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Signals for cardiomyocyte proliferation during zebrafish heart regeneration

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Abstract

The common laboratory zebrafish can regenerate functional cardiac muscle after cataclysmic damage or loss, by activating programs that direct the division of spared cardiomyocytes. Heart regeneration is not a linear series of molecular steps and synchronized cellular progressions, but rather an imperfect, relentless process that proceeds in an advantaged competition with scarring until recovery of the lost heart function. In this review, we summarize recent advances in our understanding of signaling events that have formative roles in injury-induced cardiomyocyte proliferation in zebrafish, and we forecast advances in the field that are needed to decipher heart regeneration.

Introduction

Zebrafish have become a powerful model system to study key biological events like embryogenesis, disease, behavior, and regeneration. Their external fertilization, rapid early development, and transparency of embryos benefit the study of fundamental developmental mechanisms, advances in ecotoxicology, and new drug discovery [1–3]. The ability of zebrafish to regenerate its fins was first reported in the late 1980's, although the phenomenon of fin regeneration in teleost fish had been recognized two centuries prior [4, 5]. Since then, many other tissues of zebrafish have been reported to regenerate after injury [6, 7].

Heart regeneration in zebrafish was first described in 2002 as a process in which a new wall of muscle is built through the division of cardiomyocytes (CMs), after surgical removal of a fifth of the cardiac ventricle [8]. While one of the early models invoked a progenitor cell precursor to these proliferating cardiomyocytes [9], the use of genetic fate-mapping approaches has unambiguously identified the source of muscle to be pre-existing muscle cells, stimulated by injury to divide [10, 11]. By contrast, and reviewed extensively elsewhere [12, 13], adult mammals have a limited ability to provoke cardiomyocyte division

Declarations of interest: none

upon injury, and the injured mammalian heart heals a major injury like myocardial infarction predominantly by scarring and tissue remodeling. This is considered a process of repair, not regeneration, and, while a scarred human heart can and often does perform at a sufficient level for decades, an initial injury/repair event can leave the heart vulnerable to further injury and failure.

Heart regeneration is a complex process that involves communication between multiple cell types. As opposed to morphogenesis in embryos, where many tissues grow and mature in synchrony to increase size and improve physiological function, heart muscle regeneration is more of a solo performance and does not adhere to a strict time window to perform its chief goal – to outdo the competing process of scarring. Research over the past 17 years has significantly advanced what we know of the signals exchanged during regeneration, although there remains much to learn. Whereas findings in other model systems like mice have provided many insights into the regenerative capacity of the heart, we limit this brief review to what is known of the signaling mechanisms that direct cardiomyocyte proliferation during zebrafish heart regeneration.

The cellular players in heart regeneration

To regenerate efficiently, CMs need intrinsic programs that are competent for division, as well as the ability to receive extrinsic proliferative cues. Without question, CMs are built to work. They are massive cells that rhythmically contract and are filled with sarcomeres and energy-supplying mitochondria for that purpose. Mammalian hearts have a high percentage (>50%) of polyploid CMs, with multiple diploid nuclei or single polyploid nuclei, or both, with the proportions depending on species [14]. Higher DNA content possibly enables the generation of more contractile and energizing machinery on a per-cell basis. In turn, it is just as likely that the highly differentiated structure of CMs restricts their ability to complete cytokinesis during organ growth or in response to injury. As most or all teleost and amphibian vertebrates that have been examined have a high proportion (>90%) of mononuclear, diploid CMs, the association of ploidy and regeneration is strong, and it has been fortified in several recent studies [15–17]. One of these reports examined zebrafish and described experiments inducing the expression of a dominant-negative form of the Ect2 guanine nucleotide exchange factor in CMs transiently during heart development, to yield adults with a ~50% complement of polyploid CMs [15]. The authors found that these genetically manipulated CMs participated less vigorously in regeneration than did predominantly diploid CMs. The key manipulation in this study was elegant and precisely controlled, though it is conceivable that some event in addition to multinucleation occurred under Ect2 inhibition that might contribute to the regenerative defects. Zebrafish CMs also undergo some level of dedifferentiation, a dedicated program that includes induction of cardiac transcription factors, tempering of the contractile machinery, and acquisition of a more glycolytic program [10, 11, 18], events that require further elucidation and are expected to further improve the competency of CMs to divide.

