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Towards deciphering variations of heart regeneration in fish

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Among adult vertebrates, the zebrafish presents the rather exceptional capacity to efficiently regenerate its heart after injury. This bony fish has thus become a leading genetic model organism to elucidate the natural mechanisms of successful cardiac restoration. Given its potential biomedical significance, parallel analyses between zebrafish and mammals are aiming at the identification of the permissive and restrictive factors modulating the underlying cardiomyocyte proliferation. The recent discovery that some other bony fish species have a lower regenerative competence than zebrafish opens new opportunities for comparative studies within a framework of similar animal physiology and organ structure. Here, we review recently identified modulators of cardiomyocyte proliferation in zebrafish and highlight the results obtained by this comparative approach.

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Introduction

The heart is one of the most important vertebrate organs due to its vital blood-pumping function. Consequently, a prompt and efficient mechanism to heal heart injuries would be particularly beneficial. A restoration of the myocardium to a pre-injury state obviously represents the optimal strategy. Yet, cardiac regeneration is absent in adult mammals and has only been observed in a few anamniotic vertebrates, such as zebrafish and axolotls [1•]. As the heart lacks competent cardiac stem cells, the regenerative mechanism solely relies on the proliferative capacity of pre-existing cardiomyocytes (CMs) [2–5]. Understanding how the cellular plasticity of CMs is modulated is thus of major scientific and therapeutic relevance.

In non-regenerative hearts, such as in adult mammals, damage leads to scarring, which causes varying degrees of cardiac dysfunctions including heart failure [6]. This imperfect repair seems to be a consequence of the inability of mature mammalian CMs to efficiently multiply for restoring the missing tissue [4]. Indeed, mammalian CMs substantially diminish their access to the cell cycle after the neonatal stage [7]. Nevertheless, the inherent renewal of some mature CMs can be experimentally enhanced by hypoxic conditions, pharmacological treatment, and genetic modifications of molecular pathways [8–10]. Yet, it is still debated whether mammalian CMs could reach a proliferative state sufficient for regeneration.

By contrast, the zebrafish can naturally regenerate its heart after different types of injuries, and recover the functionality of this organ, as recently reviewed in [1•,11–14]. For example, cryoinjured hearts regenerate most of their damaged tissue within 30–60 days [15], and their complete restoration may require 130–180 days [16,17]. This process is sustained by CMs that are stimulated upon injury to revert to a less differentiated state, which is thought to facilitate cell proliferation and morphogenesis [18–21]. Myocardial regeneration is accompanied by the activation of other tissue types of the heart, such as the endocardium, epicardium, blood and lymphatic vasculature, nerves and immune system [22–30]. Thus, the intrinsic features of CMs and extrinsic signals from other tissues contribute to the restorative outcome. Consequently, identifying, characterizing and controlling some of these factors constitute major scientific interest.

Cellular complexity and polyploidy of cardiomyocytes as obstacles for proliferation

Increased structural and physiological complexity of the heart has been proposed to be one of the limiting factors for regeneration in mammals [1•,13]. Because the metabolism of endothermic organisms requires more energy, mammals have acquired a more performant heart through enlargement and specialization of their CMs. Although bigger CMs can be better at generating contractile forces, their structural complexity imposes more challenges on cell division and plasticity [31]. Moreover, the mammalian cardiac hypertrophy is typically associated with a polyploidization event, meaning that the set of chromosomes is multiplied without a subsequent cell division [32]. This polyploidization coincides with the loss of proliferative capacity of the mammalian myocardium around the time of birth, suggesting a correlation between both changes [33]. The loss of the proliferative capacity can thus be considered, at least to a certain extent, as a trade-off for fostering the contractile tissue with specialized and polyploid CMs [1•].

Two recent studies tested this hypothesis in the zebrafish heart, which nearly entirely comprises diploid CMs [34••,35]. First, Gonzalez-Rosa *et al.* investigated if experimentally induced polyploidy is sufficient to affect heart regeneration in the zebrafish [35]. They established a transgenic approach to inhibit CM division during ontogenic growth by the transient overexpression of a dominant-negative form of Ect2 (dnEct2), a Rho GEF protein required for cytokinetic ring assembly. This model yielded mosaic zebrafish hearts with different proportions of polyploid CMs, which were lineage-traced through a Cre-*lox* based system. Following cardiac injury, polyploid CMs contributed to the regenerated tissue much less efficiently than diploid CMs. Moreover, hearts with a high percentage of polyploid myocardium (>50 %) failed to regenerate. Thus, polyploidization interfered with the robust proliferative capacity of the zebrafish heart.

