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Linking wound response and inflammation to regeneration in the zebrafish larval fin

HENRY HAMILTON ROEHL*

Department of Biomedical Sciences, University of Sheffield, Sheffield, South Yorkshire, UK.

ABSTRACT The study of regenerative biology aims to elucidate the innate ability of organisms to replace tissues or organs after they have been removed or damaged. The zebrafish is a powerful model for the analysis of intracellular signalling and cell behaviour and as such has made major contributions to our understanding of regenerative biology. The larval fin fold is an emerging model to understand how different signalling pathways interact to coordinate regeneration. Tissue damage causes the immediate release of signals that initiate wound closure and inflammation. Following this, regenerative cells proliferate and migrate to the damaged area. Each of these processes has been analysed using the larval fin fold model to provide a framework for how fin regeneration takes place. This review gives an overview of the current state of this field with particular emphasis on the different signalling networks that are required during fin fold regeneration.

KEY WORDS: *zebrafish, fin, regeneration, regenerative biology*

Introduction

Many of the unique experimental attributes of the zebrafish model are present before fish begin to feed, during the embryonic (0-48 hours post fertilisation (hpf)) and early larval (48hpf-120hpf) stages. At this time the fish are small, transparent and easily immobilised allowing researchers to make live microscopic observations for extended periods of time. Large numbers of eggs can be produced quickly, and larvae can be maintained for days in as little as 100 microliters of water. As most pharmaceuticals diffuse freely into the fish, pharmacological tests can be performed by simply adding compounds to the fish water. Many mutations are not homozygous viable as adult but survive until 120hpf and thus provide important tools to assess regenerative gene function in larvae. In addition, experiments done before the fish begin to feed have fewer ethical and legal considerations.

Zebrafish regeneration research has primarily focused upon adult tissues such as spinal cord, fin and heart and by comparison, there are relatively few studies that focus on larval regeneration. One reason for this may be the assumption that regeneration at larval stages utilises different mechanisms than that of the adult (Kawakami *et al.*, 2004, Yoshinari and Kawakami, 2011). Whilst the fish is rapidly growing and still developing there are many stem cells or groups of precursors cells present that are likely to contribute to regeneration. As development is ongoing, the replacement of lost tissue may simply be an extension of normal development. A further point to consider is that organs such as the heart are likely to be smaller and less complex in young fish. So, focusing on the adult may reduce the impact of developmental mechanisms and allow one to study regeneration of fully differentiated and complex tissues and organs. In other words, experiments on adult fish perhaps represent the most stringent test of regenerative capability.

Although these reasons are worth considering, the study of larval zebrafish continues to grow in popularity. The success of larval studies on damage-induced signals and the immune response have created a strong base from which to assemble the steps involved in regeneration. This review focuses on the larval fin fold and attempts to follow the initial stages of wound response and inflammation and link these to subsequent regeneration. A summary of these

Abbreviations used in this paper: citral, 3,7-dimethyl-2,6-octadienal; cloche, neuronal PAS domain protein 4 like; Csf3r, colony stimulating factor 3 receptor (granulocyte); DEAB, N,N-diethylaminobenzaldehyde; Dlx, distal-less homeobox; ErbB, erb-b receptor tyrosine kinase; Fam53b, family with sequence similarity 53, member B; FGF, fibroblast growth factor; Fyn, FYN proto-oncogene; Il1β, interleukin 1, beta; Irf8, interferon regulatory factor 8; JNK, c-Jun NH(2)-terminal kinase; Jun, Jun proto-oncogene; Lyn, LYN proto-oncogene; Msx, muscle segment homeobox; RA, retinoic acid; Runx1, runt-related transcription factor 1; Spi1b, Spi-1 proto-oncogene b; SU5402, 2-[(1,2-Dihydro-2-oxo-3H-indol-3-ylidene)methyl]-4-methyl-1H-pyrrole-3-propanoic acid; Tal1, T-cell acute lymphocytic leukemia 1; WNT, wingless-type MMTV integration site family.