The heart is not pure, uncaged muscle, but instead has a diverse cast of supporting cells. The wall of the zebrafish ventricle is, like all vertebrate hearts, lined by an outer mesothelial lining called the epicardium and an inner endothelial lining called the endocardium (Fig. 1).

The wall is vascularized and innervated, and there is a minor fibroblast component. When the heart is in an acute phase of injury, the first responders are inflammatory cells like neutrophils, macrophages, and T-cells [19–22]. Importantly, many of these non-myocardial tissues have been formally examined for their requirements during regeneration, by genetic cell ablation or genetic inactivation of a key regulator. For instance, induced genetic depletion of the epicardium and its derivatives disrupts CM proliferation and muscle regeneration, after which the process recovers as the epicardium itself regenerates from survivors [23]. Blocking rapid vascularization of the injury area by inhibiting angiogenic communication, or blocking vascularization that occurs with later cardiogenesis, also impacts muscle regeneration [21, 24]. Recently, the lympathic system has been implicated in clearance of collagen and fibrin during cardiac repair [25, 26]. Genetic ablation of regulatory T-cells, which circulate and home to the injury site, impairs heart regeneration and other examples of tissue regeneration in zebrafish [27]. As a final example, Mahmoud and O'Meara et al. found that the presence of nerves is a positive factor for cardiomyocyte proliferation and heart regeneration in both zebrafish and neonatal murine contexts [28].

These cell populations collectively have the potential to influence regeneration in many ways. This could be as a structural scaffolding: a heart with a injury ablating half of all CMs will regenerate much more quickly than a heart injured by resection or cryoinjury where only 20–25% of tissue is lost, most likely due to the spared architecture of the non-muscle linings [29, 30]. Also, likely to be key are the classic functions of the various cells, e.g. extracellular matrix deposition and vascular support for epicardial-derived cells [31–35], nutrient provision by vascular tissue, or debris clearance by macrophages and neutrophils [19, 36]. Finally, these cells can directly act as a source of signals conducive to CM proliferation.

Instructive cardiac mitogens

A simple mechanism for muscle regeneration would be this: injury-induced release of a potent signal promotes CM proliferation. In theory, one should be able to experimentally uncouple the mitogenic signal from the injury, evidence for which would be that overexpression of a factor(s) on its own induces CM proliferation when introduced to uninjured, adult cardiac tissue. Such factors can be referred to as "instructive" in this regard, following the language of developmental biology. In recent years, three diffusible or secreted factors have been found to provoke CM proliferation when overexpressed in the absence of injury. One is the extracellular factor extracellular factor Neuregulin 1 (Nrg1), which binds to the receptor tyrosine kinase ErbB4 and induces heterodimerization with ErbB2 [37]. Based on an earlier study implicating Nrg1 as a stimulant for CM proliferation in mice [38], Gemberling et al. found that induced overexpression of Nrg1 in adult zebrafish sharply increases CM proliferation after injury [39], ultimately causing cardiomegaly through hyperplasia. Blocking ErbB2 function pharmacologically with the drug AG1478 reduced indicators of CM proliferation after injury. Key questions remain from this study, most notably which specific ligands and receptors from the array of possibilities actually participate, given that no specific genetic mutations were employed. Nrg1 has been reported to be synthesized in epicardial cells as well as in T-regulatory cells [27, 39].

Vascular endothelial growth factor a (Vegfa) is a well-known inducer of endothelial cell proliferation, and angiogenesis logically tracks tissue regeneration [21, 24]. An initial study by Marin-Juez et al. reported that induced expression of an inhibitory form of Vegfa can block regenerative angiogenesis [24]. A later study found that induced cardiac expression of *vegfaa* hypervascularized the adult heart, but also led, unexpectedly, to CM hyperplasia and thickening of the muscular wall in the absence of injury [40]. As had been observed with ectopic expression of Nrg1, dedifferentiation programs such as expression of the cardiogenic transcription factor *GATA binding protein 4* (*gata4)* were activated. Upon injury, *vegfaa*-overexpression in cardiomyocytes impaired cardiac repair at the injury site, even in the presence of ectopic growth away from the wound [40]. This suggested that the location and/or amount of signal impacts the response. Moving forward, it will be critical to define how the signaling pathway leading to cardiogenesis differs from that leading to angiogenesis, and whether, for instance, the hyperplastic effect in zebrafish is analogous to the hypertrophic effect that has been assigned to Vegf ligands when delivered to cultured mammalian CMs [41].

