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Drosophila Embryos as a Model for Wound-Induced Transcriptional Dynamics: Genetic Strategies to Achieve a Localized Wound Response

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While many studies have established a paradigm for tissue repair at the level of cellular remodeling, it is not clear how an organism restricts a response only to the injured region of a damaged tissue. Skin, the largest organ in the human body, is prone to injury, and repair of epidermal tissue represents a medically relevant system to investigate.

Significance: Studies in *Drosophila melanogaster* provide a robust genetic system to identify molecular components that will positively impact repair and healing. The *Drosophila* skin consists of a single-cell epidermal layer and relies on well-conserved cellular mechanisms to coordinate gene expression during development. Many studies have established that key developmental genes promote a response to epidermal injury, but the balance between activator and inhibitor signals to coordinate a localized response remains unknown.

Recent Advances: Discovery of a genetic pathway that promotes the restriction of transcriptional response to damage only in effected regions. Interestingly, genome-wide microarray studies have identified an intersection between gene expression after aseptic injury and activation of the innate immune response.

Critical Issues: The use of a transcriptional activation reporter provides an innovative approach to uncover well-conserved components that promote the localization of a response during epidermal injury and may influence other pathological conditions of tissue damage.

Future Directions: The work reviewed in this critical review may lead to development of molecular strategies of repair and improved healing after injury or infection. The outcomes on the fundamental contribution of a transcriptional response to injury will be translatable to mammalian systems.

SCOPE AND SIGNIFICANCE

Drosophila provides a rich system to quickly assess complex biological processes. A combination of classical genetic techniques with advances in genome analyses and high-resolution microscopy enable discrete hypotheses to be tested on a diverse sample of molecular and cellular functions. The scope of this critical review is to highlight the contribution of a diverse collection of genes during a wound response to epidermal puncture injury in the *Drosophila* embryo. The significance of these studies is the identification of a novel set of well-conserved genes regulating the localization of a transcriptional response to injury.

TRANSLATIONAL RELEVANCE

In recent years, reports of patients suffering from chronic wound conditions have surpassed 6 million cases and this does not include the millions more recovering from surgical or traumatic wounds.[1] Dissection of evolutionarily conserved genetic functions

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in model organisms is a key strategy in biomedical science to understand the basic cellular and molecular mechanisms. Studies using *Drosophila* as a model organism have contributed to the discovery of well-conserved regulatory pathways—including Hox-transcription factors during developmental patterning and Toll-signaling during innate immunity. *Drosophila* provides a robust system to elucidate conserved pathways that contribute to repair and regeneration.

CLINICAL RELEVANCE

The established paradigm for distinct phases of wound repair that promote healing and regeneration highlights the contributions of wound response, inflammation, and reepithelialization.[2] Epidermal wound healing represents a powerful system to address a wide range of medically relevant pathologies.[3] The regulation of activating and inhibiting signals is required for wound repair, however, the mechanisms of limiting the signals is poorly understood.[4] This critical review provides an update on the functions of novel set of genes that regulate the localization of wound signals and may provide new directions to take clinical studies of wound care.

BACKGROUND

The single-layered epidermis in *Drosophila* provides a simple system to study an epidermal wound response after a puncture injury.[5] *Drosophila* embryogenesis is a robust developmental stage to genetically dissect the phases of wound repair, including wound response, inflammation, and reepithelialization.[6] This is in part because many or most zygotic mutants survive to the end of embryogenesis and develop epidermal barriers even when developmental patterning is defective. The development of the *Drosophila* epidermal barrier depends on the cross-linking of proteins, lipids, and chitin.[7] Reactive quinones, the end product of a dopamine biosynthesis pathway, are also required for the maturation of the *Drosophila* epidermal barrier.[8] Previous characterization of a well-conserved transcription factor grainy head (Grh), identified that Grh-target genes *Dopa decarboxylase* (*Ddc*) and *tyrosine hydroxylase* (*ple*) are not only required during *Drosophila* epidermal barrier formation, but also are transcriptionally activated around the sites of injury.[9] *Drosophila* as a model organism for wound response provides a complementary line of investigation to advance studies in mammalian wound healing. Mouse Grhlike3 gene is required epidermal barrier development and keratinocyte migration after injury.[10] In *Drosophila* there is one copy of GRH and in mouse there are three copies of GRH. *Drosophila* GRH is an essential protein, *grh* mutant embryos have weakened epidermal barrier development and do not survive outside of the protective vitelline membrane.[9] In the *Drosophila* genome, the 5′ untranslated region (UTR) of several genes contain a conserved enhancer region with GRH transcription factor binding sites. A fluorescent gene coding sequence was cloned immediately downstream of the enhancer 5′UTR and the resulting transgene generated an *in vivo* epidermal wound response reporter[11] (Fig. 1). These studies have developed a "toolkit" of many fluorescent wound reporters to monitor the *in vivo* response to epidermal injury in *Drosophila* embryos.[12] Table 1 summarizes the epidermal wound reporters in two components of the dopamine biosynthesis pathway (*Ddc* and *ple*), and two other genes required for barrier function: (1) *misshapen* (*msn*) encodes a *Drosophila* JNK kinase-kinase-kinase,[13] (2) *krotzkopf verkehrt* (*kkv*) encodes a chitin synthase.[14] The fluorescent reporters are active during the final stages of *Drosophila* embryo development. The early timing of the wound response reporter activity allows for localization patterns to be determined in mutants for essential genes that will not survive past embryogenesis (*e.g., grh*).

