Forceful closure: cytoskeletal networks in embryonic wound repair

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ABSTRACT **Embryonic tissues heal wounds rapidly and without scarring, in a process conserved across species and driven by collective cell movements. The mechanisms of coordinated cell movement during embryonic wound closure also drive tissue development and cancer metastasis; therefore, embryonic wound repair has received considerable attention as a model of collective cell migration. During wound closure, a supracellular actomyosin cable at the wound edge coordinates cells, while actin-based protrusions contribute to cell crawling and seamless wound healing. Other cytoskeletal networks are reorganized during wound repair: microtubules extend into protrusions and along cell–cell boundaries as cells stretch into damaged regions, septins accumulate at the wound margin, and intermediate filaments become polarized in the cells adjacent to the wound. Thus, diverse cytoskeletal networks work in concert to maintain tissue structure, while also driving and organizing cell movements to promote rapid repair. Understanding the signals that coordinate the dynamics of different cytoskeletal networks, and how adhesions between cells or with the extracellular matrix integrate forces across cells, will be important to elucidate the mechanisms of efficient embryonic wound healing and may have far-reaching implications for developmental and cancer cell biology.**

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INTRODUCTION

The ability of embryonic tissues to rapidly repair damage has fascinated scientists for more than a century. Embryos repair wounds with little to no inflammation or scarring, in a process conserved from fruit flies to humans. Collective cell migration drives the efficient sealing of lesions in the embryonic epidermis. Similar coordinated cell movements contribute to wound closure in adult tissues, and are critical for tissue morphogenesis and cancer cell migration. Thus, studying embryonic wound repair provides an opportunity to understand the biochemical and mechanical signals that cells use to integrate their behaviors during tissue development and in human disease (Friedl and Gilmour, 2009).

The study of embryonic wound closure offers several experimental advantages over other morphogenetic processes. First, the wound healing response is well defined in time, with a clear beginning and end. Therefore, it is possible to register multiple experiments based on the time of wounding, and the progress in wound closure can be measured based on the size of the wound. Second, if wounding assays are conducted in early embryos, breathing and muscle contraction do not interfere with live microscopy, and the process can be visualized without imaging artifacts. Third, embryonic wound repair occurs within a relatively short timescale (typically under one hour in most species), which facilitates the analysis of a greater number of samples, thus increasing the statistical power of the results. The ability to conduct many experiments within short timescales enables exploration of results that may otherwise be missed due to their variability, including the effects of wound size and geometry on the dynamics of repair (Davidson *et al.*, 2002; Abreu-Blanco *et al.*, 2012; Wyczalkowski *et al.*, 2013) or the subtle phenotypes caused by some genetic and pharmacological treatments (Zulueta-Coarasa *et al.*, 2014). Thus, embryonic

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Abbreviations used: AJ, adherens junctions; GFP, green fluorescent protein.