Another recent study has reached similar conclusions by inducing polyploid CMs in zebrafish through administration of thyroid hormone [34••]. This chemical treatment increases the frequency of binucleated CMs by fivefold in the zebrafish heart. After injury, these hearts displayed reduced CM proliferation and blocked regeneration. Thus, polyploid zebrafish CMs tend to lose their proliferative efficiency, similarly to their mammalian counterparts [36]. Interestingly, a possible link between altered thyroid hormone availability and the loss of cardiac regenerative capacity has recently been described in frogs (*Xenopus laevis)* [37]. The underlying causes of regenerative impairment due to polyploidy and thyroid hormone remain to be elucidated.

In addition, research on zebrafish has identified a number of signalling pathways, DNA-binding proteins, epigenetic factors, microRNAs and extracellular matrix proteins required for heart regeneration, as comprehensively reviewed in Ref. [11]. The interruption of these endogenous molecular factors typically impairs CM proliferation and prevents regeneration. However, identifying exogenous stimulators of CM proliferation provides a complementary approach to dissect the mechanisms underlying heart regeneration.

Boosting cardiomyocyte proliferation by vitamin D and preconditioning in zebrafish

Although zebrafish perfectly regenerate their heart after injury, two recent studies have succeeded in further improving this dramatic process by two different approaches, both resulting in an increased CM proliferative activity. In the first study, Han *et al.* demonstrated the pro-mitotic role of the steroid pro-hormone Vitamin D by administration of an exogenous analogue, called alfacalcidol [38•]. This effect was blunted by the inhibition of ErbB2 signalling, a receptor of Neuregulin 1 [39], indicating that Vitamin D partially acts through this cascade. Moreover, blockade of the Vitamin D receptor (VDR) impairs the regenerative process. During ontogenesis, genetically induced activation of VDR signalling in the heart leads to cardiomegaly, a phenotype characterized by an enlarged and thickened myocardial wall. These results highlight the importance of the endocrine system during cardiac regeneration [3,40,41]. In humans, Vitamin D signalling has been reported to protect ischemic myocardium through anti-inflammatory and anti-apoptotic mechanisms [42]. Vitamin D could, thus, have a conserved beneficial pro-regenerative function in vertebrates.

In the second study, our laboratory has shown that the Cilliary Neurotrophic Factor (CNTF) cytokine is a stimulating factor in the context of cardiac preconditioning and regeneration in zebrafish [43]. Preconditioning is a 'resilience' mechanism that is triggered by exposure to low doses of a harmful stimulus in order to enable tissues to better withstand the deleterious effects of more severe subsequent injuries [44]. We induce cardiac preconditioning in adult zebrafish by performing thoracotomy a few days before ventricular injury [45]. This procedure increases cell survival and CM proliferation during the first week of regeneration through upregulation of epicardial factors, such as CNTF [46,47]. Remarkably, a single injection of CNTF into the pericardial cavity is sufficient to trigger CM proliferation in the intact heart [43]. Mutation of the *cntf* gene suppresses the preconditioning effects after thoracotomy. In the regenerating zebrafish heart, delivery of CNTF before ventricular cryoinjury improves the initiation of regeneration via reduced cell apoptosis and boosted CM proliferation. The downstream targets of CNTF include JAK/STAT3 signalling, as the inhibition of this pathway is sufficient to suppress the effects of the cytokine. Interestingly, overexpression of a dominant negative STAT3 has been shown to restrict CM proliferation during regeneration, but not during normal growth in zebrafish [48]. In mammals, exogenous administration of the CNTF protein is known to exert protective effects for neural tissues and to promote myoblast proliferation [49]. The mechanisms of CNTF-based preconditioning are thus potentially evolutionarily conserved.

Taken together, these recent studies suggest the existence of physiological and paracrine mechanisms that can boost the proliferative programs in the zebrafish heart. The identification of additional positive modulators and a deeper comprehension of the cardioprotective machinery will provide important lines of research with the ultimate goal of mimicking such processes in mammals.

