Wound healing and inflammation: embryos reveal the way to perfect repair

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Tissue repair in embryos is rapid, efficient and perfect and does not leave a scar, an ability that is lost as development proceeds. Whereas adult wound keratinocytes crawl forwards over the exposed substratum to close the gap, a wound in the embryonic epidermis is closed by contraction of a rapidly assembled actin purse string. Blocking assembly of this cable in chick and mouse embryos, by drugs or by inactivation of the small GTPase Rho, severely hinders the re-epithelialization process. Live studies of epithelial repair in GFP-actin-expressing *Drosophila* embryos reveal actin-rich filopodia associated with the cable, and although these protrusions from leading edge cells appear to play little role in epithelial migration, they are essential for final zippering of the wound edges together—inactivation of Cdc42 prevents their assembly and blocks the final adhesion step. This wound re-epithelialization machinery appears to recapitulate that used during naturally occurring morphogenetic episodes as typified by *Drosophila* dorsal closure. One key difference between embryonic and adult repair, which may explain why one heals perfectly and the other scars, is the presence of an inflammatory response at sites of adult repair where there is none in the embryo. Our studies of repair in the PU.1 null mouse, which is genetically incapable of raising an inflammatory response, show that inflammation may indeed be partly responsible for scarring, and our genetic studies of inflammation in zebrafish (*Danio rerio*) larvae suggest routes to identifying gene targets for therapeutically modulating the recruitment of inflammatory cells and thus improving adult healing.

Keywords: embryo; wound healing; morphogenesis; scarring; inflammation; macrophage

1. INTRODUCTION

It is well known that embryos heal wounds extremely rapidly and perfectly. Any developmental biologist who has had cause to perform microsurgical manipulations on tadpoles, or chick or zebrafish embryos will agree that their capacity for tissue repair is almost miraculous. Adult wound healing, in contrast, is notoriously imperfect and generally results in fibrosis and scar contracture with poor regeneration of epidermal and dermal structures at the site of the healed wound. For this reason and owing to the relative ease with which embryonic healing can be studied, it has become a favourite paradigm of efficient tissue repair. We hope that clues from the study of how embryonic tissues heal will lead to new therapeutic strategies for improving adult wound healing. Our own studies have largely used four embryo model systems: chick, mouse, *Drosophila* and zebrafish (*Danio rerio*; figure 1)—each of these has its own particular advantages for wound healing investigations.

Tissue repair in the embryo (and to a certain extent in adults too) appears to recapitulate those cell machineries used by embryos to undergo the natural tissue movements

of morphogenesis such as gastrulation and neural tube closure. This implies that studies of morphogenetic tissue movements may offer further clues as to how wounds heal and vice versa. The beauty of studying ‘artificial’ morphogenetic movements triggered by wounding, rather than natural events like gastrulation, is their relative simplicity and the fact that they can be initiated in a controlled fashion at a known time and location on the embryo. Perhaps by studying the cascade of events initiated by wounding and leading to wound closure it may also be possible to elucidate some of the general mechanisms that regulate natural cell and tissue movements in the embryo.

Adult wound closure involves active movements of both connective tissue and epidermis. The exposed connective tissue of the wound—the granulation tissue—contracts to tug the wound edges together and, as this is happening, the epidermis migrates to cover over the exposed connective tissue. The embryo also uses a combination of connective tissue contraction and re-epithelialization movements to close a wound, but the cellular mechanisms for both movements are quite different in embryo and adult. Another major difference between adult and embryonic tissue repair concerns the extent of inflammation during healing—at adult wound sites there is always an extensive inflammatory response, but in the embryo, inflammation is minimal, if non-existent. We will discuss the significance of this difference with regard to the signals that might activate wound closure movements in the two systems and how this might be relevant to scarring.

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Figure 1. Four models for analysing the cell and molecular mechanisms of wound healing. (a) The chick embryo is surgically accessible *in ovo* and suitable for cell biology studies, but poor for genetics. (b) Mouse embryos transplanted into roller culture allow the use of transgenic and knockout mice for testing gene function. (c) *Drosophila* are genetically tractable and tissue movements can be imaged live in embryos expressing GFP-fusion proteins. (d) Relatively genetically tractable (for a vertebrate), the zebrafish larva is translucent and thus ideal for live imaging of the inflammatory response associated with repair.