Vitamin D is a third instructive signal for heart regeneration, having been initially implicated to promote CM division by a Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI)-based in vivo screen in transgenic zebrafish embryos treated with FDA-approved drugs [42]. Treatment with the vitamin D analog Alfacalcidol was sufficient to sharply boost in vivo CM proliferation in embryonic and adult CMs, and induced expression of an activated vitamin D receptor led to profound cardiomegaly in juvenile animals. A dominant-negative vitamin D receptor blocked heart regeneration when it was experimentally induced in adult CMs [42]. Interestingly, vitamin D treatment elevated the cardiac gene expression of many factors associated with ErbB2 signaling; plus, its effects on CM proliferation were disrupted by treatment with the pharmacological ErbB2 inhibitor AG1478. Vitamin D signaling, though heavily studied, is a complex pathway, and it remains unclear how signals might be regulated through processing enzymes or downstream mediators to guide regeneration. Moreover, vitamin D has been a target of several clinical research trials to define effects, if any, on cardiovascular disease, without clear evidence of specific benefits [43, 44].

These instructive factors each represent a basis for the important idea that a single factor on its own, whether an encoded protein or a hydrophobic drug, can drive the crucial event in heart regeneration. Each has the ability to (directly or indirectly) coincidentally increase proliferation indices or other biological responses in multiple cardiac cell types, thereby enabling coordinated growth of key tissues for the organ. In zebrafish, it will be essential to compare and contrast the effects these instructive factors have on molecular machinery, and to learn how these highly potent developmental factors are regulated – individually, and in shared networks - for the function of innate heart regeneration.

Permissive cardiogenic influences

Many ligands, receptors, and transcription factors have been shown to be required for zebrafish heart regeneration, although these molecules on their own have not been shown to promote CM proliferation in the absence of injury. Such mitogenic influences can be

considered “permissive”, to contrast their effects from the features of instructive CM mitogens.

Intrinsic factors acting in CMs

Several transcription factors that regulate cardiac development are re-expressed after injury (Fig. 2). Regulatory sequences of the embryonic cardiogenesis genes *gata4, nk2 homeobox 2.5 (nkx2.5), hand2, t-box 5 (tbx5), and t-box 10 (tbx20)* are activated in CMs (and in some cases other cardiac cell types) upon injury [9, 10, 45, 46]. Induced expression of a dominant-negative Gata4 in CMs impaired proliferation and heart regeneration [46], while *hand2* augmentation could increase CM proliferation after injury [45]. The others have yet to be interrogated functionally. A handful of reliable Cre-based transgenic strains are available for inducible recombination of floxed alleles in zebrafish CMs; however, there is a dire paucity of strains with *loxP* sequence-flanked gene sequences. Conditional gene deletion is a methodology that the field must advance in the coming years.

Jak/Stat3 signaling within cells can be activated by dozens of ligands including Interleukin 11a (Il11a), Interleukin *11b* (il11b), and Leukemia inhibitory factor (Lif). Transcription of the *il11a* ligand gene, the co-receptor *interleukin 6 signal transducer* (*Il6st)*, the feedback regulator *suppressor of cytokine signaling 3b* (*socs3b)*, and the transcription factor mediator *signal transducer and activator of transcription 3 (stat3)* are induced after cardiac injury, and induced expression of a dominant-negative Stat3 cassette in CMs blocked regeneration [47]. Stat3 ostensibly acts at least in part by regulating the secreted protein Relaxin 3a (Rln3a), a known transcriptional target of Stat3. The authors reported that *rln3a* is upregulated upon cardiac injury, and that systemic delivery of human recombinant RLN3 increased CM proliferation after injury [47]. Another pathway activated in CMs during regeneration involves the NF-κB transcription factor complex, which, like Jak/Stat signaling, is also essential for the mammalian hypertrophy response [48, 49]. In the presence of a dominant-negative IκBSR, which retains NfκB transcription factors in the cytoplasm, disassembly of sarcomeres, proliferation and induction of *gata4* regulatory sequences were each disrupted after injury [50]. These inhibitory effects could not be rescued by *gata4* overexpression, which implies that the downstream network of NfκB is complex; indeed, the initiating ligand has not been elucidated.