Epidermal wound response gene detection

Visualization of the wound response gene activation can be achieved by (1) *in situ* hybridization of fluorescently labeled RNA probes or (2) *in vivo* with enhancers controlling the expression of fluorescent reporter genes. For example, *Ddc* .47 is a 470 bp enhancer of *Dopa decarboxylase* cloned upstream of GFP and *ple* WE1 is a 3.0 kb enhancer of *tyrosine hydroxylase* cloned upstream of DsRed. Compound fluorescence microscopy can be used to quickly screen data and confocal fluorescence microscopy can be used for more detailed analysis. All of the wounding experiments are performed during the final stages of *Drosophila* development (stage 16 out of 17 total).[15] The developing cuticle, in late stage *Drosophila* embryos, consists of stratified layers of chitin and makes detection of RNA transcripts a difficult task. A basic protocol for fluorescent *in situ* hybridization[16] is adapted for enhancing the RNA detection. The RNA transcripts are visualized using a peroxidase enzyme amplification of the fluorescence signal.

The RNA transcripts of epidermal wound response genes can be detected within 30 min after a puncture wound. The wound reporter signal is slightly delayed because it requires the translation

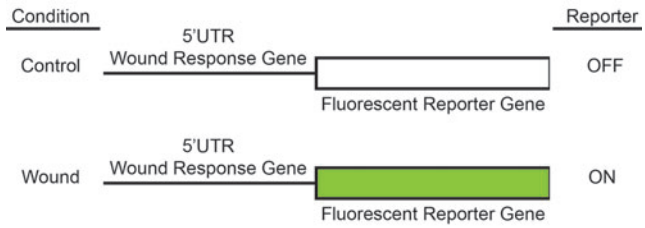

Figure 1. Architecture of epidermal wound response reporters. Activity of the reporter correlates with the condition of the embryo: control = OFF and wound = ON. The 5′UTR contains an enhancer region that is sufficient to activate fluorescent gene reporter expression and is dependent on puncture injury. Ddc, Dopa decarboxylase; UTR. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

of the transgenic GFP and DsRed fluorescence genes.[17] The localization of the *Ddc* and *ple* wound response reporters can be detected 4–6 h after puncture wounding. The epidermal wound reporter fluorescence is in the same pattern as the RNA transcripts of the wound response genes. The wound reporters provide an *in vivo* detection method for the transcriptional activation of the epidermal wound response genes. Using an *in vivo* detection is optimal for initiating genetic studies and the *in situ* detection is optimal for testing specific time points for gene expression.