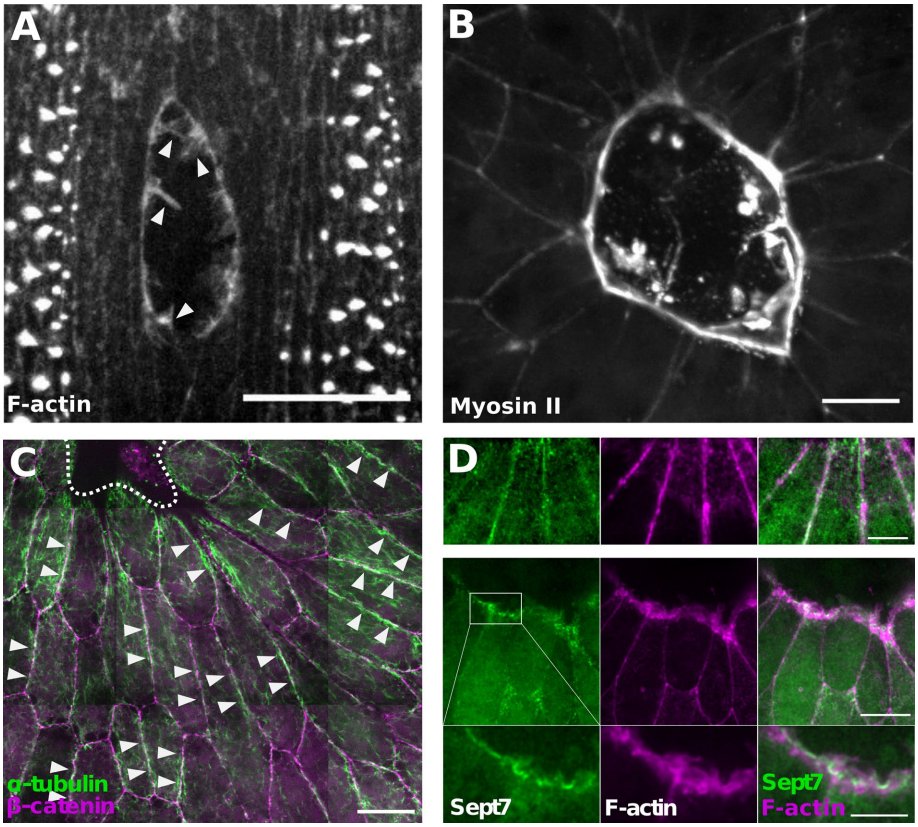

FIGURE 1: Cytoskeletal polarization during embryonic wound closure. (A) Filamentous actin (visualized using the actin-binding domain of Moesin tagged with green fluorescent protein [GFP]) contributes to the actomyosin cable around the wound, as well as to protrusions (arrowheads) during *Drosophila* embryonic wound closure. (B) Myosin accumulates at the wound edge and contributes to the formation of a contractile cable in a zebrafish embryo expressing nonmuscle myosin light-chain 12 (the myosin II regulatory subunit) tagged with GFP. (C) Immunofluorescence staining in a *Xenopus* embryo showing microtubule (green) alignment perpendicular to the wound edge (arrowheads). β-Catenin (magenta), an AJ component, is present in all cell–cell boundaries with the exception of the wound edge (dotted line). (D) The septin subunit Sept7 accumulates both at the wound edge and along cell–cell boundaries perpendicular to the wound edge. (A–D) Scale bars, 20 μm. (C, D) Reprinted with permission (Shindo *et al.*, 2018).

wound healing constitutes an excellent system for the detailed, quantitative characterization of tissue morphogenesis in real time and with high statistical confidence.

The cytoskeleton provides structure and organization within and across cells, while also generating forces that propel cell movements. Cytoskeletal networks include actin, microtubules, septins, and intermediate filaments (Fletcher and Mullins, 2010; Mostowy and Cossart, 2012). Actin filaments interact with myosin motors to generate contractile forces, while microtubules bear compressive forces as well as facilitate transport of intracellular cargo. Septins form structures of different geometries that can bind to both actin networks and microtubules, as well as to the cell membrane, thus providing a means for creating heterogeneous cytoskeletal networks that can interact with the cell surface. Finally, intermediate filaments may provide elasticity and durability to the cell. Cytoskeletal networks are anchored at adherens junctions (AJs), which maintain epithelial cell connections (Mege and Ishiyama, 2017), providing a physical link between the cytoskeletons of neighboring cells. Here, we review the roles that different cytoskeletal networks play during embryonic wound closure, and we propose that cross-talk between different cytoskeletal components may be a critical factor for cells to generate and bear the mechanical stresses associated with the rapid, coordinated cell movements that drive embryonic repair.

A CONTRACTILE ACTOMYOSIN CABLE FORMS ALONG THE WOUND MARGIN

Upon wounding, the cells immediately adjacent to the wound polarize their cytoskeleton. Both actin and myosin accumulate at the interface with the wounded cells, forming a supracellular cable around the wound (Figure 1, A and B). Actomyosin polarization and supracellular cable assembly as mechanisms of wound closure were first demonstrated in the chick embryo (Martin and Lewis, 1992; Brock *et al.*, 1996), and subsequently in mouse (McCluskey and Martin, 1995), frog (Davidson *et al.*, 2002), fruit fly (Kiehart *et al.*, 2000; Wood *et al.*, 2002), and zebrafish (Hunter *et al.*, 2018) embryos, as well as in cnidarians (Kamran *et al.*, 2017), adult worms (Xu and Chisholm, 2011), and the adult mouse cornea (Danjo and Gipson, 1998). Notably, human intestinal epithelial cells also repair damage through the assembly of an actomyosin cable around the wound, both in culture (Bement *et al.*, 1993) and in vivo in patients with inflammatory bowel disease (Russo *et al.*, 2005), suggesting that the mechanisms of wound closure observed in the embryo may represent an ancient, general repair mechanism of epithelial monolayers.

