The wounded worm
Using C. elegans to understand the molecular basis of skin wound healing

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Introduction

The ability to heal wounds is an ancient and conserved function of epidermal epithelial layers. The importance of skin wound healing to human life and biology has long been evident, however many of the molecular mechanisms underlying wound repair remain little understood. In the past several years, analysis of the C. elegans innate immune response to fungal infection of the epidermis has led to investigations of the ability of the C. elegans skin to respond to damage. In a recent paper we used live imaging to investigate the cell biological basis of wound repair in the adult C. elegans epidermis. We found that needle or laser injury of the skin triggers a large and sustained increase in epidermal calcium. Epidermal calcium signals appear to specifically promote actin-dependent processes of wound closure. The innate immune and wound closure responses act in parallel to promote survival after injury. Our findings indicate that wounding triggers multiple signals in the C. elegans skin. C. elegans offers a tractable model to dissect how epidermal epithelia activate coordinated responses to repair damage.

Comparing C. elegans and Mammalian Skin

The skin layers of mammals and nematodes, while structurally very different, exhibit certain fundamental similarities (Fig. 1). Both are epithelial tissues; differentiated mammalian skin is composed of a multilayered stratified squamous epithelium (epidermis) and an underlying connective tissue layer (dermis), whereas the nematode skin is formed by a simple epithelial layer. The mammalian epidermis is generated from specialized keratinocytes that divide in the basal layer and whose progeny undergo progressive differentiation into the corneocytes of the stratum corneum (SC). The SC forms the primary
permeability barrier of the skin and is composed of corneocytes embedded in a lipid-rich extracellular matrix.\textsuperscript{5,6} The \textit{C. elegans} cuticle is in some senses analogous to the SC; it is an extracellular compartment composed of cross-linked collagens, insoluble proteins termed cuticulins, and associated glycoproteins and lipids.\textsuperscript{7} External to the collagenous cuticulins, and associated glycoproteins collagens, insoluble proteins termed analogous to the SC; it is an extracellular cuticle is in some senses \textit{C. elegans}. Among other salient differences, most of the larval or adult \textit{C. elegans} skin is formed by a single large syncytial cell (termed hyp7); blast cells known as seam cells generate new epidermal cells that fuse with hyp7 allowing post-embryonic growth.

**Figure 1.** Comparison of mammalian and \textit{C. elegans} skin layers. (A) Mammalian skin consists of the epidermis and dermis, separated by a basement membrane. The epidermis is composed of several cell layers, including the basal layer (BL) resting on the basement membrane, and the differentiated cell layers of the spinous layer (SL), granular layer (GL) and the stratum corneum (SC). The SC is a lipid-rich layer composed of cholesterol, free fatty acids, ceramides and collagen, which together provide the permeability barrier function of the skin. (B) \textit{C. elegans} skin consists of the epidermis and cuticle. The epidermis is a simple epithelium whose basal surface rests on a basement membrane. The apical surface of the epidermal epithelium secretes the cuticle, a collagenous extracellular matrix. The cuticle is a flexible barrier layer that is composed predominantly of cross-linked collagens. External to the cuticle is a lipid rich epicuticle that may also function in the permeability barrier.

### The Cutaneous Innate Immune Response to Injury

Although countless \textit{C. elegans} skin wounds have been caused by DNA or RNA microinjection experiments, it is only recently that the ability of \textit{C. elegans} to survive and repair such drastic trauma has been examined. Evidence that the nematode skin can play an active role in injury responses first came from studies of the fungus \textit{Drechmeria coniospora}, one of many cuticle-penetrating nematode pathogens whose route of infection is via the skin. Pioneering work from the Ewbank lab revealed that \textit{Drechmeria} infection induces expression of antimicrobial peptide genes (AMPs) in the epidermis; many of these AMPs had been annotated as neuropeptide-like (\textit{nlp}) genes, but appear to have a protective role. AMP induction after infection requires the Toll-interleukin receptor like domain protein TIR-1 and the PMK-1 p38 mitogen-activated protein kinase cascade.\textsuperscript{8,9} Further studies of the response to \textit{Drechmeria} infection identified additional signaling components acting upstream of the TIR-1/p38 cascade, including the G protein GPA-12/Gz12, phospholipase C/PLC-3, and TPA-1/PKC8.\textsuperscript{10} Infection also activates expression of caenacin AMPs through a neuroendocrine transforming growth factor-\textbeta (TGF-\textbeta) signaling pathway.\textsuperscript{10}