In addition to transcription factors themselves, epigenetic regulation, like chromatin remodeling or histone modification, have been implicated in zebrafish heart regeneration [51]. Transgenic myocardial inhibition of Brahma-related gene-1 (Brg1), a component of the ATP-dependent chromatin remodeling complex SWI/SNF, inhibited injury-induced CM proliferation, potentially due to increased expression of cyclin-dependent kinase inhibitors *cdkn1a* and *cdkn1c* [51]. RNAseq and ChiP-seq screens using purified *gata4*-expressing CMs revealed that Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (Ezh2), a component of the polycomb repressor complex 2, suppresses expression of structural genes involved in sarcomere formation by H3K27-tri-methlation [52]. When a transgenic histone H3 with a mutated methylation site was ectopically expressed, CMs at the wound site retained a mature state – with intact sarcomere structure and reduced expression of embryonic *cardiac myosin heavy chain*. This study combines with others that have identified

broad changes in histone regulation during zebrafish heart regeneration [53, 54]. The initial signals that trigger large-scale and local changes in chromatin structure during heart regeneration await elucidation.

Extrinsic factors from neighboring cells

An injured zebrafish heart engages a host of secreted signals from neighboring non-myocardial cells (Fig. 2). Retinoic acid, its production controlled by the enzyme Retinaldehyde dehydrogenase 2 (Raldh2), is synthesized by the epicardium and endocardium within hours of injury [55]. Broad transgenic inhibition of retinoic acid receptors (RARs) impairs CM proliferation, although requirements for RARs have yet to be attributed to a specific cell type. Signaling by release of the membrane-bound transcription factor Notch has also been implicated in communication between the myocardium and epicardium or endocardium [56, 57]. Although Notch is dispensable for activation of injury markers in endocardial cells, blocking Notch signaling via the Notch inhibitor Dominant negative mastermind-like (DN-MAML) in endothelial cells (including the endocardium) was reported to decrease CM proliferation [57]. Transcriptome analysis of injured DN-MAML-expressing hearts identified Wnt antagonists Wnt inhibitory factor 1 (Wif1) and Notum1b as likely Notch targets. Notably, pharmacological suppression of Wnt by the small-molecule antagonist IWR-1-endo partially rescued the phenotype caused by conditional loss of Notch, and Wnt inhibition at injury sites was proposed to be required for normal CM proliferation [57].

Myostatin and activin Inhibin subunit beta aa (Inhbaa) are TGFβ ligands that are antagonistically regulated during tissue repair [58, 59]. While *myostatin* expression is reduced in the ventricular wall, *inhbaa* is upregulated in cells at the wound after cryoinjury [58]. Interestingly, either transgenic *myostatin* overexpression (OE) or *inhbaa* knock-out resulted in decreased CM proliferation, whereas knock-out of *myostatin* or *inhbaa*-OE caused hyperplasia and hypertrabeculation with late stage pericardial edema, respectively. The authors reported that Myostatin and Inhbaa bind to two distinct Activin receptors respectively, and these in turn lead to the activation of distinct Smads [58]. *inhbaa*-OE caused an increase in CM proliferation independently of Nrg1-Erbb2-signaling; thus, it would be interesting to determine how heart regeneration is impacted by coincident overexpression of both *inhbaa* and Nrg1. Wu et al investigated potential roles for Bone morphogenetic protein (BMP) signaling during regeneration, after observations that *bone morphogenetic protein 2b* (*bmp2b)* RNA levels increased in the wound border zone and epicardium after cryoinjury [60]. Induced global overexpression of *bmp2b* decreased the wound size, while overexpression of BMP-inhibitor *noggin3* delayed muscle repair. Transgenic *bmp2b* increased CM proliferation only slightly in injured hearts, and had no effect on CM proliferation in uninjured hearts; however, Noggin inhibition of BMP signaling limited CM de-differentiation and cell cycle entry. Signaling pathways like Insulin-like growth factor (Igf) and Sonic Hedgehog (Shh) have also been implicated in CM proliferation by various loss-of-function studies, with the epicardium in each case reported as a key ligand source [61, 62]. Notably Sugimoto et al. used inducible Cre-based techniques to disrupt the *shha* gene specifically in epicardial and epicardial-derived cells, providing elegant genetic