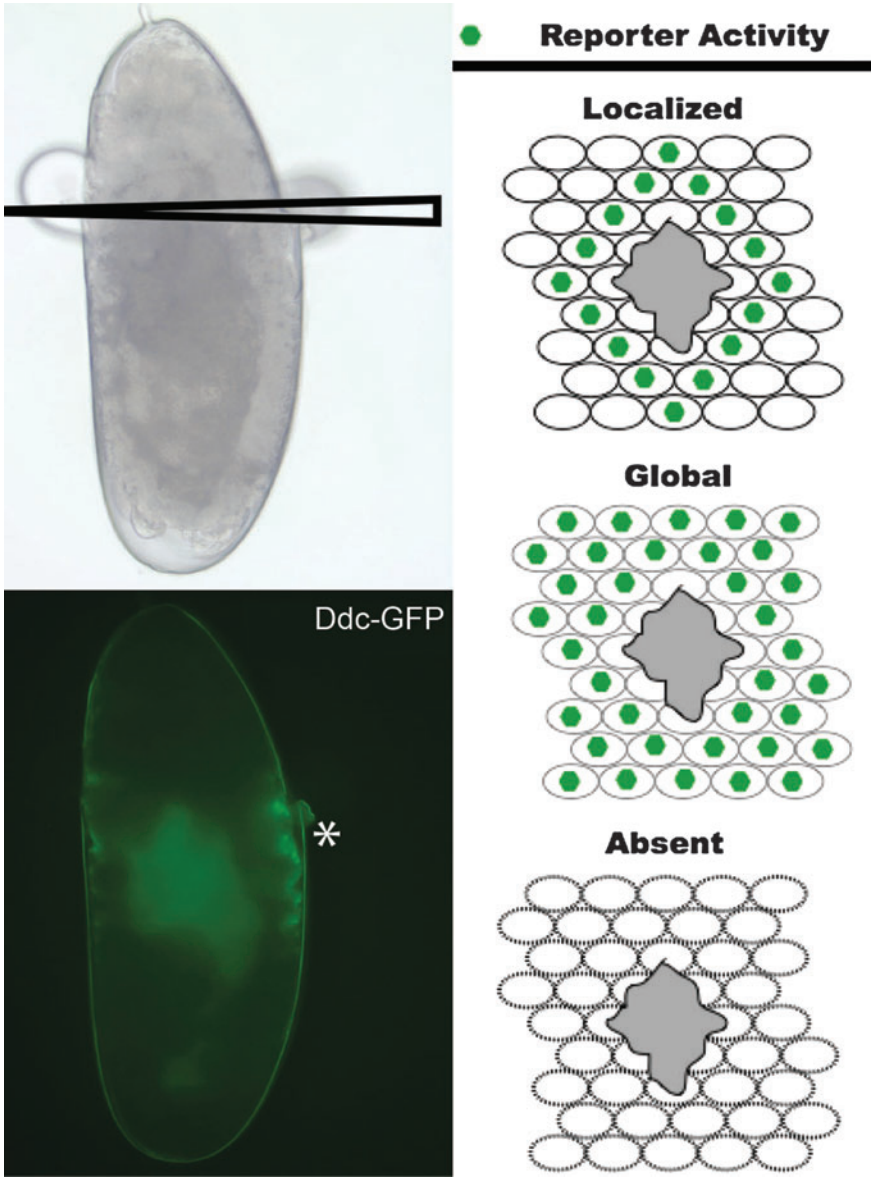

Figure 2. Microinjection injury and phenotypes of epidermal wound response reporters. Brightfield image with a line superimposed over the embryo to show the microinjection injury. Fluorescent image of the Ddc-GFP wound response reporter activity in the cells surrounding the site of puncture injury. A representation of the epidermal cells with an injury site; summarizing "localized," "global," or "absent" pattern of reporter activity. Asterisk marks the site of puncture injury. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Table 1. Drosophila *epidermal wound response reporters*

Epidermal Wound Response Reporters	*Fluorescence Protein*
Dopa decarboxylase (Ddc), cuticle protein	GFP
Tyrosine hydroxylase (ple), cuticle protein	DsRed
Misshapen (msn), JNK-KKK	DsRed
Krotzkopf verkehrt (kkv), chitin synthase	DsRed