Insights from comparative studies of heart regeneration in different fish species

CM proliferation is the key process for heart restoration. In order to understand the molecular causes regulating CM proliferation, a promising approach is to determine the similarities and differences between the injured hearts of regenerative and non-regenerative organisms. An example of such comparisons is the recent creation of a

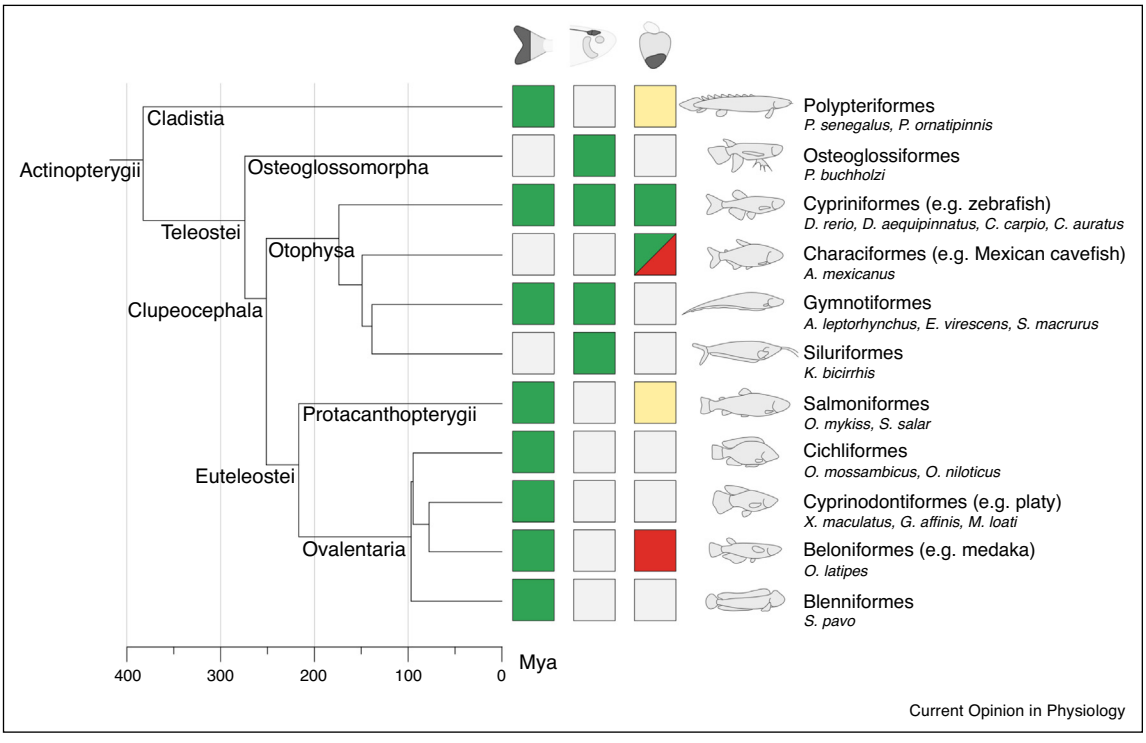
common platform for transcriptomic databases to facilitate cross-experiment analyses [50]. This interspecies approach is elegantly represented by a study in which zebrafish regeneration-specific enhancers are activated in the post-injury tissues of neonatal mice, suggesting a shared potential [51]. However, the important taxonomic distance between zebrafish and mammals might lead to organismal and genomic differences that hinder the identification of factors specifically linked to regeneration.

A number of fish species have been shown to efficiently restore a variety of organs in addition to their heart [52]. Interestingly, while regenerative capacities seem to be highly conserved for some body parts (e.g. fins), others vary between species (e.g. heart, Figure 1). Therefore, bony fishes provide a morphologically coherent and genetically focused context for interspecies research.

One of the first reported interspecies analysis on heart regeneration was performed between zebrafish and medaka (*Oryzias latipes*) [53], two species that separated during the early teleost radiation approximatively 250 million years ago [54] (Figure 1). After cardiac injuries, medaka react with high mortality, excessive fibrosis and health impairments [53,55••]. In addition to these phenotypes, low CM proliferation and a lack of revascularization are observed, indicating an absence of heart regeneration. Interestingly, the regenerative failure was also associated with delayed macrophage recruitment, along with suppressed neutrophil clearance. Using interspecies transcriptomic analyses, Lai and colleagues identified a reduction in the immune response compared to zebrafish [55••]. The authors then confirmed the significance of this difference by improving cardiac regeneration in medaka through polyIC injections. This treatment, which stimulates the Toll-like receptor signaling, results in enhanced CM proliferation, neovascularization and scar resolution. These findings not only suggest a crucial role for the immune system in pro-regenerative programs, but they also demonstrate the value of interspecies analysis to identify modulators of regeneration.

