ECM protein in healing wounds. In adult wounds, type I collagen is the dominant isoform present and is deposited in large fibers and organized bundles, which provide a strong scaffold upon which the wound repair proceeds (Clark, 1996; Beanes et al., 2002). This stiff matrix regulates cellular migration and promotes the differentiation of fibroblasts into myofibroblasts (Huang et al., 2012). Type III collagen is far more prevalent in fetal wounds and forms a finer and more reticular collagenous lattice than is observed in adult wounds (Larson et al., 2010). While collagen is a major constituent of the fetal wound during repair, many other factors impact the amount, organization and isoforms of collagen expressed. These will be discussed further in the following sections.

Hyaluronic Acid

HA is found throughout the body and has a number of biological assignments; among them cushioning and lubricating joints, osmotic buffering in the kidneys, and tissue hydration control in the skin (Chen and Abatangelo, 1999). Adult wounds only produce HA during the early stages of granulation tissue formation (Clark, 1996). In contrast, high molecular weight HA is abundant in embryonic wounds throughout the repair process. HA encourages fibroblast migration and influences the type of collagen that is laid down, shifting toward the production of type III collagen (Clark, 1996; Chen and Abatangelo, 1999). Because fetal HA is fully hydrated, it also prevents the passage of large proteins and bacteria through steric exclusion (Ogston and Phelps, 1961). This hydration also causes HA to occupy more extracellular volume and limits contact inhibition of proliferating cells, allowing faster recuperation of the damaged cell population (Namazi et al., 2011). HA can act as a free radical scavenger and this protective characteristic appears to be increased in higher molecular mass forms of the molecule (Presti and Scott, 1994). The HA matrix has inhibitory effects on angiogenesis and lymphocyte infiltration, which may account for the decreased inflammation seen in embryonic wounds. Conversely, low molecular weight forms of HA, which tend to be more prevalent in adult injuries, have been reported to encourage angiogenesis and lymphocyte infiltration (Clark, 1996; Savanti et al., 2000).

Tenascin-C and Fibronectin

Tenascin-C is a six subunit oligomer belonging to a family of deadhesive proteins (Clark, 1996). It prevents apoptosis despite the weak ECM adhesion it provides and allows cells to more freely change shape (Murphy-Ullrich, 2001). Tenascin-C has also been shown to promote migration and differentiation of myofibroblasts in cardiac wound healing (Tamaoki

et al., 2005). Based on these properties, tenascin-C could promote greater organization of the ECM and allow for faster cell migration into the wound.

Fibronectin is a broadly acting glycoprotein that plays structural, adhesive, and bridging roles in wound healing (Schultz and Wysocki, 2009). For instance, fibronectin functions as a conduit for fibroblast migration into the granulation tissue (Greiling and Clark, 1997). While it is initially deposited from the plasma, it is later synthesized locally (Clark et al., 1983). Fibronectin also serves as a repository of growth factors that ensures the extended activity of factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and TGF- β , in the wound site (Macri et al., 2007). Fragments of fibronectin contribute to an inflammatory response by activating macrophages, with the smallest fragments eliciting the greatest response (Feghali and Grenier, 2012).

During embryonic development, tenascin-C and fibronectin act in opposing roles, by supporting motility and attachment, respectively (Longaker et al., 1991; Whitby et al., 1991). Injured embryonic tissues upregulate these proteins earlier in the wound healing progression than occurs in the adult. Tenascin-C is first detected in the embryo, approximately 1 hr postinjury, while fibronectin is found upregulated around 4 hr after injury. In adult tissues, the expression of tenascin-C and fibronectin is delayed 24 and 12 hr following wounding (Longaker et al., 1991; Whitby et al., 1991). This order of expression is important, as tenascin-C antagonizes the action of fibronectin and other adhesive proteins that are required for cell adhesion and efficient wound closure (Clark, 1996).

Periostin

Periostin is a broadly acting ECM component of the fasciclin family that has been studied in many tissues (Kudo, 2011). In mouse skin, it is expressed mainly in the basement membrane and dermal-epidermal junction during early development (E13.5). It becomes more prominent in expression during late gestation (E19), and then diminishes and localizing around hair follicles (P9). Expression in the basement membrane is recapitulated in adult wounds with upregulation around wound borders (Zhou et al., 2010).