***Address correspondence to:** Henry Hamilton Roehl. Department of Biomedical Sciences, University of Sheffield, Firth Court, Western Bank, Sheffield, South Yorkshire, United Kingdom, S10 2TN. Tel: 44 114 222 2351. E-mail: h.roehl@sheffield.ac.uk - https://orcid.org/0000-0001-6497-2405

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findings is presented in Fig. 1. Other emerging larval models that are not discussed here are neural regeneration (Liu *et al.*, 2016, Lush and Piotrowski, 2014, Meyers *et al.*, 2012, O'Brien *et al.*, 2009a, O'Brien *et al.*, 2009b, Ohnmacht *et al.*, 2016, Rieger and Sagasti, 2011, Rosenberg *et al.*, 2014, Sieger *et al.*, 2012), muscle regeneration (Knappe *et al.*, 2015, Pipalia *et al.*, 2016, Rodrigues *et al.*, 2012, Seger *et al.*, 2011) and liver regeneration (He *et al.*, 2014, Huang *et al.*, 2014). In addition to research specifically framed to investigate regeneration, there are many studies that could also be considered to address questions of regeneration that are not discussed here. These include experimental biology approaches such as cell ablation, mutations that result in degeneration such as muscular dystrophy and studies of responses to toxicity.

Fin fold regeneration

Overview

The median fin fold is a transient structure that begins to form during somitogenesis (~15hpf) and is resorbed and replaced by the dorsal, caudal and anal fins during metamorphosis (Parichy *et al.*, 2009). It is made up of a folded layer of epidermis that extends dorsally, caudally and ventrally from the posterior two thirds of the body. The epidermis is one to two cells thick during the time points that are generally analysed for regeneration. It is supported by actinotrichia which are collagen-rich fibrillar structures that are found in a layer directly underneath the epithelia. In the centre of the fin fold there are fin mesenchymal cells that associate with actinotrichia, sensory axons as well as scattered pigment cells and leucocytes. Excision of the caudal end of the fin fold is done with a fine scalpel between the 48 and 72hpf and it takes 2-4 days for regrowth to complete (Kawakami *et al.*, 2004, Lisse *et al.*, 2015, Yoshinari and Kawakami, 2011) (Fig. 2). Regeneration of limbs in amphibians is characterised by formation of a thickened epithelium (wound epithelium) that is marked by expression *dlx* genes. The wound epithelium overlays densely packed proliferative cells (the blastema) that will give rise to new tissue and express *msx* genes. Similarly, by 24 hours post excision (hpe) the wound epithelium of the fin fold is marked by expression of *dlx5a* and the blastema by expression of *msxc* and *msxe* (Kawakami *et al.*, 2004). Cells that will give rise to the blastema originate from areas around the notochord and begin their migration distally within an hour of wounding (Mateus *et al.*, 2012). These cells become rounded and their nuclei become condensed, but unlike blastemal cells in other systems, they do not proliferate rapidly: damage-induced cell division in the fin fold is more diffuse spreading throughout the posterior trunk (Kawakami *et al.*, 2004, Mateus *et al.*, 2012).

Transcription-independent damage signals

When the skin is breached, signals released in the first seconds and minutes ensure that the wound is rapidly closed to restore the internal environment and to reduce the risk of pathogen infiltration (Cordeiro and Jacinto, 2013, Niethammer, 2016). Osmotic shock and mechanical damage along the margin of the wound cause the immediate release of damage associated molecular patterns (DAMPs) such as ATP that communicate to the immune system (de Oliveira *et al.*, 2015, de Oliveira *et al.*, 2014). In addition, within seconds intracellular calcium (Ca^{2+}) is elevated at the margin, and this increase is then propagated to over 200μm deep into the fish (Enyedi *et al.*, 2016, Yoo *et al.*, 2012). Ca^{2+} causes the release of