Genetic regulation of an epidermal wound response

Recent reports on the regulation of *Drosophila* wound healing have focused on the wound closure phenotype, allowing discovery of many pathways regulating cell migration.[18,19] The fluorescent wound reporter localization pattern provides a direct assay of transcriptional activation of the barrier repair genes after microinjection injury (Fig. 2). The fluorescent wound reporter localization was used in an unbiased screen of ~5,000 mutant genes to identify a novel set of well-conserved genes required for the localized expression of epidermal wound-inducible genes[20] (Table 2). Subsequent studies to complement the genetic screen, used a microarray to survey the transcriptional profiles of gene expression after puncture injury.[21] In previous microarray studies using a laser-mediated wounding technique in *Drosophila* embryos, the enrichment levels of gene expression for control compared to wounded samples was not significantly different.[22] To address the issue of a puncture wound resulting in a small area of damaged cells compared to the whole embryo, a novel protocol was developed for a simultaneous puncture and chemical microinjection of a serine protease, Trypsin. Serine proteases play an important role in the signal transduction pathways controlling dorsovental patterning, innate immune response, and localization of the epidermal wound response.[20,23,24] The combination of puncture injury and trypsin-mediated activation resulted in the identification of a novel set of eight genes that are transcriptionally upregulated in the epidermal cells surrounding a puncture injury in the *Drosophila* embryo (*Ady43A*, *Ets* at 21C, *jun-related antigen* [*jra/jun*], *kayak* [*fos*], *Relish*, *rhomboid*, *spatzle*, and *dorsal*).[21]

Table 2. *Genes that regulate the localization of epidermal wound response reporters*

New Genes Required for Localized Wound Reporter	*Phenotype*
Flotillin-2, lipid raft-associated protein (cg32593)	Global
Src42A, protein tyrosine kinase (cg7873)	Global
Dual oxidase, NADPH oxidase and peroxidase (cg3131)	Absent
Wurst, Clathrin-associated protein (cg9098)	Global
Ghost, COPII vesicle component (cg10882)	Absent
Varicose, septate junction- associated protein (cg 9326)	Global
Drosophila homolog of yeast *Maki* (cg11412)	Global
Shroud, short-chain dehydrogenase/reductase (cg12068)	Absent

Comparing the conservation of genes upregulated in recent microarray studies in mammalian wound repair with the results from the *Drosophila* microarray presented in this critical review studies highlight key similarities and differences between the distinct model systems. A prominent example of conserved gene regulatory profiles was observed in the significant upregulation of the FOS and JUN family genes.[21,25–27] In addition, downstream effectors of the JNK signaling pathway have well-described mutant phenotypes that disrupt wound healing in the *Drosophila* embryo.[28,29] During the reepithelialization stage of wound repair, mammalian cells undergo a process of cellular proliferation.[30] In contrast, *Drosophila* embryonic epidermal cells resolve a wound through migration of neighboring cells.[31] A distinct mode of transcriptional regulation between mammalian and *Drosophila* wound repair can be observed in the regulation of genes promoting cell cycle. For example, several cyclin genes were significantly downregulated after puncture in *Drosophila* embryos.[21] A similar class of cyclin genes were significantly upregulated after mammalian keratinocyte scratch wounding.[26] It is interesting that the recent microarray studies in the *Drosophila* embryo identified *Src42A* as being upregulated after puncture injury, however, *Flo-2* was not identified.[21] This result suggests a sensitivity limitation of the microarray experiment and the challenge of detecting transcriptional changes in gene like *Flo-2*, which is highly expressed in all tissues in the developing embryo.[20]

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

From the list of novel genes identified in the initial genetic screen, three genes (*Flotillin-2*, *Src42A*, and *Dual Oxidase*) were of significant interest because of their previously published roles in regeneration, signal transduction, and wound response signaling. Flotillins were originally identified as a novel class of membrane-associated proteins in detergent resistant fractions purified from mammalian cells.[32] Another group characterized flotillins as being upregulated in regenerating optic neurons of injured goldfish retinal ganglion cells.[33] Flotillins are highly conserved among metazoan animals, 80% similarity among vertebrates and invertebrates.[34] Previous work to characterize *Drosophila Flotillins* suggest a molecular mechanism in cell–cell communication.[35,36] The "global" pattern of the wound reporter in the loss-of-function *Flo-2* mutant embryo and the "absent" pattern of the wound reporter in the gain-of-