Periostin interacts directly with type I collagen, fibronectin, and tenascin-C. Periostin, tenascin-C, and fibronectin, forming a framework for the cross linking of collagen. Periostin also interacts with Bone morphogenetic protein (BMP)-1 to directly upregulate lysyl oxidase activity—the primary mediator of collagen crosslinking—and encourages BMP-1 incorporation into fibronectin matrices (Maruhashi et al., 2010). Observations in both periostin and tenascin-C knock-out mice suggest that both interact to organize the ECM architecture into the characteristic fibrous mesh seen in unwounded tissues (Norris et al., 2007; Kudo, 2011). While the most significant action of periostin in adult wound healing is in crosslinking collagen, it also stimulates differentiation of fibroblasts into myofibroblasts (Norris et al., 2007; Elliott et al., 2012). After myocardial infarction, increased periostin expression is associated with tight scar formation (Hakuno et al., 2010). One study in adult mouse reported that exogenous periostin induced proliferation of cardiomyocytes following cardiac injury reducing scarring and promoting a regenerative repair of the heart, similar to that seen in lower vertebrates such as the zebrafish and embryos (Kühn et al., 2007). However, subsequent work has questioned whether periostin promotes regenerative healing

of the adult myocardium (Lorts et al., 2009). The response of periostin expression to injury in the embryo is not well characterized.

GROWTH FACTORS

TGF- β

TGF- β has received much attention in the field of dermal repair and is recognized as one of the broadest-acting cytokines involved in the injury response. TGF- β is secreted from platelets, neutrophils, macrophages, and a variety of other cells. For a description of TGF- β effects in other tissues see (Clark, 1996). Ferguson suggested that the relative abundance of different TGF- β isoforms is important in promoting a switch from scarring to scar-free wound healing in mammals. This hypothesis was based on the observation that TGF- β_3 is more prevalent in non-scarring, embryonic wounds while adults expressed more elevated levels of TGF- β_1 . In a key experiment, Ferguson et al. reported that knocking down TGF- β_1 or TGF- β_2 with neutralizing antibodies or via application of exogenous TGF- β_3 promoted scar-free healing (Shah et al., 1995; Ferguson and O'Kane, 2004). TGF- β_1 is known to upregulate collagen synthesis and reduce MMP activity in fibroblasts. This change in balance between factors promoting collagen production and MMP peptidases mediating its breakdown, results in net accumulation of collagen in the wound (Clark, 1996; Shek et al., 2002). Embryonic fibroblasts are less responsive to TGF- β_1 induced upregulation of collagen, likely due to reduced TGF- β receptor density and signal transduction responsiveness (Rolfe et al., 2007; Namazi et al., 2011).

TGF- β is also thought to act in cardiac wound healing in the adult heart through its role in downregulating the renin-angiotensin-aldosterone system (RAAS), which governs myocardial collagen synthesis (Palatinus et al., 2010; Stein et al., 2010). Stein and coworkers have shown that chronic RAAS inhibition leads to reduced interstitial and patchy fibrosis and reduced vulnerability to ventricular arrhythmias in mice (Stein et al., 2010). The role of the TGF- β signaling axis in the cardiac injury response in embryos is not clear.

Interleukins

While originally thought to be specific for leukocytes (white blood cells), the IL family has since proved to be a ubiquitous set of cytokines that are highly influential in wound healing. IL-8 recruits neutrophils to sites of inflammation and IL-6 both recruits and directly activates monocytes/macrophages. Wounding stimulates a rapid increase in IL-6 and IL-8, which persists until 72 hr in the adult but disappears by 12 hr in the embryonic wound. In addition, fetal fibro-blasts secrete less IL-6 and 8 (Liechty et al., 1998, 2000a). IL-10 is a member of the IL family that inhibits IL-6 and 8. IL-10 levels are increased in embryonic wounds and this upregulation is thought to actively suppress inflammation (Liechty et al., 2000b). Variations in IL secretion can also determine the phenotype of macrophages that are recruited to the wound (Gordon, 2003). IL-1 has been shown to modulate procollagen production in cultured dermal fibroblasts derived from adult tissues with high concentrations stimulating production and lower doses inhibiting production (Heckmann et al., 1993). In the heart, IL-1 increases the expression of MMP-1, 3, 9, and 10 and reduces the expression of metalloproteinases that suppress neovascularization (Turner et al., 2010).