Figure 2. Histology of wound repair in a chick embryo. (a) A resin section through a closing wound on the dorsal surface of a chick embryo's wing bud. The bilayered epithelium is sweeping forward (left to right), over a naked wound mesenchyme. (b) Scanning electron micrograph of a similar wound reveals the superficial layer of epithelial cells (the periderm) as the sheet moves forward (upwards) to cover the wound mesenchyme. (c) A confocal image of an optical section through the basal layer of the epithelium reveals a bright actomyosin cable in the leading edge cells stained with an anti-myosin antibody.

Figure 3. Parallels between wound healing and morphogenesis in *Drosophila* embryos. (a) Still from a movie of wound closure in a *Drosophila* embryo expressing GFP-actin throughout its epithelium. The leading edge cells of the wound epithelium express a clear actin cable and numerous filopodia and lamellar protrusions. (b) Transmission electron micrograph at the point where wound edges meet one another to reveal interdigitation of filopodial protrusions from opposing epithelial cells. (c) Dorsal closure in the *Drosophila* embryo is driven by a very similar assembly of actin cable and filopodial protrusions that appear to knit together epithelial edges at anterior and posterior zipper fronts (region indicated in inset). (d) As during wound healing, interactions between filopodia of opposing epithelial cells precedes cell-cell adhesion. (e-g) Schematic series to illustrate how interdigitation of actin-rich filopodial protrusions may prime permanent adhesions between opposing epithelial cells during dorsal closure and wound healing (adapted from Jacinto *et al.* (2001)).

Figure 4. Early transcriptional events during morphogenesis and wound healing. (a) The gene, pucker, is expressed by leading edge cells as they undergo dorsal closure (see figure 3c). (b) Immunocytochemistry for *c-fos* reveals transient staining from 30 min post wounding in epithelial cells and, to a lesser degree, the wound mesenchyme (top right). (c) *c-fos* mRNA is expressed in the front three or four rows of cells peaking at *ca.* 10–30 min post-wounding the mouse embryo. (d) Another immediate early gene, *krox 24*, is induced within 5 min of tissue wounding, and its expression domain also extends up to three cell diameters back from the central wound site. Figure (a) courtesy of Dr Alfonso Martinez-Arias, Cambridge.

2. RESULTS AND DISCUSSION

(a) *Re-epithelialization of an embryonic wound is driven by actin purse-string contraction*

At the cut edge of an adult cutaneous wound a monolayer of basal keratinocytes sweeps forward across a provisional matrix of fibronectin, vitronectin and other matrix molecules at the interface between the wound dermis and the fibrin clot. Cells within the front few rows extend lamellipodia and alter their integrin expression; specifically they upregulate fibronectin/tenascin and vitronectin binding integrins and relocalize their collagen/laminin binding integrins so that the epidermal sheet can attach down and

drag itself forward over the wound substratum (reviewed in Grinnell 1992; Martin 1997; Werner & Grose 2003).

Our early studies in chick and mouse embryos revealed how the epithelium swept forward over the wound substratum (figure 2a,b), but suggested that lamellipodial crawling was not the migratory mechanism used to cover an embryonic wound. Transmission electron microscopy shows that wound epithelial cells remain adherent to the underlying basal lamina, which appears to be drawn along with the epithelial cells as they move forward (McCluskey *et al.* 1993). Fluorescently tagged phalloidin, used to visualize the distribution of filamentous actin, reveals a thick cable of actin in the basal epidermis at the leading edge of

the marginal cells encompassing the wound—contraction of this cable almost certainly provides the force required to draw the wound edges together in a purse-string-like manner (Martin & Lewis 1992). Indeed, when new assembly of filamentous actin is blocked by cytochalasin D, wounds completely fail to re-epithelialize (McCluskey & Martin 1995). As well as a filamentous actin cable, other components of the contractile machinery, including myosin II (figure 2c), are also assembled in a coordinate manner, as are the proteins—for example e-cadherin—that enable the intracellular cable to link with neighbouring cells via adherens junctions (Brock *et al.* 1996).