function *Flo-2* mutant embryo suggest that *Flo-2* is both necessary and sufficient to limit the spread of the epidermal wound response reporter activity. A similar "global" pattern of RNA localization were observed in *Flo-2* mutants with *in situ* results of *Ddc*, *ple*, *msn*, and *kkv*.[20] The *Flo-2* mutant embryos do not have a wound healing phenotype and survival rates after wounding for both the loss-of-function and gain-of-function *Flo-2* mutants show similar rates to wounded *wild-type* controls. Of particular interest to the study of signal transduction during the wound response is the previous report that flotillins require phosphorylation mediated by Src-family kinases to maintain localization at the plasma membrane of cultured mammalian neuronal cells.[37] Src-family kinases are evolutionarily conserved group of protein tyrosine kinases that promote cellular proliferation and were recently shown to integrate wound response signals during zebrafish tissue regeneration.[38]. Consistent with the previous reports that flotillins depend on the function Src-family kinase activity, similar "global" pattern of epidermal wound reporter localization are observed in *Src42A* loss-of-function mutant embryos and "absent" pattern of the wound reporter in the gain-of-function *Src42A* mutant embryo. However, loss-of-function *Src42A* mutants to do complete embryogenesis and survival rates after wounding cannot be determined. Future studies that focus on the cellular localization of Flo-2 epidermal cells surrounding the site of injury may define a role for Src phosphorylation and signal transduction during the transcriptional activation of wound response genes. A balance in signal localization may improve how an organism coordinates an "activator" response for repair and an "inhibitor" response for scarring or inflammation. The *Drosophila* embryo provides a developmental system to correlate the studies of wound reporter localization in combination with cellular localization. One outcome of these studies is that Flo-2 can contribute to a better understanding of wound signal dynamics and lead to improving tissue repair treatments.

During injury, damaged cells produce multiple signals to elicit a wound response. Additional work using a model of zebrafish regeneration developed the use of a hydrogen peroxide sensor correlated with the migration of inflammatory cells to the site of injury.[39] Similar results were observed in *Drosophila* and genetic tests indicated the enzyme dual oxidase (Duox) is required for the hydrogen peroxide production and stimulation of the inflammatory response.[40] Recent studies from *Drosophila* showed that Duox can also promote a calcium flux at the site of epidermal injury.[41] Additional genetic analyses combine the localization of the epidermal wound response reporters with the activity of chemical compounds (*e.g.,* Serine protease—trypsin, hydrogen peroxide, and Src-Kinase inhibitor—SU6656). Interestingly, the overexpression of *Flo-2* and *Src42A* are sufficient to inhibit not only the activation of a local puncture wound response, but also the global activation of a chemical-mediated wound response (*e.g.,* trypsin and hydrogen peroxide).[20] A model is proposed for novel interactions between *Flo-2*, *Src42A*, and *Duox* and (Fig. 3).

New insight about the mechanism for localization of the wound response reporters in the cells surrounding injury can be inferred from the other novel genes identified in the genetic screen (Table 2) and with support from online resources (flybase.org).[42,43]

Several of the genes that have mutant phenotypes in wound response localization also have phenotypes in tracheal tube formation. Similarities between the localization of wound reporters and tracheal tube development exist between processes that communicate signals to coordinate

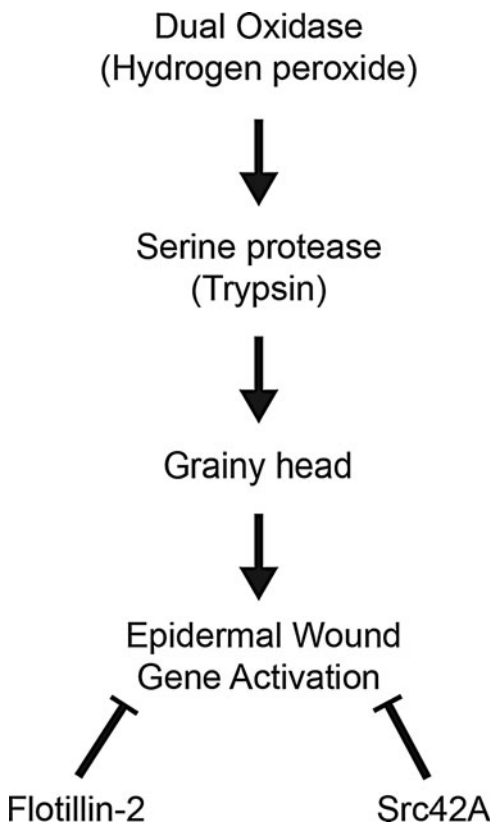

Figure 3. Key components of genetic pathway regulating epidermal wound response reporters. Epistatic relationships were determined using genetic analyses of double mutants and chemical-mediated wound response reporter activation. The pathway is not linear and receives overlapping signals from multiple components.