PDGF and Other Signaling Factors

PDGF is one of the earliest growth factors to participate in both adult and embryonic wound healing responses as it is released from platelet granules (Haynes et al., 1994; Clark, 1996). It acts as a chemoattractant for inflammatory cells and fibroblasts. Increasing levels of PDGF have been shown to increase both the number of fibroblasts and the amount of collagen deposition seen in embryonic wounds (Haynes et al., 1994). Wounds at early developmental stages tend to show faster clearing of PDGF, with no detectable presence 48 hr post injury (Whitby and Ferguson, 1991). This clearance may play a role in both the limited immune response of embryonic wounds, as well as the distinct patterns of collagen deposition at these stages.

Fibroblast growth factors (FGFs) are a family of cytokines with many activities including the regulation of cell proliferation, differentiation, and migration. FGFs are ubiquitously important for morphogenetic processes throughout the embryo, with their role in limb development being particularly well characterized (Metcalf and Ferguson, 2007). FGF 2, 7, and 10 are down-regulated in fetal wounds, while FGFs 1, 2, 5, and 7 are all upregulated during adult wound healing (Dang et al., 2003b). It is unclear whether this differential expression contributes to scar-free healing in the embryo.

Other growth factors also play a role in wound repair and regeneration. VEGF is increased in wounds and promotes angiogenesis. Exogenous additions of VEGF have been shown to promote healing of diabetic wounds (Frank et al., 1995). However, VEGF alone is not sufficient to form mature vessels (Risau, 1997). The addition of Angiopoietin-1 with VEGF prompts improved vessel development and maintenance with normal branch structure and pericyte populations (Benest et al., 2006). Epidermal growth factor (EGF) appears to have assignments in the healing of many tissues including the colon, skin, mammary gland, and liver. It also stimulates keratinocytes to produce HA (Pienimäki et al., 2001). HGF encourages the production of granulation tissue and vascularization after injury (Toyoda et al., 2001). Treatment with HGF has been reported to induce embryonic-like healing of skin wounds in adult diabetic mice (Yoshida et al., 2004).

MMPs and Tissue Inhibitor of Metalloproteinases

MMPs belong to a family of peptidases that include several collagenases, gelatinases, and stromelysins (Woessner, 1991; Clark, 1996). The expression of MMPs by macrophages and other cells is found in similar patterns of localization in adult and embryonic tissues, but a lower frequency of cells express these enzymes in adult tissues (Bullard et al., 2003). MMP-9 has been shown to activate TGF- β by cleaving latency associated peptides and can thus act to increase levels of active TGF- β at the wound site in adult tissues (Yu and Stamenkovic, 2000). In turn, TGF- β_1 also decreases matrix degradation through increased inhibition of MMPs via elevated expression of tissue inhibitor of metalloproteinase (TIMPs) by fibroblasts. (Bullard et al., 2003). TIMPs are expressed in greater quantities in adult wounds and act to stabilize the scar ECM (Lorenz et al., 2001). MMP-13 plays a role in the formation of granulation tissue and its absence has been reported to delay the differentiation of myofibroblasts (Toriseva et al., 2012).

Dang and colleagues reported that late in gestation, when wounds tend to resolve in scar tissue, MMP-1, 2, and 14, as well as TIMP-2 are increased. However, wounds at earlier gestational stages that heal in a scar free manner both show more rapid and greater levels of MMP-1 and 9 upregulation than in wounds than wounds at developmental stages (Dang et al., 2003a). Given that MMPs are upregulated upon wounding by early responding cells in both developing and adult tissues, MMPs appear to be central actors in the acute response to injury of both the embryo and mature adult (Bullard et al., 2003).

