

# Chapter 10

## Cardiac Regeneration in Zebrafish

Chi-Chung Wu and Gilbert Weidinger

**Abstract** Heart failure caused by cardiomyocyte loss after ischemic tissue damage is a leading cause of death worldwide, since adult mammals cannot regenerate heart injuries. While some new cardiomyocytes are produced in adult mammals during normal ageing and after infarction, this occurs at insufficient rates for effective cardiac regeneration. Zebrafish, on the contrary, are able to regenerate multiple organs including the heart. Injuries induce complex cellular and molecular responses in endocardium, epicardium and myocardium, which robustly regenerate in a coordinated manner, resulting in full morphological and functional recovery. In particular, differentiated cardiomyocytes re-enter the cell cycle and proliferate to regenerate the myocardium. Thus, the zebrafish has emerged as an important model to study mechanisms of naturally occurring cardiac regeneration. Here, we describe zebrafish heart injury techniques and review current data on mammalian cardiomyocyte turnover and production in response to injury as well as our current knowledge of the cellular and molecular mechanisms of zebrafish heart regeneration.

**Keywords** Zebrafish • Heart regeneration • Cardiomyocytes • Cell proliferation • Epicardium • Endocardium

### 10.1 Introduction

#### *10.1.1 Cardiomyocytes Are Insufficiently Replaced After Injury of the Adult Mammalian Heart*

Myocardial infarction (MI) is a leading cause of death worldwide. Compared to other organs in mammals (e.g. lung, liver, bone, skin), the adult heart displays very little regenerative capacity in response to injury. In particular, lost adult mammalian cardiomyocytes (CMs) are largely not replaced after MI or other disorders resulting

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C.-C. Wu • G. Weidinger (✉)  
Institute for Biochemistry and Molecular Biology, Ulm University,  
Albert-Einstein-Allee 11, 89081 Ulm, Germany  
e-mail: [gilbert.weidinger@uni-ulm.de](mailto:gilbert.weidinger@uni-ulm.de)

in CM death, leading to muscle loss, irreversible scarring and eventually heart failure (Laflamme and Murry 2011). In contrast, adult zebrafish can completely regenerate their heart via CM replacement, resulting in scar-free recovery (Poss et al. 2002; Schnabel et al. 2011; Gonzalez-Rosa et al. 2011; Chablais et al. 2011; Wang et al. 2011). Intriguingly, neonatal mice can regenerate the myocardium after partial surgical resection as well, but this ability is lost by 7 days after birth (Porrello et al. 2011). While complete functional heart regeneration requires not only restoration of lost muscle tissue, but also coordinated replacement of the other tissues of the heart, in particular of endocardium, epicardium and coronary vasculature, a central question in heart regeneration research has been how CMs can be restored. Genetic lineage-tracing experiments have indicated that during neonatal mouse and adult zebrafish heart regeneration CMs are replaced via proliferation of pre-existing CMs, and not from stem or progenitor cells (Kikuchi et al. 2010; Jopling et al. 2010; Porrello et al. 2011). In adult mammals, CMs have long been believed to be terminally differentiated and thus unable to proliferate (Zebrowski and Engel 2013). Instead, much of the growth of the postnatal mammalian heart occurs via enlargement of pre-existing CMs, i.e. hypertrophy (Li et al. 1996; Yoshizumi et al. 1995). Thus, one explanation for the differential ability of the adult zebrafish and mammalian heart to regenerate would be the idea that CMs retain the ability to re-enter the cell cycle and proliferate in the zebrafish, while such capacity is lost during ontogeny in mammals.

However, there is now good evidence that new CMs do form during adult mammalian life, including humans. Taking advantage of the integration of atmospheric  $^{14}\text{C}$ , which was generated by nuclear bomb tests during the Cold War, into cells born during that time, Frisén and colleagues estimated the age of CMs in humans. They showed that human CMs are being renewed during adult life, but at a very low rate of 1 % per year at the age of 25 and 0.45 % at the age of 75 (Bergmann et al. 2009). Thus, approximately 45 % of all CMs are exchanged during a human lifetime while 55 % remain from neonatal growth (Bergmann et al. 2009).

While it is not possible to identify the cellular source of newly forming CMs in the adult human heart, several elegant studies in mice have addressed whether CM renewal during aging and the low-rate CM replacement after cardiac injury are due to proliferation of existing CMs or due to CM differentiation from progenitor cells (Ali et al. 2014; Ellison et al. 2013; Hsieh et al. 2007; Malliaras et al. 2013; Senyo et al. 2013, 2014; van Berlo et al. 2014). Unfortunately, the conclusions drawn by several studies based on genetic lineage-tracing of CMs or cardiac progenitor cells differ considerably. While most reports using different means to track the fate of differentiated CMs found that renewal of CMs during homeostasis is due to proliferation of existing CMs (Senyo et al. 2014), one study concluded that progenitor cells contribute to CM formation after cardiac injury, since the progeny of differentiated CMs got diluted with other cells in injured hearts (Hsieh et al. 2007). In contrast, other studies using lineage-tracing of differentiated CMs and multi-isotope imaging mass spectrometry concluded that the limited CM replacement after injury is due to CM proliferation (Senyo et al. 2013; Ali et al. 2014). However, c-kit positive cardiac progenitor cells have been reported to contribute significantly to CM

production after cardiac injury as well (Ellison et al. 2013). In contrast, another study found that c-kit positive cells form negligible numbers of CMs both during homeostasis and after injury (van Berlo et al. 2014).