expansion of wound response or tube size. In addition, several proteins that promote clearance removal of cellular materials in the interior of the tracheal tube may determine the turnover of the wound response. Wurst is a transmembrane protein that promotes liquid-clearance and air-filling during tracheal tube development and achieves this essential conversion through clathrin-associated endocytosis.[44] *Wurst* mutant embryos have a "global" pattern of wound reporter localization and suggest that defects in endocytosis and clearance of a wound signal may contribute to the phenotype. Additional support for endocytosis as a cellular process to limit the spread of a wound signal come from research on the role of flotillins in cell signaling during regulatory processes involved in regeneration.[45] Both *Flo-2* and *wurst* mutant embryos have the "global" pattern of wound localization after injury. Recent work on flotillin function in both plants and bacteria highlight a role in endocytosis.[46,47] Rab proteins are key components of the endocytic pathway and have a well-established role in coordinating development[48] and may provide an intersection between wound response localization and endocytosis. Future studies to determine the contribution of endocytic pathway components and their distinct localization patterns during epidermal wound response in the *Drosophila* embryo may provide further insight into cellular mechanism promoting wound repair.

An important function of a critical review is promoting scientific transparency and maintaining a constant review of the research literature. New genomic data can resolve closely mapped genes that were previously thought to be alleles of the same gene (*e.g., kayak* and *shroud*). Based on a previous study, the *kayak-shroud* allele was in the *Drosophila* FosD gene.[49] Initial studies observed an epidermal wound response reporter phenotype in mutant embryos of the *kayak-shroud* allele.[11] Subsequent studies determined that the *shroud* allele was a mutation in a neighboring gene, encoding a short-chain dehydrogenase/reductase.[50] It is clear that *shroud* plays an important role in regulating wound repair from the original study of the wound reporter localization defects.[11] The broader role of hormone biosynthesis in the context of wound repair remains to be determined. Recent studies have found new evidence to support the claim that *kayak* plays an important role in regulating not only the localization of the epidermal wound response,[21] but also promotes epidermal cell migration during wound healing.[19,51]

During the procedure of microinjection with a glass pipette, no additional techniques are employed to preserve a sterile environment and the result is a localized pattern of wound reporter activation (Fig. 4). However, the direct puncture of a *Drosophila* embryo in a liquid culture of Gram-negative bacteria, *Erwinia carotovora carotovora*,

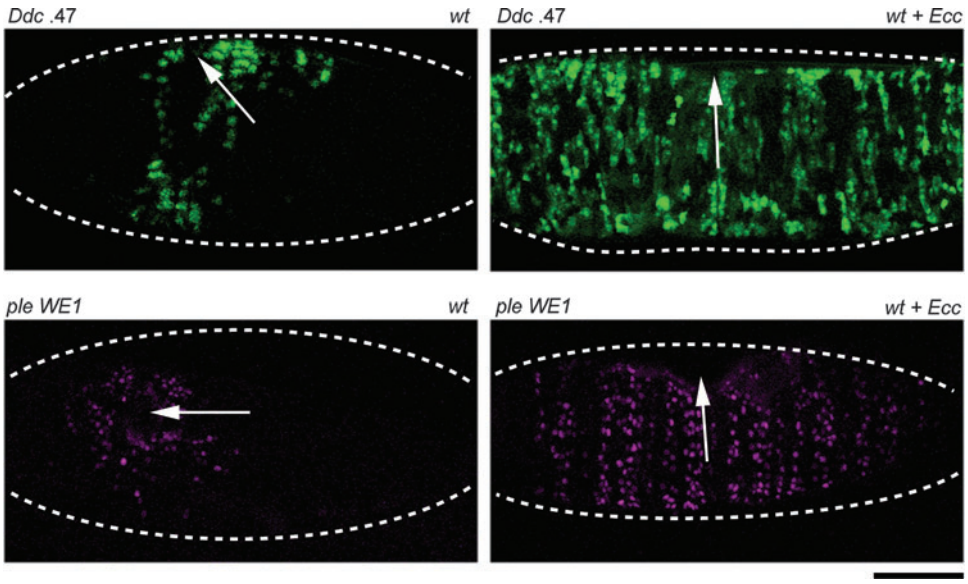

Figure 4. Aseptic and septic injury. Fluorescent images of embryos with Ddc.47 and ple WE1 wound response reporters. *wild type* (*wt*) is an aseptic condition and is the "standard" protocol for puncture injury. The aseptic condition activates a "localized" pattern of the wound response reporters. *wt+Ecc* is a septic condition and is achieved by injuring the embryo while submerged in a liquid culture of *Erwina carotovora carotovora.* The septic condition activates a "global" pattern of the wound response reporters. Dashed lines outline the embryo. Arrows mark the site of puncture injury. Scale bar = 50 μm. *Ecc, Erwinia carotovora carotovora*; ple, tyrosine hydroxylase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