INTERCELLULAR JUNCTIONS

Connexin43 (Cx43) is the most abundant connexin in the skin, being expressed by keratinocytes, fibroblasts, and endothelial cells (Qiu et al., 2003). And is also plentiful throughout the body, especially in cardiac tissues (Guo et al., 1992; Vozzi et al., 1999). Similarly, Cx43 shows relatively pervasive expression in developing mammals (Ruangvoravat and Lo, 1992). In normal skin wounds, Cx43 is rapidly downregulated and matched by a complementary increase in Cx26 and Cx30 (Brandner et al., 2004). There are also concomitant changes in Cx43 phosphorylation patterns (King and Lampe, 2005).

It has been speculated that such changes in connexin expression and phosphorylation could create communicational compartments that participate in coordination of wound closure. Cx43 may also have important effects via noncoupling-based mechanisms, including via regulatory and protein-protein interaction domains on its cytoplasmic tail which may affect cell adhesion and migration (Palatinus et al., 2012). Hemichannels composed of Cx43 mediating communication between the cell cytoplasm and extracellular environment also appear to have critical assignments in the wound healing response (Pollok et al., 2011).

Targeting Cx43 function by antisense targeting or mimetic peptides was reported to promote reduced inflammation and scarring following wounding of adult mice, (Qiu et al., 2003; Mori et al., 2006; Ghatnekar et al., 2009) paralleling certain aspects of embryo wound healing. The effects of targeting Cx43 in wound healing in utero are unclear, although it has been reported that wound repair is accelerated in neonatal mice that are null for Cx43 gene expression (Kretz et al., 2004).

Results from Mendoza and coworkers suggest that Cx43 plays a direct role in the regulation of cytoskeleton components during fibroblast migration. Knock down of Cx43 and *N*-cadherin (the adhesive component of adherens intercellular junctions) enhanced migration rates of dermal fibroblasts and keratinocytes (Mendoza-Naranjo et al., 2012). Interestingly, it was found that the decreased expression of these two proteins, individually, also increased activation of RhoGTPases, RhoA and Rac1, which have been shown to initiate actin polymerization and stabilize it, respectively (Machacek et al., 2009). Francis et al came to related conclusions, cells from Cx43 knock-out mice are deficient in cell motility associated with unusual actin fiber organization and a loss of polarity (Francis et al., 2011). Kandyba and coworkers provided evidence of a role for Cx43 in re-epithelialization and keratinocyte migration in a 3D culture model system mimicking adult mouse skin (Kandyba et al., 2008). Cx43 has long been known to have roles in cell motility in morphogenesis and development

(Lo et al., 1999; Matsuuchi and Naus, 2012). Whether Cx43 modulates the behavior of migrating cells in response to injury in embryos awaits investigation.

As mentioned previously, closure of embryonic skin wounds involves an actin cable “purse-string” (Martin and Lewis, 1992). The authors of this study noted the localization of cadherins in clusters around the wound margin, marking the sites between adjacent cells which were connected by adherens junctions (Hartsock and Nelson, 2008). Adherens junctions play a critical role in organization and polymerization of actin bundles, as such it seems likely that these intercellular junctions have assignments in wound closure in the embryo.

LESSON FROM EMBRYONIC WOUND HEALING FOR TISSUE ENGINEERING

The goal of tissue engineering is to develop safe and clinically effective biological substitutes that restore, maintain, or improve tissue function in patients. Unfortunately, before tissue-engineered solutions can be implemented, significant work remains to be done on limiting complications resulting from the wound healing and immune responses of patients. In this respect, natural selection has perhaps engineered the coolest tissue engineered solution devised so far—avoiding graft versus host disease during gestation when a mammalian embryo becomes implanted in its mother’s womb. This and other lessons learned from the embryo might be adapted to improve the performance and tolerance of tissue engineered devices. In this section of the review, we will touch on some areas relevant to this discipline; again with emphasis on skin wound healing. The section will conclude with a note of caution on the obstacles that remain to clinical implementation of tissue-engineered devices for treating patients.