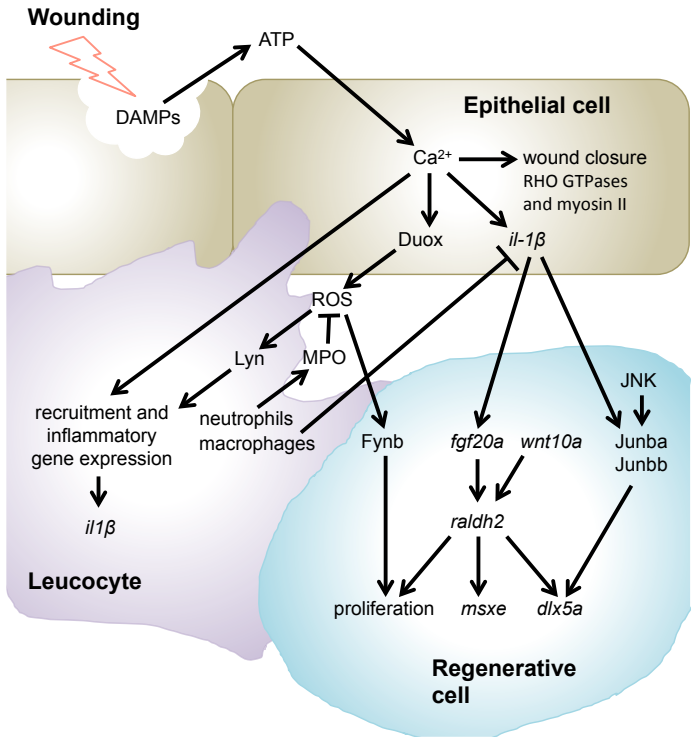

Fig. 1. A model of signaling network interactions during fin fold regeneration. *See the text for details.*

eicosanoids which act to attract leucocytes (Enyedi *et al.*, 2016). Evidence from other models suggests that Ca^{2+} also acts through Rho family GTPases to reorganise actin and myosin II in epithelia to draw the wound closed (Cordeiro and Jacinto, 2013, Niethammer, 2016). Within minutes, reactive oxygen species (ROS) are synthesised by the flavoenzyme dual oxidase (Duox) at the wound margin and then diffuse up to 200μm into the fish (Niethammer *et al.*, 2009, Rieger and Sagasti, 2011). The predominant form of ROS is likely to be H_2O_2 as other species are too reactive to diffuse this far. Like the other signals, ROS act on the immune system to attract leucocytes to the wound and do so by activating a Src family kinase (SFK) called Lyn (Niethammer *et al.*, 2009, Yoo *et al.*, 2011). As neutrophils arrive at the wound they release myeloperoxidase which breaks down H_2O_2 to dampen ROS signalling (Pase *et al.*, 2012). These three early signals may form a linear pathway (Cordeiro and Jacinto, 2013, de Oliveira *et al.*, 2015, de Oliveira *et al.*, 2014, Niethammer, 2016). This sequence begins when damaged cells release ATP which then activates purinergic receptors in neighbouring intact cells, that causes elevation of intracellular Ca^{2+}, which activates Duox causing synthesis of ROS. Although this cascade is probably central in the early response it is perhaps an oversimplified view of the first few minutes after wounding. It is likely that there is feedback between these pathways, as well as other early pathways that contribute to the initial response.

Regeneration signalling pathways

After the initial response to wounding, the organism needs to recruit cells to rebuild the missing tissue. These cells need to proliferate, be patterned, go through morphogenesis and differentiate. As in development these processes are likely to involve many

signalling pathways. Although the fin fold is a very simple tissue, it is already apparent that the networks involved in restoration of the tissue are complex and numerous.

Developmental signalling pathways have understandably been heavily investigated for their roles in fin fold regeneration. The FGF receptor inhibitor SU5402 has been used to show that FGF signalling promotes damage induced cell proliferation (Kawakami *et al.*, 2004). Mathew *et al.,* found that the RA synthesis gene *raldh2* is induced by 4hpe and that RA synthesis inhibitors DEAB and Citral also block *msx* and *dlx* gene expression as well as damage-induced proliferation (Mathew *et al.*, 2009). Mathew *et al.,* also investigated the role of the FGF and WNT/βcatenin pathways using SU5402 as well as a dominant negative transgene to target WNT/β-Catenin signalling. Their results suggest the model that FGF and WNT/β-Catenin pathways activate RA signalling which patterns the regenerating fin fold and drives proliferation. How these interactions relate to the initial formation of the fin fold is currently unclear as fin fold development is an understudied area.

Upstream of developmental signalling there are pathways involved with stress response and inflammation. Expression of the JNK signalling components *junba*, *junbb* and *c-jun* is strongly induced within 30 minutes of damage indicating that they are immediate early genes (Ishida *et al.*, 2010). Phosphorylation of Jun proteins occurs by the wound within minutes of excision and inhibition with the JNK inhibitor SP600125 blocks proliferation and regeneration. Similarly, the cytokine *il1β* is upregulated in the epidermis by 3hpe and it is required for cell proliferation as well as expression of *fgf20a* and *junba* (Hasegawa *et al.*, 2017). *il1β* is also known in other systems to be transcriptionally activated by the JNK pathway (Newton and Dixit, 2012), so it is possible that the upregulation of *junba* is part of a positive feedback loop.