can elicit a "global" pattern of wound reporter activation (Fig. 4). The comparison of puncture-injury and trypsin-mediated injury gene expression profiles provides additional evidence that aseptic injury regulates innate immune response.[21] A recent article from the Medzhitov lab, an expert in the field of mammalian innate immunity, provides evidence that increased tissue repair can contribute to decreased mortality after secondary infection with bacterial pathogens in a mouse model of influenza infection in the upper respiratory tract.[52] Future studies with *Drosophila* puncture injury may provide a direct model to test the hypothesis that a localization of damage signals will promote tissue repair.

SUMMARY

The *Drosophila* embryo has enlisted a well-conserved ensemble of genes to promote a local response to epidermal injury and suppress "global" response. Recent studies from cardiac injury studies in zebrafish provide additional support for the important role of balancing activating and inhibiting signals during heart regeneration.[53] Additional studies in planaria tissue regeneration demonstrate a wide range of inductive signals activate the neoblast cell population and contribute to a wound response pattern.[54]

The significance of the *Drosophila* epidermal wound response reporter method is to combine the protocol for puncture/microinjection with visualization of a transcriptional response to epidermal injury in *Drosophila* embryos. Survival after puncture wounding is greater than 95% in wild-type embryos.[12] One of the merits of using transcriptional activation as a method to study wound repair is that the results provide more insight into a mechanism of how signals are transduced from the site of injury to the neighboring cells. Broader use of the aseptic wound puncture protocol in genetic and chemical screens will allow identification of new regulators of the transcriptional response to epidermal wounding, and further the translation of wound healing discoveries into mammalian models of injury treatments. However, one of the demerits of the transcriptional activation method is that the results do not directly link to phenotypes in wound healing or repair. For example, *Drosophila Flotillin-2* mutant embryos heal after epidermal puncture injury.[20]

The studies highlighted in this critical review will provide insight on the epidermal wound response in *Drosophila* by answering questions pertaining to the cellular mechanism of wound signal localization and the intersection of wound repair and innate immunity. Results from these studies on *Drosophila* wound response can further impact other models of regeneration at both the level of animal systems (*e.g.,* zebrafish) and human organ systems (*e.g.,* skin). Recent studies in zebrafish show a similar role for oxidative species (*e.g.,* hydrogen peroxide) in promoting wound repair and regeneration that complement the preliminary studies with a role for the enzyme Duox in wound response activation.[20,39] In addition, the original report on the role of flotillins in optic nerve regeneration in goldfish has been confirmed in zebrafish and morpholino experiments show a specific requirement for flotillin activity during this process.[55] For the application of the *Drosophila* wound response results to human models of regeneration and repair, many models of both developmental pathologies as well as induced injury cases exist. The recent availability of a *Flo-1* knockout mouse model will allow bridging experiments to be performed and test the efficacy of the result from *Drosophila* studies.[56] New insights into the complex regulation of the transcriptional response after injury in the *Drosophila* embryo will improve paradigms for tissue repair and regeneration in clinically relevant models of cellular damage.

TAKE-HOME MESSAGES

- A localized transcriptional response to injury is regulated by a set of well-conserved genes.
- Barrier formation genes promote development of protective layers and establishing a response to cellular damage.
- *Drosophila* studies provide insight into novel functions of genes during the processes controlling a response to epidermal injury.
- Wound care may be improved by applying activators or inhibitors of a localized wound response pathway.
- The balance of activating a wound response and suppressing an immune response may improve wound repair.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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Abbreviations and Acronyms

Ddc = Dopa decarboxylase
Duox = dual oxidase
Flo-2 = flotillin-2
Grh = grainy head
ple = tyrosine hydroxylase
UTR = untranslated region