Cell–cell junctions play an important role in maintaining tissue integrity and organizing the cytoskeleton during wound closure. In the *Drosophila* embryonic epidermis, the actomyosin cable forms following the disassembly of the AJs along the wound edge, in a process driven by Src-dependent endocytosis (Hunter *et al.*, 2015, 2018; Matsubayashi *et al.*, 2015), and maintained through transcriptional down-regulation of E-cadherin (Carvalho *et al.*, 2014). Blocking endocytosis or overexpressing the core AJ component E-cadherin prevents the formation of the actomyosin cable, and down-regulating E-cadherin while endocytosis is partially blocked rescues actin assembly around the wound (Hunter *et al.*, 2015; Matsubayashi *et al.*, 2015). These results suggest that AJ internalization from the wound edge precedes cable formation (Figure 2). However, the reasons why AJs must be disassembled to form an actin cable are not understood. One possibility is that removal of AJ complexes provides physical space for the polymerization of actin networks (Carvalho *et al.*, 2014). Alternatively, AJ components may need to be recycled to form specialized junctional structures that promote actin assembly around the wound (Matsubayashi *et al.*, 2015). Finally, it is also plausible that AJ disassembly is an efficient mechanism to "break down" the preexisting actin cortex and generate short actin filaments and monomers for the assembly of wound-associated actin networks (Hunter *et al.*, 2015). Experiments using photoconversion approaches and superresolution microscopy to track changes in the localization of AJ components and

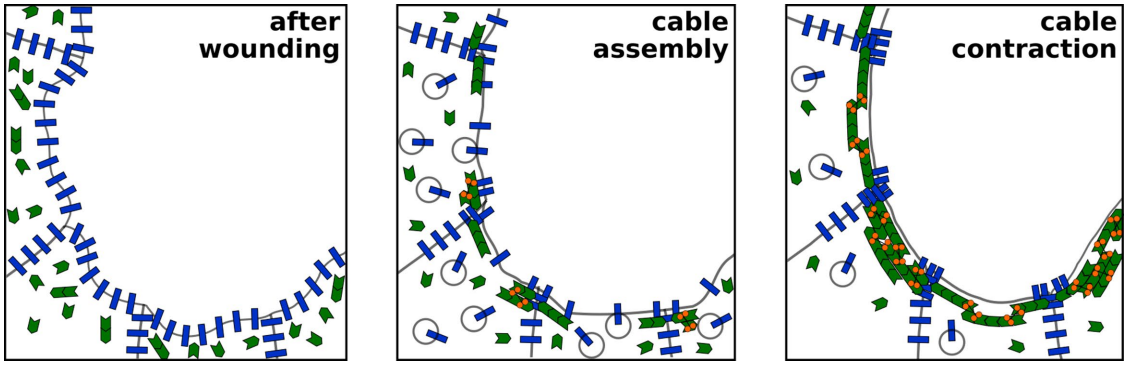

FIGURE 2: Actomyosin cable assembly requires adherens junction redistribution. (Left) Immediately after wounding an embryonic epithelium, AJs (blue) are almost continuous along all edges of the cells, and actin (green) is not polarized. (Center) Shortly after wounding, AJ components including E-cadherin, β-catenin, and α-catenin are removed from the wound edge, in a process mediated by polarized endocytosis. AJ components relocalize to former tricellular junctions around the wound, where actin polymerization (green) and myosin assembly (orange) begin. (Right) AJ removal from the wound edge continues as the actomyosin cable further assembles into a heterogeneous network around the wound and contracts, coordinating cell movements.

actin shortly after wounding will help distinguish between these models.