The findings that skin infections triggered innate immune responses raised the question of whether such responses were pathogen-specific or a more generic response to damage. These thoughts motivated the development of epidermal wounding procedures involving either small puncture wounds from microinjection needles, or localized damage caused by laser irradiation.\textsuperscript{11} Current needle wounding procedures involve puncturing the cuticle and hyp7 in the posterior body; the epidermal basement membrane and internal organs may also be damaged. Femtosecond laser wounds are more likely confined to the hyp7 cell, although the exact mechanism of damage remains to be fully explored. Thus, current \textit{C. elegans} wounding experiments essentially address the repair capacity of a single large syncytial cell as opposed to wound responses of multicellular epithelia typically studied in other organisms.\textsuperscript{11} Wild type animals display a normal life span after such wounds, and indeed both types of wound can induce AMP expression, often to levels comparable to that seen after infection. Epidermal innate immune responses to injury are also well known in the mammalian skin, which massively upregulates AMPs after injury, independent of infection or inflammation.\textsuperscript{12} In \textit{C. elegans}, wound-induced AMP expression requires many of the same signaling components as infection-induced expression, although some factors such as the kinase NIP1-3 are only required for the response to infection.\textsuperscript{7} Additional studies of \textit{dapk-1} (death associated protein kinase) mutants revealed a syndrome of late-onset epidermal phenotypes comprised of constitutive upregulation of epidermal AMPs and progressive hypertrophy of the cuticle,\textsuperscript{13} suggesting that \textit{dapk-1} mutants might...
provide a model for spontaneous epidermal wounding. Taken together, these studies indicated that puncture or laser wounds or the “spontaneous wounds” of *dapk-1* mutants can induce epidermal innate immune responses, raising the question of how other aspects of wound repair are regulated.

**Wounding Triggers an Epidermal Calcium Signal**

To understand the initiating events in epidermal responses to damage we focused on calcium signaling, long known to be important in embryonic wound healing and in single cell wounding.14,15 Using the genetically encoded Ca²⁺ sensor GCaMP3 expressed in the adult epidermis, we found that wounding triggered a rapid elevation of epidermal Ca²⁺ that can persist for at least 1 h (Fig. 2A). When Ca²⁺ elevation is blocked by soaking worms in the Ca²⁺ chelator BAPTA-AM, the ability of animals to survive needle wounding is dramatically reduced, suggesting that persistent Ca²⁺ activation after wounding is required for wound healing. Where does the epidermal Ca²⁺ come from? Internal stores of Ca²⁺ in the epidermis may contribute to the Ca²⁺ wave, as we find that the Ins(1,4,5)P₃ receptor ITR-1 is required for epidermal Ca²⁺ responses. We also identify an epidermal signal transduction pathway that includes the epidermal TRPM channel GTL-2, the Gaᵢ₁ quiescent PLCγ, and its effector PLCβ.16 Wounding may trigger parallel G protein cascades involving GPA-12 and EGL-30, and its effector PLCβ as required for epidermal Ca²⁺ signaling. Epidermal activity of GTL-2 has also recently been implicated in regulating neuronal excitability,16 raising the possibility that wound-triggered epidermal Ca²⁺ elevation could also have non-autonomous effects.

A signaling cascade involving Cdc42 and Rho, via a Ca²⁺ dependent mechanism for skin wounds to close, in the absence of actin cables. In either case, actin polymerization is an essential initial step for wound closure. The small GTPase Cdc42 is essential for actin polymerization after wounding (Fig. 2C). Loss of the actin nucleating factors ARX-2 (ARP2/3) or WSP-1 (WASP),23 causes decreased formation of filopodia and slower wound closure, suggesting that in the absence of filopodial protrusions, actin cables are less efficient at closing the wound. Indeed, actin cables and filopodial protrusion appear to have opposing roles, in that wounds close faster by filopodial protrusion when the purse-string is removed. These observations raise the question of why opposing mechanisms might be activated by wounding: possibly a balance between filopodial protrusion and purse-string mediated closure is important for other aspects of wound healing such as scar formation. Wounding of single cells activates both Cdc42 and Rho, via a Ca²⁺ dependent signal.24 We speculate that wounding the *C. elegans* epidermis also triggers local activation of these small GTPases. If so, an important future goal will be to define factors responsible for GTases activation in response to wounding, and to understand how these themselves are regulated in the epidermis.

**Calcium Signals Trigger Actin-Dependent Wound Closure**

The actin cytoskeleton is critical for wound closure in many models.20 Our study suggests Ca²⁺ signals are required for wound closure in the adult *C. elegans* epidermis, likely promoting direct actin polymerization (Fig. 2B). Small GTases of the Rho family regulate the formation of actin structures such as actin cable, filopodia and lamellipodia.21 Drosophila embryonic wounds are closed by a contractile actomyosin cable, which acts like a purse string, and whose contractility is dependent on the Rho GTPase, Rho1.20 Unexpectedly, although contractile actin cables appear to form after wounding *C. elegans* skin, our results suggest that a “purse-string” closure mechanism is not essential for wound closure. In animals with reduced activity of Rho or of nonmuscle myosin II, the number of filopodia is increased but the area of actin accumulation is reduced (Fig. 2C), suggesting that protrusive activity is increased and that the wound can close faster than in the wild type. Purse-string dependent closure may be more critical in wounds of multicellular epithelia than in the syncytial epidermal wounds in *C. elegans*.22 Nevertheless our results suggest that changes in filopodia number and shape may be an alternative, compensatory mechanism for skin wounds to close, in the absence of actin cables.