Linking damage signals to regeneration

How does an organism sense that a tissue or an organ is missing, what molecules trigger regeneration and what determines whether the wound is simply healed over to leave a scar or replaced? The answers to these questions may hold the key to initiating human regeneration and have understandably been the focus of much research on the larval fin fold. Two candidates that may link the early wound response to regeneration have emerged in the last few years.

Inflammatory signals and leucocytes are thought to play a complex role in regeneration in many systems (Eming *et al.*, 2017). Analysis of the fin fold has yielded conflicting results over the last decade, but recent data has begun to support a nuanced role for inflammation during the initiation of regeneration. Neutrophils are attracted to the wounded fin fold within minutes and their presence peaks at about 4hpe, while macrophage numbers peak several hours later. Initially, two studies that used a *pu.1* morpholino to target macrophages and neutrophils found that leucocytes are dispensable for regeneration (Mathew *et al.*, 2007, Yoo *et al.*, 2012). In contrast, another study that compared the individual roles for leucocytes by removing macrophages with an *irf8* morpholino and neutrophils with a *runx1* mutation found that loss of neutrophils increased regeneration and loss of macrophages slowed regeneration (Li *et al.*, 2012). Similarly, transgenic ablation to reduce macrophages also slowed fin fold regeneration (Petrie *et al.*, 2014) and a recent study that utilised *tnfr1* knockdown by morpholino suggested a role for macrophage-derived TNF signaling during fin regeneration (Nguyen-Chi *et al.*, 2017). In agreement with these findings, glucocorticoid treatment which blocks inflammation reduces proliferation (Mathew *et al.*, 2007, Sharif *et al.*, 2015).

A series of papers from the Kawakami lab offer a molecular model that may help explain these apparently conflicting results (Hasegawa *et al.*, 2017, Hasegawa *et al.*, 2015, Yoshinari *et al.*, 2009). Their group ablated leucocytes using the *cloche* and *tal1* mutants as well *spi1b*, *irf8* and *csf3r* morpholinos. They found that between 12-24hpe, cell death is elevated and cell division is reduced and showed that this is due to a reduction in the numbers of macrophages. Surprisingly they found that these defects are accompanied by a strong and persistent elevation of *il1β* expression. As discussed above, *il1β* is expressed in the damaged epithelium and is required for activation of regeneration, so why then would it be upregulated when regeneration is reduced? To investigate further, Hasegawa *et al.,* first showed that ectopic upregulation of *il1β* under control of a heat shock promoter is sufficient to induce cell death in the tail of unoperated larvae. They were also able to show that the deleterious effects macrophage depletion could be rescued by knock-down of *il1β* with morpholinos. In addition they found that glucocorticoid treatment inhibits expression of *il1β* in the epithelium and also rescues macrophage depletion. This elegant set of experiments suggests that macrophages may act upon regeneration by resolving *il1β* expression. This, along with the role of neutrophils in dampening ROS signalling, suggests that leucocytes may not initiate regeneration, but rather that they provide negative feedback to early damage-induced pathways to ensure the appropriate regenerative response. Since *il1β* is known in other systems to be activated by Ca^{2+} and DAMP signalling