The supracellular cable originates from punctate AJs that form around the edge of the wound (Figure 2) and may serve as anchor points for the cable (Danjo and Gipson, 1998; Matsubayashi *et al.*, 2015). The mechanisms of assembly of the discrete AJs at the wound edge, and whether they are the only sites where the actin cable is anchored, remain unclear. Furthermore, the role of other cell–cell adhesive structures during wound repair has not been established. Recent work demonstrates the importance of occluding junctions, which primarily control transport of molecules across the epithelium. Loss of occluding junctions results in altered tissue mechanical properties, abnormal cellular rearrangements around the wound, and defective actomyosin dynamics at the wound edge, thus delaying wound closure (Carvalho *et al.*, 2018). These results highlight the importance of investigating the contributions of different adhesive structures to cytoskeletal organization during embryonic wound repair.

The actomyosin cable around wounds is a dynamic, contractile structure that coordinates cells through mechanical signals transmitted by AJs. The cable forms in a Rho-dependent manner (Brock *et al.*, 1996; Wood *et al.*, 2002; Abreu-Blanco *et al.*, 2012) and encircles the wound. However, the cable is not homogeneous in composition, with segments of increased actin and myosin localization and others with reduced actomyosin levels (Figure 3, green and orange) (Bement *et al.*, 1993; Zulueta-Coarasa and Fernandez-Gonzalez, 2018). Actomyosin heterogeneity drives efficient wound closure by allowing the staggered contraction of different segments of the wound edge, thus minimizing the resistance they encounter as they reduce their length. When a segment of the wound edge contracts, myosin is rapidly recruited to the neighboring, stretched segments in a process that involves mechanically gated ion channels (Zulueta-Coarasa and Fernandez-Gonzalez, 2018). In addition, tension stabilizes myosin in the segments of the wound edge that are actively contracting, thus providing a ratcheting mechanism indispensable for rapid wound closure (Kobb *et al.*, 2017). The mechanisms by which tension stabilizes myosin at the wound edge, and the channel or channels that facilitate myosin recruitment to the wound margin, remain unknown.

ACTIN-BASED PROTRUSIONS ZIP THE WOUND IN THE FINAL STAGES OF WOUND CLOSURE

Actin also assembles into protrusive structures that contribute to wound repair (Figure 1A). Long, thin filopodia made up of actin bundles, and broad lamellipodia that consist of an actin meshwork (Figure 3, green), were first identified in wound healing assays in vitro (Nobes and Hall, 1999; Fenteany *et al.*, 2000). High-resolution live imaging and genetic analysis demonstrated that filopodial and lamellipodial protrusions are required to fully close wounds in the embryonic epidermis (Wood *et al.*, 2002; Abreu-Blanco *et al.*, 2012; Li *et al.*, 2013). The assembly of lamellipodia and filopodia is typically mediated by the Rho GTPases Rac1 and Cdc42, respectively. Rac1- and Cdc42-based protrusions are present throughout wound closure, and they may drive crawling behaviors that contribute to tissue repair (Abreu-Blanco *et al.*, 2012). Protrusions are up-regulated in the final stages of wound healing when they can reach across the lesion and interdigitate with other protrusions, suggesting a role in the final sealing of the wound (Wood *et al.*, 2002; Abreu-Blanco *et al.*, 2012; Li *et al.*, 2013). Reducing Cdc42 activity, which eliminates protrusion formation, leads to wounds that do not fully close (Wood *et al.*, 2002; Abreu-Blanco *et al.*, 2012), consistent with a role of protrusions as "wound zippers."

Actomyosin protrusions could also mediate proper cell matching after wound repair, as evidenced by the role of protrusions in other morphogenetic processes. Dorsal closure, for example, is the sealing of a discontinuity in the epidermis of the *Drosophila* embryo that employs several of the same mechanisms as wound repair. Dorsal closure is driven by the collective movements of the cells in the two epidermal sheets flanking the discontinuity. Protrusions originating from the cells at the leading edge of each epidermal sheet knit the epidermis together, maintaining the proper alignment of gene expression patterns across the seam (Jacinto *et al.*, 2000). Additionally, in *Drosophila* heart development, two rows of cardiac progenitors meet to form the primitive heart tube, and Cdc42-dependent protrusions are required for the two rows of migrating progenitors to line up properly (Zhang *et al.*, 2018). The mechanisms that facilitate recognition of matching cells through filopodial interactions are not completely understood, although patterned expression of adhesion molecules may facilitate cell pairing. It is therefore possible that during wound closure,