evidence that this tissue is a significant source of Hedgehog ligand during heart regeneration [63].

Outlook

It is now evident that experimental disruption of many candidate factors individually can have no apparent effect on heart regeneration – the authors know this firsthand, and such results do not make their way easily into publications. Genetic redundancy is a generally fascinating but still confounding issue, and mechanisms of compensation are likely in play with a subset of these factors [64]. Evolution of the genetic toolset for adult zebrafish can address this, as well as the challenges of tying results like those summarized in this review into coherent regulatory networks. When groups use the same injury models and methods of genetic manipulation, it is more straightforward for them to reproduce findings and perform tests of epistasis.

We emphasize that a key area to pursue more deeply is how signals, especially potent, instructive signals, are induced and restricted at the level of chromatin structure, gene regulatory elements, and transcription factor-DNA complexes. This is reviewed more extensively elsewhere [65]. As a method to identify signals, high-resolution proteomes of heart regeneration would be of great interest, but defining them effectively has challenges, such as the limited amount of tissue and dominance of profiles by contractile and mitochondrial proteins. Recently developed technologies might help unveil the regeneration proteome at new detail, including large-scale assessment of protein modification and protein-protein interaction dynamics [66–70]. Before a clear equation for regeneration is derived, it in fact is likely that sufficient nuggets will have been mined from the study of heart regeneration in laboratory models to already initiate effective therapies. The discovery of instructive influences in zebrafish, and recent similar discoveries in mice, suggests that such interventions employing potent triggers are on the horizon [14, 71–74].

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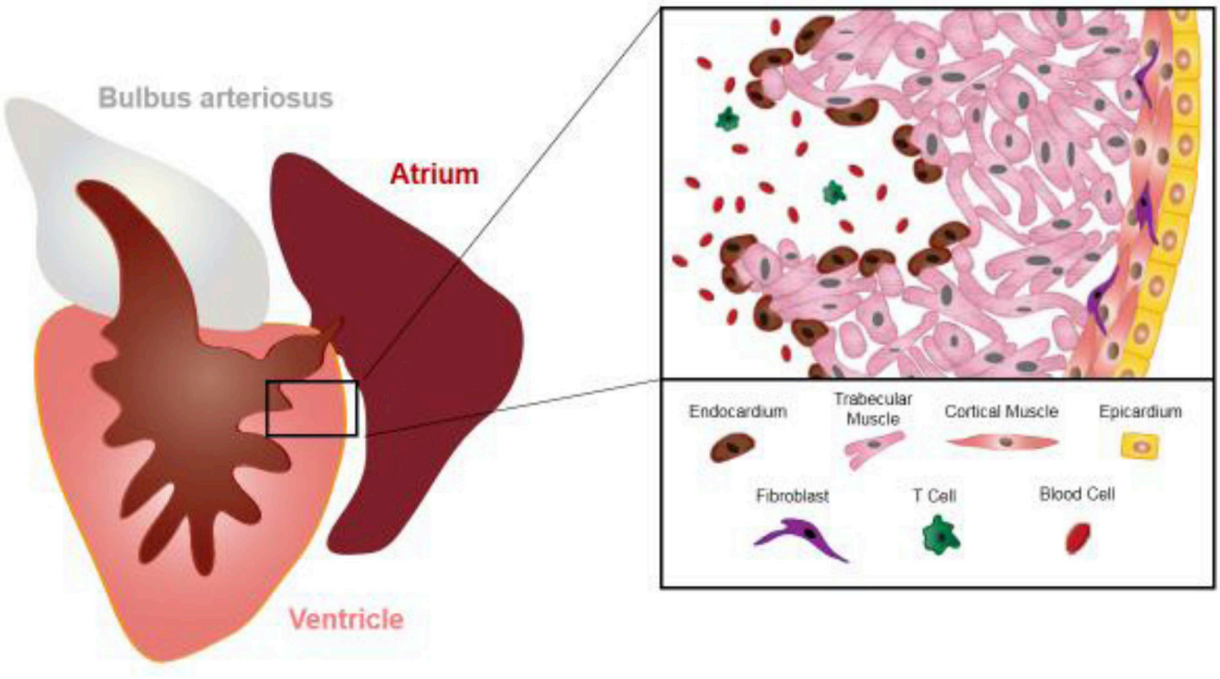