As assembly of the actin cable is so rapid (visible in leading edge cells within just 2 min of wounding) it would seem that at least the early stages of cable formation must be due to re-deployment of existing actin, myosin and junctional proteins. By loading the Rho blocking enzyme, C3 transferase, into chick wound edge cells *in ovo*, we showed that Rho activity is essential for assembly of the wound-induced actin purse string, whereas analogous Rac-blocking experiments do not interfere with the wound response, suggesting that Rho is indeed the master switch that mediates purse-string assembly at the embryonic wound margin (Brock *et al.* 1996). We still do not know what the initiating wound cue is, but our best guess is that it may be a mechanical signal; cells stretched along the wound margin as it initially gapes open may be mechanically stimulated to organize their actin in alignment with the force of stress, thus setting up the purse string that will subsequently cause the epithelium to close. Indeed, there are now clear data showing how Rho can be activated in cells by mechanical signals, and in *Drosophila* embryos mechanical cues have now been shown to operate as fundamental triggers directing local morphogenetic and patterning events (Farge 2003).

(b) Studying the actin machinery of wound healing live in *Drosophila* embryos reveals more than just a purse string

More recent studies using transgenic ActinGFP-expressing *Drosophila* embryos, wounded by means of a laser beam, have allowed live confocal imaging of the dynamic actin machinery as it assembles and draws the wound epithelium closed. Concomitant with the assembly of an actin cable, we also see dynamic filopodial protrusions extending from leading edge epithelial cells (figure 3a). These protrusions occasionally touch down on the substratum ahead of them, but show no sign of actively adhering and tugging the epithelium forwards. Genetic approaches using either small GTPase mutants or dominant negative transgenes for Rho and Cdc42, provide a means to analyse in real time the functions of actin cable and filopodia, respectively. In Rho mutants, a cable fails to assemble but, after a lag phase of several hours, cells compensate for the absence of a purse string, by tugging on their neighbours by means of the exuberant filopodia and lamellae they assemble in place of the cable (Wood *et al.* 2002). Actin-rich protrusions enable a wound to eventually close, even in the absence of a cable, by numerous foci where cells zip together. Blocking the activity of Cdc42 by means of a dominant negative transgene suggests that a failure to assemble filopodia does not hinder the rate of epithelial wound closure, but it does dramati-

cally block the final knitting together of the wound edge fronts as they finally meet one another, and so these wounds never completely close (Wood *et al.* 2002). It appears that final fusion is dependent on interactions between the actin-rich protrusions from opposing epithelial fronts (figure 3b).

(c) There are significant parallels between epithelial wound closure and dorsal closure in a fly embryo

Co-assembly of an actin cable and filopodial protrusions is not unique to wound edge epithelial cells. During the naturally occurring epithelial hole closure movements of dorsal closure during *Drosophila* embryogenesis and ventral enclosure of the *Caenorhabditis elegans* embryo, both these actin machineries are seen (figure 3c; Young *et al.* 1993; Raich *et al.* 1999; Jacinto *et al.* 2000; Hutson *et al.* 2003). The function of the cable is revealed in Rho-mutant *Drosophila* embryos and in Zipper mutants, null for non-muscle myosin. In both these mutants, dorsal closure generally proceeds and epithelial leading edges advance towards one another but the edges are flaccid rather than taut as in wild-type embryos. Counter intuitively, expression of the dominant negative Rho transgene in engrailed-expressing stripes of the epithelium causes these cells—which fail to assemble cable—to advance faster than their wild-type neighbours, suggesting that the cable operates as a constrainer of leading edge cells as well as a contractile purse string (Jacinto *et al.* 2002a; Bloor & Kiehart 2002). The function of the filopodia is quite clearly to drive adhesion of the two epithelial edges to one another. Blocking their assembly in engrailed stripes of epithelium by expression of dominant negative Cdc42 results in these stretches of epithelium failing to fuse properly in the midline (Jacinto *et al.* 2000). In *C. elegans*, the filopodia exhibit puncta of α -catenin at their tips and prime transient adhesions which mature into the permanent adherens junctions that form along the midline epithelial seam when gastrulation is complete—if these are prevented from forming then the embryo ruptures during the later elongation/expansion phase (Raich *et al.* 1999; Simske & Hardin 2001). These epithelial adhesions in fly and worm are preceded by a complex interdigitation of the filopodia from opposing epithelial cells (figure 3d–g), much as has been shown for the adhesions that form between keratinocytes in culture as they reach confluency (Vasioukhin *et al.* 2000).