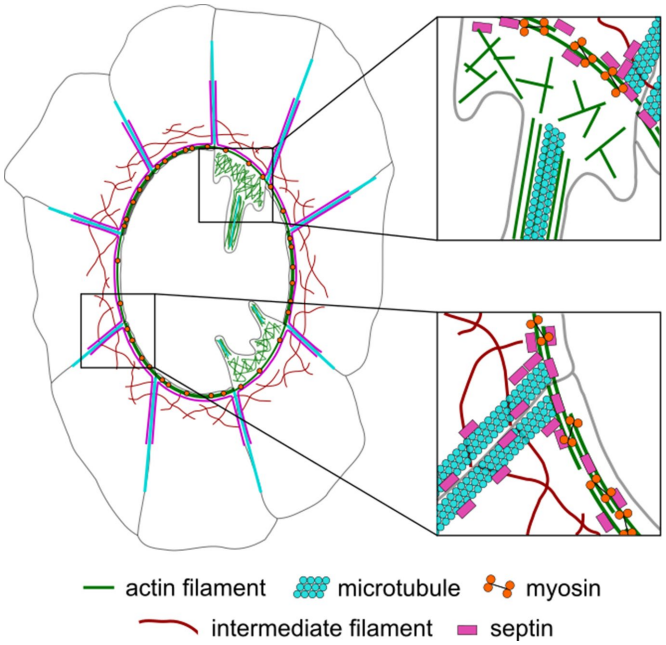

FIGURE 3: Multiple cytoskeletal networks contribute to embryonic wound closure. Different cytoskeletal components contribute to wound healing in a variety of model organisms. Upon wounding, actin (green) and myosin (orange) become polarized in the cells adjacent to the wound, accumulating at the wound edge and forming a supracellular actomyosin cable around the wound. Cable contraction generates forces transmitted across cells by adherens junctions (not shown) and coordinates cell movements. Actin also forms protrusions that promote cell crawling and seamless wound zipping. Microtubules (cyan) are also present in protrusions, where they may facilitate cargo transport. In parallel, microtubules at cell–cell boundaries may aid in the elongation of the cells as they extend into the wound. Septins (magenta) localize to the wound edge and cell–cell boundaries, where they may coordinate actin and microtubule networks, as well as membrane reorganization. Finally, intermediate filaments (red) accumulate close to the wound edge, where they may exchange mechanical signals with the extracellular matrix.

protrusions allow cells to meet across the lesion and knit together seamlessly, both at cellular and molecular scales.

RELATIVE CONTRIBUTIONS OF THE ACTOMYOSIN CABLE AND ACTIN-BASED PROTRUSIONS

Supracellular actin cables and actin-based protrusions contribute differently to wound repair in different systems, and formation of one or the other can depend on wound area and geometry (Begnaud *et al.*, 2016). In cell sheets in vitro, protrusive activity disappears when the discontinuity has a nonadherent substrate, suggesting that the prevalence of actin protrusions depends on the ability to form cell–matrix adhesions (Vedula *et al.*, 2015). In systems that rely on both actomyosin cable and protrusion formation, wound closure is impaired when either mechanism is blocked. Without protrusive activity, the cable still forms and wounds reduce their area, but do not complete closure (Wood *et al.*, 2002; Abreu-Blanco *et al.*, 2012). On the other hand, loss of the actomyosin cable results in wounds that still close, albeit more slowly, and display morphogenetic scars indicating a loss of proper tissue architecture (Ducuing and Vincent, 2016). Blocking both cable formation and protrusive activity results in wounds that fail to close (Abreu-Blanco *et al.*, 2012). Recent mathematical simulations suggest a need for both actomyosin cable contraction and protrusions for rapid wound closure (Staddon *et al.*, 2018). While the cable provides collective guidance cues that coordinate cell movements, protrusive activity may promote cell shape changes and the exchange of neighbors to locally fluidize the tissue, dissipating active stress and facilitating cell migration into the wound. The model suggests that cells rely on different modalities of migration to minimize the duration of wound closure depending on the mechanical properties of the tissue, and the area and shape of the wound (Staddon *et al.*, 2018), consistent with experimental findings (Vedula *et al.*, 2015; Begnaud *et al.*, 2016). Recent work demonstrated that the ability of cells to exchange neighbors accelerates wound closure (Razzell *et al.*, 2014; Tetley *et al.*, 2018), although actin-based protrusions have not yet been implicated in the cellular rearrangements associated with wound repair. Thus, both the actomyosin cable and actin protrusions are important for wound healing, and their relative contributions may depend on physical tissue properties and wound morphology.