Stem Cells

The multipotency of stem cells is one of the more alluring characteristics of the embryo for tissue engineers. There are many challenges to using stem cells derived from developing and adult tissues in tissue engineering. Implanted stem cells typically have low survival rates when placed into the hypoxic and hostile environment of an adult wound (Vunjak-Novakovic et al., 2011). This being said, a number of stem cell based therapies are presently in preclinical stages for treatment of skin wounds [reviewed (Wu et al., 2010; Gauglitz and Jeschke, 2011)]. These include the stem cells from a variety of sources, such as bone marrow, umbilical cord blood, adipose tissue, and skin/hair follicles, each of which have been utilized to isolate stem cells and tested for their ability to modulate the healing response of acute and chronic wounds.

Approaches based on stem cells engineered to express recombinant genes is one area of growing interest. In one relatively novel development at the intersection of growth factors and stem cells, Cho and coworkers demonstrated that treating adipose derived stem cells with TGF- β_1 produces a conditioned media that induces the expression of type I collagen, MMP-1, and migration of fibroblasts (Cho et al., 2010). The authors proposed that this conditioned media might be used as a priming solution for scaffolds to provide a quick burst

of growth factors to initiate the wound healing process. Fibrocytes have been reported to be reprogrammed to promote remodeling rather than scarring by inducing local fibroblasts to increase MMP-1 expression and proliferate (Medina and Ghahary, 2010).

Additionally, prospects for enhancing stem cell therapies with new biomaterials and scaffolds are attracting attention. Novel techniques, including bioprinting and electrospinning, as well as scaffolds incorporating cell-matrix, cell-cell, mechanical cues, and soluble factors are being tested for potential in beneficially directing stem cell behavior during wound healing (Hodgkinson and Bayat, 2011).

Manipulation of Growth Factors and Matricellular Proteins

Recapitulating aspects of the embryonic signaling milieu has been a longstanding rationale for those working to beneficially reengineer the wound healing process; (Ferguson and O’Kane, 2004; Martin, 2004; Mori et al., 2008; Rhett et al., 2008) indeed, the exogenous application of developmentally active signaling molecules during wound healing is not limited to our species. EGF has key and evolutionarily conserved roles in the development of skin and dermal appendages (Sanson, 2001; Atit et al., 2003; Wang et al., 2006). Mammals, including lions, are said to “lick their wounds” and studies in mice have shown that EGF in saliva accelerates the healing of burns, possibly by attenuating the initial inflammatory response (Hutson et al., 1979).

Fergusson and coworkers pursued the hypothesis that altering the ratio of TGF- β isoforms in adult wounds to match those found in the embryo may improve skin wound healing. This hypothesis was the rationale for these workers bringing a TGF- β_3 -based therapy to human trials (Occeleston et al., 2008). Phase II trials of Justiva, a proprietary formulation of TGF- β_3 were promising. Unfortunately, Phase III trials failed to show benefit in scar revision surgeries (Justiva, 2011). This failure was discouraging for the field, it nonetheless remains that the preclinical evidence for anti-fibrotic effects of targeting TGF- β signaling pathways is relatively persuasive. Suppression of endogenous TGF- β_1 has been proposed to account for the fibrosis-reducing effect of overexpression of HGF mediated by a viral gene vector in cutaneous wounds (Ha et al., 2003). Nitric oxide has been shown to downregulate TGF- β and have a protective effect in skeletal muscle healing (Filippin et al., 2009). More directly, knockdown of TGF- β_1 by antisense RNA was shown to be effective in decreasing scarring after skin injury (Choi et al., 1996).

Growth factors have been incorporated into the polymer scaffolds used in tissue engineering (Ekaputra et al., 2011; Florczyk et al., 2012; Garg et al., 2012). Choi et al demonstrated that human placenta-derived ECM is an effective skin graft. In this protocol, the placenta was decellularized, homogenized, and formed into a porous sheet. Placement on full thickness rat wounds yielded improved regeneration, including newly formed hair follicles and keratin layer. Many growth factors and ECM proteins present during development, including collagen, TGF- β_1 , FGF, and PDGF, remained present in the matrix after processing and were thought to contribute to the observed treatment effects (Choi et al., 2012). Manipulations of so-called matricellular proteins including SPARC (secreted protein, acidic and rich in cysteine), CCNs, thrombospondins, osteopontin, and periostin, have been reported to reduce inflammation, promote accelerated wound closure and/or scarless healing

characteristics, similar to that occurring in injured embryonic tissues (Agah et al., 2002; Puolakkainen et al., 2005; Mori et al., 2008; Jun and Lau, 2011; Ohtsuka et al., 2012).