In addition to similarities in cytoskeletal assemblies, there are clear parallels between embryonic repair and morphogenesis at the signalling level. Dorsal closure in flies is dependent on signalling by the Jun kinase cascade; indeed many of the mutants that fail in dorsal closure are components of this signalling pathway (reviewed in Harden 2002; Jacinto *et al.* 2002a,b). Activation of the Jun kinase cascade leading to AP-1 activation and transcriptional upregulation of decapentaplegic (a TGF β family member), and puckered (a dual specificity phosphatase; figure 4a), is mirrored in wounded rat embryos where we see immunostaining for *c-fos* in leading edge cells within minutes of wounding (figure 4b; Martin & Nobes 1992). Expression of *c-fos* (figure 4c), and other immediate early genes including *krox 24* (figure 4d; Grose *et al.* 2002), is transient and succeeded by transient expression of TGF β 1

in the front few rows of wounded epithelial cells (Martin *et al.* 1993). In *Drosophila* there is now good evidence that defects in jun kinase signalling perturb healing (Ramet *et al.* 2002), and more recent studies have shown that jun kinase signalling plays a key role in driving at least one vertebrate morphogenetic movement, that of eyelid closure which superficially resembles *Drosophila* dorsal closure. Indeed, mice with an epithelium-specific knockout of *c-jun* are born with open eyes and also show some wound healing defects (Zenz *et al.* 2003).

(d) Embryonic wounds undergo connective tissue contraction but they do not scar

Many hours after wounding adult skin the normally sessile dermal fibroblasts in the neighbourhood of the wound become activated and are recruited to the wound site where they proliferate and lay down matrix to fill in the connective tissue defect. This new connective tissue becomes heavily invaded by a network of capillary sprouts from pre-existing blood vessels of the wound bed in one of the rare instances of angiogenesis in adult tissues (reviewed in Martin 1997; Werner & Grose 2003). The vascularized wound connective tissue is termed granulation tissue and is responsible for the considerable wound contraction seen during adult skin healing.

It is generally thought that, although the early migrating adult wound fibroblasts are competent to generate some of the tensile forces necessary to initiate contraction, the chief contractile cell of adult granulation tissue is the myofibroblast, a specialist cell that differentiates from normal wound fibroblasts but more closely resembles a smooth muscle cell with expression of α -smooth muscle actin and capacity for generating strong contractile forces. The signals at the wound site that trigger this conversion from fibroblast to myofibroblast are believed to be a combination of growth factors such as TGF β (Desmouliere *et al.* 1993) and mechanical cues, related to the forces resisting contraction (reviewed in Grinnell 1994).

Our own *in ovo* studies of wound healing in chick embryos, labelling small groups of exposed mesenchymal cells at the wound margin with a lipophilic dye, DiI, allowed us to trace mesenchymal movements during the wound closure period and revealed a significant contraction of the initially exposed mesenchyme by the time the wound had become fully re-epithelialized (Martin & Lewis 1992). Similar experiments on wounded, limb-bud stage (E11.5) mouse embryos grown in culture show that the initially exposed wound mesenchyme contracts to about half of its original area by the time the wound has fully closed, suggesting that re-epithelialization and connective tissue contraction contribute equally to the total wound closure effort (McCluskey & Martin 1995), just as had previously been shown for adult wounds (Abercrombie *et al.* 1954). However, quite unlike adult contractile granulation tissue, it appears that embryonic connective tissue contraction does not require conversion of fibroblasts to myofibroblasts, because throughout the period of wound healing in a mouse embryo we see no cells staining positive for the myofibroblast marker, α -smooth muscle actin at the wound site except for that associated with blood vessels (McCluskey & Martin

1995). We presume that at least one of the signals triggering embryonic wound mesenchyme to contract is a paracrine TGF 1 cue released by the adjacent wounded epidermis (Martin *et al.* 1993).