OTHER CYTOSKELETAL ELEMENTS CONTRIBUTE TO EPITHELIAL WOUND REPAIR

Microtubules, septins, and intermediate filaments have not been as intensely studied as the actin cytoskeleton during embryonic wound closure. In *Drosophila* embryos, microtubules are present within filopodial protrusions at wound edges (Abreu-Blanco *et al.*, 2012), where they may provide pushing force and facilitate the transport of adhesion receptors or guidance cues along the protrusion (Figure 3, cyan). In addition, in *Xenopus* embryos, microtubules form bundles along cell cortices perpendicular to the wound edge (Figure 1C) (Shindo *et al.*, 2018). Disrupting microtubules results in reduced elongation of the cells adjacent to the wound edge, suggesting that microtubule bundles may stabilize the lateral edges of cells as they elongate into the wounded region (Shindo *et al.*, 2018).

Septins have been implicated in cell migration in a variety of cell types, and further investigation may reveal a conserved role in wound healing. Septins can interact with both actin filaments and microtubules, as well as the cell membrane. Notably, Sept7, which is a subunit of septin heterooligomers, localizes to the lateral cell edges and to the wound edge in *Xenopus* embryos (Figure 1D) (Shindo *et al.*, 2018). Sept7 is required for the reorientation and organization of microtubules during wound closure, as well as for contraction of the actomyosin cable around the wound (Shindo *et al.*, 2018), suggesting that septins may coordinate the rearrangements of different cytoskeletal networks during embryonic wound repair (Figure 3, magenta).

The role of intermediate filaments in wound closure is not well understood. In cell culture models of wound healing using human epithelial cells, vimentin is up-regulated in the cells at the leading edge (Figure 3, red), and reduction of vimentin expression reduces migration speed (Gilles *et al.*, 1999). Indeed, both vimentin and keratins continue to be implicated in regulating cell motility. The interaction of cell–matrix adhesions with intermediate filaments in *Caenorhabditis elegans* provides mechanosensitive cues that promote epithelial morphogenesis (Zhang *et al.*, 2011). Therefore, intermediate filaments and the hemidesmosomal adhesion structures that they interact with may prove necessary for cell migration during embryonic wound repair.

CONCLUSION

The cytoskeleton as a whole is indispensable for successful wound healing, providing the necessary structural support and contractile

forces to drive forward movement and coordinate cell behaviors. Although we currently have a general idea of how actomyosin structures contribute to wound closure, the specifics of the formation, regulation, and maintenance of these structures remain unclear. For example, the actomyosin cable may be anchored at AJs at cell–cell boundaries around the wound, but a single filament cannot bridge the gap between these punctate adhesion structures. Actin-binding proteins, such as filamin or actinin, could cross-link multiple shorter filaments together. Alternatively, proteins that bind both actin filaments and the cell membrane, such as ERM family proteins or septins, may provide anchor points between AJs. Additionally, the role of actin-based protrusions is not well characterized in vivo, with respect to whether they form cell–matrix or cell–cell adhesions that assist in driving forward migration, or if protrusions "zip" the gap between two cells. Finally, we have only begun to investigate the role of other cytoskeletal networks that may influence the structural stability of the tissue during wound closure. The cross-talk between different cytoskeletal components is likely important in bearing the mechanical loads that cells experience during wound healing. Uncovering the role of the cytoskeleton in coordinating cell movement for wound repair will contribute to the broader understanding of complex morphogenetic processes during embryonic development and how collective cell behaviors contribute to the spread of disease.

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