Figure 1. Schematic representation of the zebrafish heart and key cell types.

The zebrafish ventricle retains a heavily trabeculated anatomy, with a thin outer wall of cortical muscle. Several cardiac cell types and their general location within the heart are shown. “All cardiac cell types (e.g. vascular tissue, nerves) are not depicted in this simplified cartoon”.

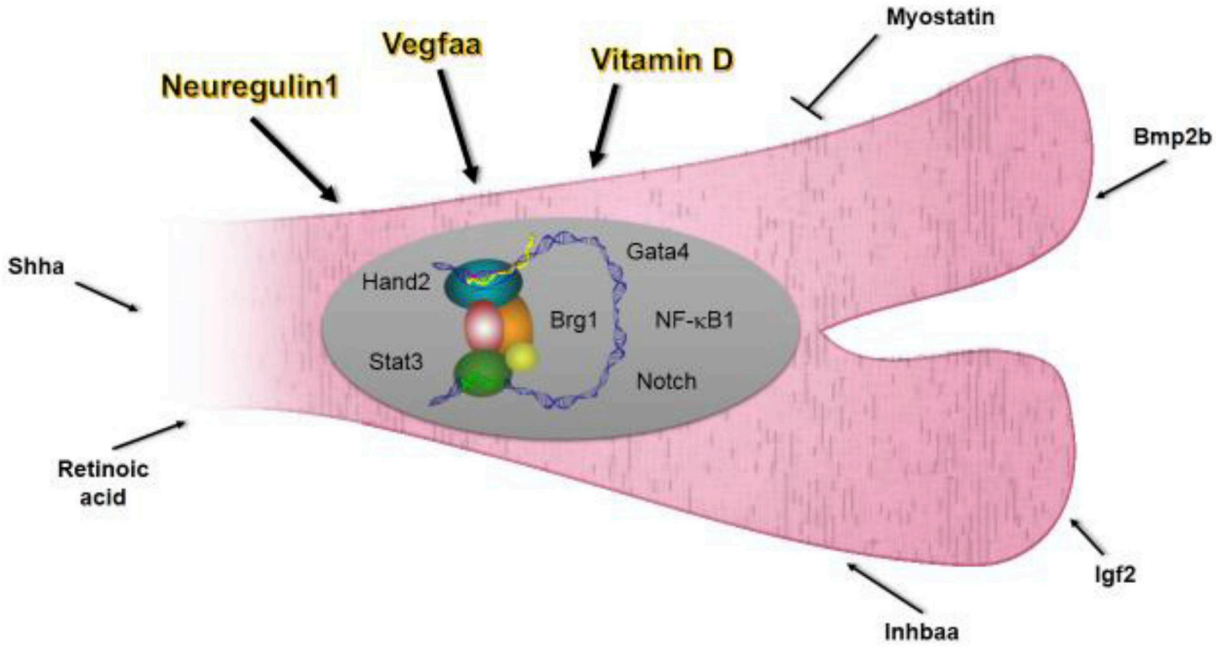

Figure 2. Schematic of pathways implicated in zebrafish heart regeneration.

Pathways described here are shown as either intrinsic or extrinsic effectors. Instructive factors are highlighted and have bolded arrows. Putative cellular sources of extrinsic factors: Retinoic acid - endocardium, epicardium; Shha - epicardium; Nrg1 - epicardium, T-cells; Vegfaa – endocardium, epicardium; Bmp2b - epicardium; Igf2 - epicardium, endocardium.