(e) Inflammation does not occur following embryonic wounding

The inflammatory cascade at an adult wound site is initially activated by release of various cytokines and growth factors from degranulating platelets at any site where blood leaks from damaged blood vessels. These early signals are believed to initiate and orchestrate the tissue movements of re-epithelialization and connective tissue contraction and possibly to trigger the characteristic wound angiogenic response. The same signals also serve to recruit into the wound site the major inflammatory cells responsible for phagocytosing pathogens and other cellular debris; subsequently these inflammatory cells, primarily neutrophils and then macrophages, become the major sustained source of further growth factors and cytokines at the wound site after the early platelet signals have abated (reviewed in Martin 1997). All of these inflammatory responses are missing, or significantly reduced, at the embryonic wound site. First of all, in early embryos there are no platelets. Megakaryocytes, the progenitors of platelets, do not even begin to differentiate until about E13.5 in the mouse (Rugh 1990). The various inflammatory cell lineages make their first appearance outside the haematopoietic organs only a few days earlier. Macrophages are the first to appear and are seen leaving the yolk sac and entering mesenchymal tissues from about E10 (Morris *et al.* 1991). Other studies show that differentiated neutrophils appear in the foetal circulation somewhat later still (Rugh 1990). Our own studies in wounded mouse embryos and fetuses, using macrophage-specific antibodies, show that even at stages when macrophages have begun patrolling tissues they are not generally recruited to wounds until after the wound has closed. In limb-bud-stage (E11.5) embryos we found that macrophages were not actively recruited to the wound site at any time during the 24 hour healing period. A similar lack of macrophage recruitment to the healing wound site persists until relatively late in development at E14.5, when the limbs are nearly fully patterned and coincident with the first stage in foetal development when wounds heal with scars (Hopkinson-Woolley *et al.* 1994). Not surprisingly, because macrophages are one of the major sources of growth factors at the wound site, these potent signals are expressed at much lower levels and more transiently in the wounds of young embryos (Whitby & Ferguson 1991; Martin *et al.* 1993); this change in growth factor profile at the wound site may well partly explain the transition from perfect healing to healing with a scar as the embryo develops towards late foetal stages. These observations suggest that depleting the levels of some growth factors at the adult wound site might benefit repair and bring the process closer to that seen in scar-free healing in the embryo. Indeed, several strategies for reducing the levels of TGF β 1 in wounds made to the back skin of adult rats, including neutralizing antibodies, have successfully reduced scarring at the wound site (Shah *et al.* 1992, 1994, 1995).

Figure 5. Wound healing in the wild-type versus PU.1 null 'macrophageless' mouse. (a) Two to three days after an incisional wound to a wild-type neonatal mouse and the epithelium has just resealed beneath the wound scab. Deep to the epithelial layer is a disorganized wound connective tissue with abundant inflammatory cells. This tissue will go on to form a scar. (b) An equivalent wound in a PU.1 null sib has also re-epithelialized and has a more mature wound epidermis, sitting above a wound connective tissue that appears similar to that of unwounded skin.

(f) Wound healing studies in the PU.1 null 'macrophageless' mouse reveal that inflammation is not necessary for repair and may be causal of fibrosis

To test how necessary the inflammatory response is for orchestrating tissue repair we have studied wound healing in the PU.1 null mouse, which is genetically incapable of raising an inflammatory response because several haematopoietic lineages, including macrophages and neutrophils, are absent or severely delayed in their differentiation (McKercher *et al.* 1996) in these mice. Previous studies in which macrophages had been depleted by anti-macrophage serum and steroid injections had shown significant delays in repair of wounds in guinea-pigs (Leibovich & Ross 1975), but this steroid treatment may have knocked down more than just leucocytes as it is known that steroids block upregulation of immediate early genes in response to growth factor inductive signals and, as we have already discussed, these may be crucial activators of the repair process. In fact a genetic knockdown of the inflammatory response appears to enhance rather than perturb repair in PU.1 null mice. We see increased vascularity at the wound site and somewhat faster re-epithelialization of the wound surface, as well as an apparent failure to form a scar (figure 5; Martin *et al.* 2003). There appear to be fewer cells dying at the PU.1 null wound; this is in part owing to the absence of neutrophils which constitute the bulk of cells that die in wild-type wounds, but it may also be due to the reduced presence of cells actively killing otherwise healthy wound cells as they deliver cytotoxic-free radical bursts to kill microbes. The reduced mass of dying cells is cleared, although less efficiently than in the presence of macrophages, by 'stand-in' fibroblast phagocytes in PU.1 null wounds (Martin *et*