In either case, actin polymerization is an essential initial step for wound closure. The small GTPase CDC-42 is essential for actin polymerization after wounding (Fig. 2C). Loss of the actin nucleating factors ARX-2 (ARP2/3) or WSP-1 (WASP),23 causes decreased formation of filopodia and slower wound closure, suggesting that in the absence of filopodial protrusions, actin cables are less efficient at closing the wound. Indeed, actin cables and filopodial protrusion appear to have opposing roles, in that wounds close faster by filopodial protrusion when the purse-string is removed. These observations raise the question of why opposing mechanisms might be activated by wounding: possibly a balance between filopodial protrusion and purse-string mediated closure is important for other aspects of wound healing such as scar formation. Wounding of single cells activates both Cdc42 and Rho, via a Ca²⁺ dependent signal.24 We speculate that wounding the *C. elegans* epidermis also triggers local activation of these small GTPases. If so, an important future goal will be to define factors responsible for GTases activation in response to wounding, and to understand how these themselves are regulated in the epidermis.

**Remodeling, Scar Formation and Repair of the Permeability Barrier**

The later stages of wound repair, known as remodeling in mammalian models, involve reformation of the extracellular matrix and permeability barrier and the formation of scars.3,25 Intriguingly, scar-like autofluorescent structures are also seen after needle or laser wounding adult *C. elegans* epidermis (Fig. 2B), and are visible for several days at the wound site.3 The exact origin or composition of these structures remains to be determined; they form independently of the PMK-1 p38 cascade. Ultrastructural analysis reveals that scar structures are associated with thickened basal layers of cuticle, suggesting that wounding might induce local secretion of cuticle during repair. Interestingly, *dapk-1* mutants display progressive hypertrophy of cuticle at the head region, including local accumulation of cuticle collagens and spontaneous autofluorescent scar-like material.13 As DAPK-1 can negatively regulate wound closure, DAPK-1 may provide a genetic entry point to understand how scar formation, cuticle secretion, and wound closure are coordinately regulated after wounding. Remarkably, mutations in *sydn-1*, a regulator of mRNA polyadenylation,26 strongly and specifically suppress these aspects of the Dapk-1 epidermal phenotype. The mechanism of SYDN-1 suppression may shed light on DAPK-1’s functions in the epidermis, as may the identity of other
as-yet uncloned suppressors of dapk-1 (T.I.H. and A.D.C., unpublished).

Wounding and Gene Expression

In C. elegans, needle or laser wounding triggers the elevated transcription of a large number of genes encoding AMPs. The Stat-like transcription factor STA-2 is required for AMP induction in response to wounding and is localized to endocytic vesicles in the epidermis. It will be interesting to determine whether localization of these potential signaling vesicles is affected by wounding. It will also be important to address whether transcriptional regulators implicated in wound responses in other animals also play roles in C. elegans wound responses. For example, in mammals, the JUN N-terminal kinase (JNK) and the transcription factor activator protein 1 (AP1) regulate gene expression after wounding. The function of AP1 in wound healing is conserved in Drosophila and mammals. Another transcription factor, Grainy head, has a remarkably conserved role in epidermal wound repair. The C. elegans Grainy head ortholog GRH-1 is expressed in the epidermis and is required for embryonic cuticle integrity. It seems likely that GRH-1 is involved in epidermal wound healing in adults, although this remains to be explored. Gene expression profiling of wounded animals may be feasible, allowing a comprehensive analysis of the epidermal transcriptome’s response to damage.

Figure 2. C. elegans epidermal wound responses. (A) Epidermal GCaMP fluorescence elevation after femtosecond laser wounding. Pcol-19-GCaMP3(juIs319). Lateral views of adult epidermis in mid-body before, immediately after and 1 h after laser wounding; x marks site of laser wound. Spinning disk confocal, intensity code; scale, 10 μm. (B) Needle wounding triggers actin assembly around the wound site. Pcol-19-GFP::Moesin (juIs352) labels actin filaments in adult epidermis. At 24 h an autofluorescent “scar” (red) is visible at the wound site and the actin structures have disappeared. x marks site of needle wound; laser scanning confocal images; scale, 10 μm. C, Graphical summary of mutant or RNAi phenotypes of genes implicated in C. elegans wound-induced actin dynamics.

Outlook

The C. elegans epidermis has a robust repair mechanism that is likely critical for survival in an environment rich in skin-penetrating pathogens and sources of mechanical damage. We end by noting that many interesting areas of wound healing biology remain to be explored in C. elegans. For example, what is the role of the lipid-rich epicuticle in the permeability barrier; is it resynthesized after wounding? How does aging affect wound repair in C. elegans? Do C. elegans embryos repair skin wounds, and if so do the mechanisms resemble those of the adult? Finally, does epidermal damage trigger other organismal responses, for example in the nervous system? The relative simplicity of C. elegans and its famous genetic tractability suggest that rapid progress should be possible in understanding such aspects of wound repair.

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