Thus, while further studies are needed to clarify how CMs are formed in the adult mammalian heart, there is a growing consensus that the rate of CM formation under homeostatic conditions and in response to heart injury is low, too low for effective myocardial regeneration after heart injury (Senyo et al. 2014).

### ***10.1.2 Mammalian Cardiomyocyte Proliferation Can Be Experimentally Induced***

Despite the uncertainties about the cellular basis of CM turnover in adult mammals, the above mentioned studies suggest that adult mammalian CMs are able to proliferate and could potentially be augmented therapeutically. In support of this idea, injection of the growth factor Neuregulin 1 (Nrg1) has been shown to induce proliferation of CMs in both healthy and injured adult mouse hearts, to reduce scar size and to improve functional recovery after MI (Bersell et al. 2009). Likewise, induction of constitutively active ERBB2, a tyrosine kinase receptor of Nrg1, in neonatal, juvenile or adult mouse CMs was found to lead to cardiomegaly caused by CM hypertrophy and proliferation, while ERBB2 induction after MI improved functional recovery (D’Uva et al. 2015).

Interestingly, several studies indicated that proliferation might be actively inhibited in adult mammalian CMs. First, overexpression of several miRNAs was found to stimulate both DNA synthesis and cytokinesis in neonatal mouse and rat CMs in culture and in adult animals (Eulalio et al. 2012). Second, deletion of components of the Hippo signaling pathway, which controls organ size and suppresses cell proliferation, has been shown to induce adult CM renewal via cell cycle re-entry and mitosis (Heallen et al. 2013). In addition, removal of Hippo components in P7 mice, a time at which natural heart regeneration is compromised (Porrello et al. 2011), followed by apical resection at P8 resulted in significant muscle regeneration from spared CMs and improved functional recovery (Heallen et al. 2013). Together, these studies suggest the potential to augment cardiac regeneration by stimulating adult mammalian CM proliferation *in vivo* through supplying pro-proliferation factors and/or removing natural inhibitors.

## **10.2 Zebrafish Heart Regeneration – Injury Models**

A promising way to uncover factors that are able to stimulate CM proliferation after heart injury would be to elucidate the molecular basis underlying naturally occurring adult heart regeneration in non-mammalian vertebrates. Cardiac regeneration

after injury has predominantly been studied in zebrafish, but also in salamanders (Oberpriller and Oberpriller 1974; Piatkowski et al. 2013). Interestingly however, it has been reported that heart regeneration does not occur in medaka, a teleost fish model organism that otherwise shares many features with zebrafish (Ito et al. 2014). Thus, it is unclear whether heart regeneration is a common feature amongst teleost fish and other lower vertebrates, and more data will be needed to address the intriguing question of how heart regeneration evolved or whether cardiac regenerative capacity was lost during evolution in certain lineages.

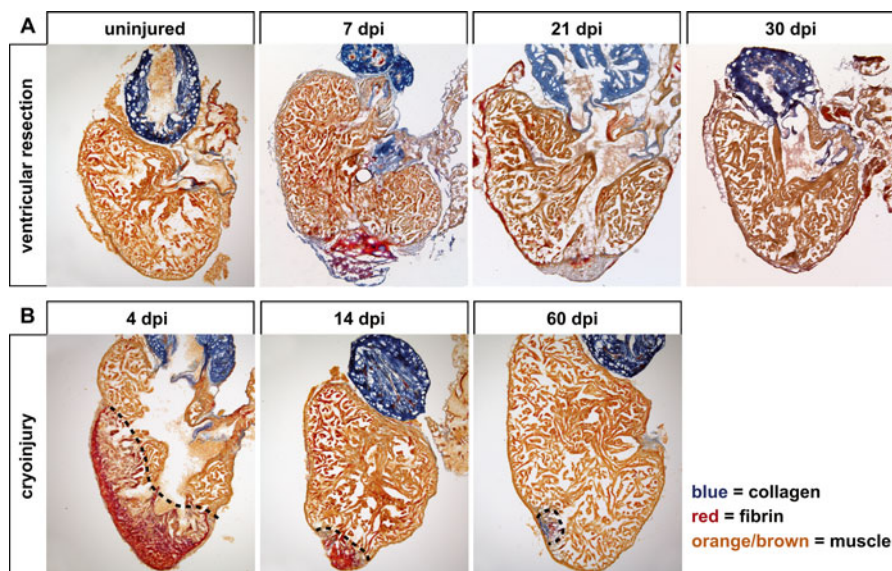
Since the discovery of heart regeneration in zebrafish, this model has developed rapidly over the past years, owing to its robust regenerative capacity and its propensity for genetic manipulation (Gemberling et al. 2013). In this chapter, we summarize the current knowledge about the cellular basis and molecular regulation of zebrafish heart regeneration.