al. 2003). The cytokine and growth factor profile at wound sites in the PU.1 null mice is, not surprisingly, very different to that in equivalent wild-type sibs. For example, IL6, a pro-inflammatory cytokine known to be robustly expressed in wild-type wounds (Mateo *et al.* 1994), is almost absent in 'macrophageless' PU.1 null wounds, and TGF β 1 mRNA levels are reduced compared with those in wild-type sibs (Martin *et al.* 2003). Clearly, these mice offer good opportunities for dissecting the genetics of inflammation and for determining the downstream consequences of inflammation that may lead to fibrosis and scarring at the adult wound site.

(g) Live studies of leucocyte migration in zebrafish larvae will allow us to dissect the genetics of inflammation

To really understand the cell biology of any biological process it is best to watch it happen *in situ*, in real time. Clearly, this is not possible in the mouse, but because of the translucency of zebrafish larvae, differential interference contrast (DIC) optics allows one to observe the movement of leucocytes towards wounds. Each step in the inflammatory cascade—from the moment when leucocytes first adhere to the endothelium, through their extravasation between endothelial cells of the vessel wall, their chemotaxis towards the site of damage, their phagocytic behaviour at the wound site, and finally their resolution as the inflammatory response ceases—can be captured. Moreover, combining this amenability for live imaging with the relative genetic tractability (at least for a vertebrate), of zebrafish, there are clear opportunities in this system for determining not just whether a gene is involved in the inflammatory response, but at precisely which step in inflammation its protein product is required.

This analysis of inflammation will become considerably more elegant as several lines of leucocyte-specific green fluorescent protein (GFP) fish, which we are currently constructing, come online. Using these new fish we plan to dissect out essential components of the chemotactic signalling and reception machinery that draws leucocytes to the wound site as well as of the apparatus that enables them to diapedese through a blood vessel wall and crawl towards these cues. With the recent advent of the morpholino gene 'knock down' strategies in zebrafish (Heasman 2002), we can target specific candidate 'inflammation' genes and test their function. Notably, one of the chemokine signals that guides leucocytes to wounds has recently been shown to play a key role in guiding germ cell migration in zebrafish embryos (Doitsidou *et al.* 2002), suggesting that here too we will see a conservation in signals regulating tissue repair and embryo morphogenesis and patterning.

3. CONCLUSIONS: TOWARDS PERFECT HEALING IN ADULT TISSUES?

Studying wound healing in embryos offers a simple model of tissue repair and provides us with genetic opportunities to dissect the various cell behaviours that underlie this process. The efficient repair achieved by embryos as they heal wounds shows that perfect tissue regeneration is possible and offers a target for designing therapies to improve adult healing in the clinic. It appears that at the crudest level, inflammation—or at least the influx of neutrophils and macrophages—is not an essential prerequisite for efficient healing, so long as microbial infection is controlled with antibiotics. Now we need to understand the cell biology and genetics of inflammation better to identify gene targets for subtle modulation of the emigration and cytokine/growth factor release by the various leucocyte lineages, because total knockdown of inflammation is clearly not going to be an optimal treatment. Of course, designer drugs that modulate aspects of the inflammatory response will not only be useful in management of wound healing but also in countless other pathologies, such as Crohn's and various fibrotic diseases of lung and kidney, and even heart disease and cancer (Watts & Satsangi 2002; Tanino *et al.* 2002; Taubes 2002; Coussens & Werb 2002), as all of these are at least partly driven by inflammation gone awry. One other significant benefit of studying embryonic repair is the observation that repair and morphogenesis mirror one another in many regards. If we learn more about the ways in which epithelial tissues migrate forwards to close a wound and how they finally fuse together to form a perfect seam, we will be closer to understanding the more complex morphogenetic events, like neural tube closure and palate fusion, that can be so catastrophic when they fail.

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