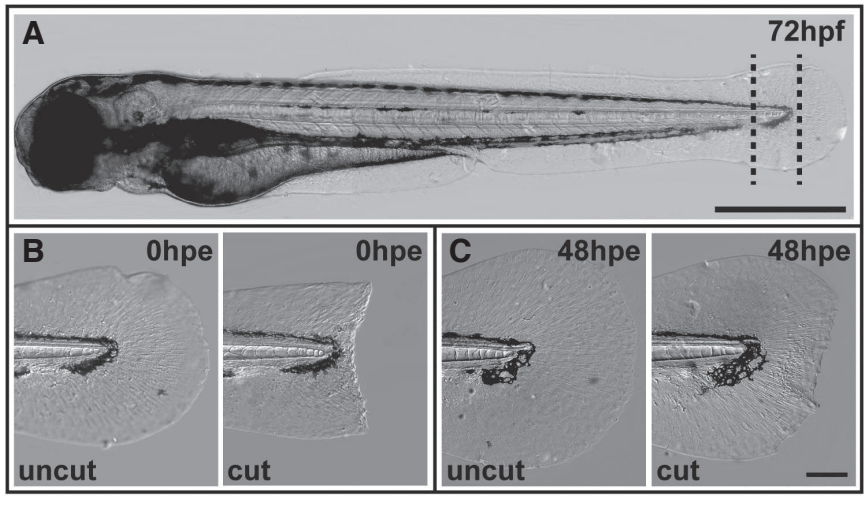

Fig. 2. Larval zebrafish fin fold regeneration. (A) *Larval fish at 72 hpf showing the possition for excision of the fin fold (dotted line on the right) and the possition for tail excision (dotted line on the left). Scale bar equals 500 μm.* **(B)** *Close-up images of the fin fold before and after excision. Larvae are 72 hpf.* **(C)** *Close-up images of the fin fold after 48 hours of recovery when the fin has almost completely regenerated. Larvae are 120 hpf. Scale bar, 100 μm.*

(Newton and Dixit, 2012), *il1β* is may be an inflammatory signal that serves to link damage to regeneration.

The second candidate for initiation of regeneration is ROS signalling. Rieger *et al.,* used *duox* morpholinos to show that when ROS synthesis is blocked, axonal regeneration within the fin fold does is reduced (Rieger and Sagasti, 2011). The Huttenlocher lab went one step further by dissecting the role of SFKs acting downstream of ROS in the fin fold (Yoo *et al*., 2012). They found that the Duox inhibitor DPI and the SFK inhibitor PP2 both block leucocyte recruitment and regenerative cell proliferation. However, when they knocked down individual SFKs they found that *lyn* morpholinos only affect leucocytes and *fynb* morpholinos only affect proliferation. This uncoupling of the inflammatory and regenerative activities of ROS suggests that *fynb* acts as part of the trigger for regeneration. Inhibition of ROS/SFK signalling was only effective within the first hour after wounding, at a time when *il1β* is also functioning. Whether these two pathways interact or form a linear pathway has not yet been investigated.

Tail regeneration

The tail excision assay is closely related to fin fold excision except that the end of the tail is also removed (Rojas-Munoz *et al*., 2009). The cut is made transversely where there is a gap in the melanophores which line the ventral edge of the trunk (see Fig. 2A). The end of the neural tube and notochord as well as muscle and blood vessels are removed and recovery takes up to four days. One of the hallmarks of tail regeneration is the protrusion of notochord cells which form a clump of cells that sit under the wound epithelium during regeneration. This clump of cells forms immediately after excision and is also seen during axolotl and tadpole tail regeneration. These cells are sometimes referred to as the blastema, but there is no evidence to support this assumption. Forward genetic screens have identified mutations that affect the ErbB2 and ErbB3 receptor tyrosine kinases which disrupt tail regeneration (Rojas-Munoz *et al*., 2009). In addition to this study, other papers have identified chromatin remodelling components, the Wnt/β-catenin signalling modifier Fam53b and nitric oxide synthases as being expressed during larval tail regeneration (Kizil *et al*., 2014, Kizil *et al*., 2009, Lepiller *et al*., 2009, Pfefferli *et al*., 2014). It is likely that these pathways are also active during fin fold regeneration but a direct comparison has not been made.

Conclusion

Although great progress has been made in understanding how the fin fold regenerates, there are still several key questions that remain unanswered. One fundamental issue is that of the source of the cells that reform the fin. Do stem cells or precursor cells form the blastema? Is there dedifferentiation of mature cell types or is dedifferentiation even necessary? The majority of the tissue lost is epidermal, and these cells are proliferative and move to cover the wound after excision (Mateus *et al*., 2012). Similarly, fin mesenchymal cells, pigment cells and leucocytes are highly migratory and may simply move to replace the lost tissue. Lineage studies of the cells that make up the blastema are needed to identify the cellular identity of new tissue. A second important question is whether regeneration of the fin fold involves the same processes as the initial development of the fin. The Wnt/β-catenin, RA and FGF pathways all act during anterior/posterior axis elongation and it is possible that their roles during regeneration are identical to their roles during development. Alternatively, these pathways could be playing roles that are unique to regeneration. A third crucial area for analysis is how is regeneration initiated. So far the Il1β and ROS/Fynb pathways are the best candidates to link wounding to regeneration. But a molecular mechanism for how these pathways have evolved to play this role has not emerged. Topical questions are what are the direct, molecular targets of these pathways during regeneration, and are these targets also affected by non-regenerative signalling. The tools to address these issues are readily available and it is likely that in the next few years the answers to most if not all of these questions will be revealed.

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