A second and particularly interesting study has recently been elaborated using intraspecies comparisons of the Mexican cavefish (*Astyanax mexicanus*) [56••]. This species, which had a common ancestor with zebrafish approximately 180 million years ago (Figure 1), includes

Figure 1



Phylogeny of selected orders of bony fishes and their reported regenerative capacities. A portion of the phylogenetic tree of Actinopterygii (ray-finned fishes) is depicted and overlaid with relevant taxonomic groups and the corresponding evolutionary time expressed in million years ago (Mya). Orders, which were selected as having at least one species with a reported regenerative capacity, are displayed on the right site of the figure. The first species listed in each order was used to create the schematic drawing of fish. For each order, colour-coded boxes indicate the reported regenerative capacities for the fin, the nervous system and the heart. Colour-code is green for efficient regeneration, yellow for suspected regeneration, red for shown absence of regeneration and grey for absence of published data. Phylogeny is based on Ref. [54], regenerative capacities on Refs. [52,53,56••,59–61,63,64].

two distinct morphotypes. In addition to the typical river *A. mexicanus*, a subpopulation has evolved to inhabit cave ponds where they got trapped following transient floodings between 1 and 3 million years ago [57]. These fish have adapted their behavior, metabolism and morphology to dark environments. Surprisingly, the cave fish have not only lost their eyes and skin pigmentation but also their ability to undergo heart regeneration [56••]. By comparing non-regenerative cave population with the regenerative river population using quantitative trait locus analysis, the authors have identified three loci putatively linked with cardiac healing. One of these genes is *lrrc10*, a leucine-rich repeat protein. However, further analyses are needed to determine the mechanistic causes of the diminished cardiac regeneration. The Mexican cavefish represents a very promising model to study the fast evolutionary loss of heart regeneration capacity and thus a platform to identify central modulators of this process.

Genetic changes associated with adaptation to life in darkness could possibly be linked to the loss of systemic factors required for stimulation of CM proliferation. In particular, it would be interesting to investigate whether cavefish display an insufficiency in Vitamin D, as suggested by the aforementioned work in zebrafish [38•]. Indeed, sunlight exposure is probably required for the synthesis of this hormone in fish, as rainbow trout reared in the dark for two years develop a deficiency in Vitamin D, which can be rescued by exposure to artificial light [58]. Thus, the Mexican cavefish might have evolved metabolic adaptations that are not compatible with this cardiac pro-regenerative pathway. It would be interesting to test whether exogenous administration of Vitamin D would restore heart regeneration in cavefish, and if constant darkness prevents cardiac restoration in zebrafish. More generally, it will be highly relevant for myocardial research to assess and understand the evolution and modulation of the regenerative capacity among fishes (Figure 1).

Currently, heart regeneration has been assessed in a limited number of fish species. In this context, it is important to note that because negative results are notably more difficult to publish, other species of fish might have been sampled but their absence of regeneration never reported. Cypriniformes is the most assessed phylogenetic order with two additional fish species shown to regenerate their hearts after cautery injury, namely the giant danio (*Devario aequipinnatus*) and the goldfish (*Carassius auratus*) [59,60]. Together with the data from the Characiformes *A. mexicanus*, these results could suggest that heart regeneration is conserved in Otophysa fishes (Figure 1). More distantly, CM proliferation has been detected in the Salmoniformes *Salmo salar* and Polypteriformes *Polypterus senegalus*, but assessing the efficiency of the regenerative process as a whole will require further analysis in these species [61,62].

Conversely, it is not yet known if the absence of heart regeneration observed in medaka is conserved beyond the Beloniformes order. To investigate this question, our laboratory is currently studying heart regeneration in a species of the related Cyprinidontiformes platyfish (*Xiphophorus maculatus*) (Figure 1). Together with studies in more distant orders, such as Cichliformes or Blenniformes, our results could help determine whether a lack of heart regeneration is common to Ovalentaria fishes. The examination of representative species of additional phylogenetic groups will illuminate new factors modulating heart regeneration.

Conclusions

Understanding the molecular mechanisms underlying CM proliferation in vertebrates is of major scientific and therapeutic interest. Zebrafish is the leading genetic model organism in this field, and the study of its dramatic regenerative capacities has brought numerous insights into myocardial research. To identify conserved modulators of cardiac regeneration, zebrafish data are typically compared with those of non-regenerative species, and of mammals in particular. However, the distant phylogenetic separation between these two taxa is linked with major physiological and anatomical differences potentially unrelated to heart regeneration. In this context, comparisons between closely related species with varying degrees of regenerative capacity are of particular interest. Recent studies have demonstrated that heart regeneration is variable between fishes from different taxonomic families, as well as between distinct morphotypes. The resulting comparative interspecies and intraspecies analyses suggest that the cardiac restoration is also regulated by currently undetermined organismal factors [26]. Sampling of additional fish species will open new avenues to approach this topic and, in the long term, might bring clues for novel medical strategies.

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Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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