Connexin 43

Targeting of intercellular junctional proteins also appears to have promise in recapitulating aspects of embryonic wound repair (Gourdie et al., 2006; Rhetts et al., 2008; Palatinus et al., 2010; Mendoza-Naranjo et al., 2012). Antisense oligonucleotides reducing Cx43 expression were reported to reduce inflammation and scar tissue and accelerate healing in acute skin wounds (Qiu et al., 2003). Cx43 anti-sense has also been shown to promote reduced scarring after cutaneous burn injury, (Coutinho et al., 2005) enhance wound closure in a rodent diabetic model, (Wang et al., 2007) and provide benefit to spinal cord and central nervous system injuries (Cronin et al., 2006, 2008).

Synthetic peptides mimicking functional domains of the Cx43 molecule have also been studied in wound healing applications and yielded outcomes resembling those observed for antisense targeting. Peptides matching the extracellular docking domains of connexins have provided encouraging results for healing of acute and diabetic wounds (Wright et al., 2009; Pollok et al., 2011; Wright et al., 2012). A mimetic peptide based on the Cx43 carboxyl terminal (CT) domain was shown to reduce inflammation, speed the rate of wound closure and reduce area of scarring in rodent models of skin wound healing (Ghatnekar et al., 2009). This study pointed to peptide effects on Cx43 gap junction remodeling in the epidermis bordering the wound following injury. The CT mimetic peptide was also found to reduce Cx43 gap junction remodeling in the injury border zone following injury to the heart, as well as reducing inducible arrhythmias (O'Quinn et al., 2011).

CHALLENGES TO IMPLEMENTING SCAR FREE TISSUE-ENGINEERED REPAIR

In a further encouraging result it was reported that the Cx43 CT peptide reduced immune reaction and decreased scar capsule formation around silicone implants (Soder et al., 2009). It remains to be seen whether a similar benefit will occur for more complex tissue engineered devices—for example, constructs containing stem cells. Addressing this question points to a broader challenge for the field of tissue engineering. As it stands at present, we remain unable to integrate many of the novel biomaterials, scaffolds, tissue-engineered constructs, and engrafted stem cells generated by tissue engineering research safely into patients without risking complications due to the host immune system response. Fibrotic tissue and scarring around tissue-engineered grafts is manifestation of the host immune response. Immune-suppressant drugs provide one avenue to approach this issue, but as is the case with allo-transplantation of organs, a lifetime on powerful immune-suppressing drugs carries the risk of diabetes, hypertension, and skin cancer for patients (Parham, 2009 pp 455–475).

The use of autologous cells derived from the patient could provide a route to mitigating the host response to novel tissue-engineered constructs. However, wound healing processes, including inflammation, fibrotic tissue deposition and scar remodeling will still ensue

following placement of any graft into a patient, irrespective of the origins of its constituent materials and cells. Moreover, there is evidence that certain autologously derived cells are rejected following engraftment in vivo. Induced progenitor cells (iPSCs) are a type of stem cell that can be derived from adult tissues and reprogrammed to grow into different types of mature cells, including nerves, muscle, and bone (Takahashi and Yamanaka, 2006). However, in a study published recently, it was reported that iPSCs were rejected in mice, even when autologous cells were transplanted back into the individual mouse from whom which they were made (Zhao et al., 2011).

In sum, while exciting progress has been made, the challenges ahead should not be underestimated.

CONCLUSION

The embryonic wound represents a unique environment, the fuller understanding of which could lead to breakthroughs in regenerative medicine and tissue engineering. These wounds have a unique signaling environment, differentially arrayed complement of ECM proteins and show immune responses to injury that is distinct from those seen in adult tissues. Many promising clinical approaches that exploit aspects of this foundational knowledge are already being tested in preclinical and clinical settings; however, the secrets of the scarless repair and regeneration mechanisms occurring in the embryo are yet to be fully disclosed. The hypothesis that uncovering these mechanisms could provide breakthroughs for the field of regenerative medicine and tissue engineering remains a tantalizing prospect.

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