### ***10.2.1 Ventricular Resection***

Zebrafish hearts consist of one atrium, one ventricle and a prominent non-muscular extension of the aorta, termed the bulbus arteriosus. The ability of zebrafish to regenerate the heart was first reported by Keating and colleagues (Poss et al. 2002). After surgical removal of around 20 % of the apex of the ventricle, the injury was found to be rapidly sealed by a fibrin clot. This fibrinous wound tissue increased in size till 7–9 days post injury (dpi). Evidence for CM cell cycle activity was obtained, and a contiguous wall of muscle was restored by 30 dpi (see Sect. 10.3 below). By 60 dpi, the fibrin clot disappeared and the size and shape of the ventricle appeared to be normal, with little or no collagen deposition, indicating that, in contrast to adult mammals, permanent scarring does not occur (Fig. 10.1a) (Poss et al. 2002). To date, ventricular resection remains a frequently used injury model, but several studies have developed additional ways to damage the heart, which better mimic some aspects of mammalian cardiac injury.

### ***10.2.2 Cryoinjury***

Acute loss of myocardial tissue after MI is typically caused by ischemia and subsequent CM death throughout the affected area. After clearance of dead cells and matrix debris from the infarct zone, damaged tissue is replaced by scar tissue characterized by collagen deposition. While zebrafish effectively regenerate the resected heart, the correlation between ventricular resection in zebrafish and MI in mammals is limited. For instance, ventricular resection does not cause massive cell death and thus no cell debris removal before muscle replacement is required. In addition, scar tissue formed after resection mainly comprises fibrin with little or no collagen deposition. Therefore, the value of ventricular resection as a translationally relevant



**Fig. 10.1** Regeneration of ventricular myocardium in the resected and cryoinjured zebrafish heart (a) Acid fuchsin orange G (AFOG) staining on longitudinal sections of uninjured and resected hearts at different times after injury. At 7 days post injury (dpi), the injured area is sealed by a fibrin clot (*red*). The wound tissue progressively reduces in size from 7 to 21 dpi. At 30 dpi, the resected apex is filled with muscle (*orange/brown*) and no obvious wound tissue and collagen deposition (*blue*) is observed, indicative of complete morphological regeneration. (b) Acid fuchsin orange G (AFOG) staining on longitudinal sections of cryoinjured hearts at different time points after injury. At 4 dpi, the injured area is characterized by fibrin deposition (*red*). The wound tissue progressively reduces in size from 4 to 60 dpi where little or no collagen deposition is observed. *Dashed lines*, wound boundary

cardiac injury model has been questioned and cryoinjury has been employed in zebrafish as an alternative to better model certain aspects of MI (Chablais et al. 2011; Gonzalez-Rosa et al. 2011; Schnabel et al. 2011).

In these models, either a small piece of dry ice (Schnabel et al. 2011) or a thin metal filament cooled by liquid nitrogen is applied to the surface of the ventricle (Chablais et al. 2011; Gonzalez-Rosa et al. 2011). This treatment was found to cause extensive cell death and to result in an injury affecting approximately 25 % of the ventricle size. Infiltration of immune cells was observed as early as 1 dpi (Schnabel et al. 2011), while morphological regeneration (muscle restoration and wound tissue resolution) took longer than that after ventricular resection, around 60–130 days. Cellular responses including elevated CM proliferation and reactivation of developmental gene programs in the epicardium and endocardium were found to be largely similar to those seen in the resection model (see Sect. 10.3 below; Gonzalez-Rosa et al. 2011; Schnabel et al. 2011). In contrast to ventricular resection, scar formation with extensive collagen deposition was observed as early as 3 days after cryoinjury (Gonzalez-Rosa et al. 2011). Intriguingly however, the

collagen-scar was progressively resolved and scar-free regeneration occurred (Fig. 10.1b) (Gonzalez-Rosa et al. 2011; Schnabel et al. 2011; Chablais et al. 2011). Transient scar formation has been suggested to be functionally important for regeneration in zebrafish, implying that scarring and regeneration are not necessarily mutually exclusive (Chablais and Jazwinska 2012). However, whether the molecular composition of the transient scar in zebrafish differs from that of permanent mammalian scars is unknown, and the molecular basis underlying scar resolution remains to be elucidated.

### 10.2.3 Genetic Ablation

#### 10.2.3.1 Cardiomyocyte Ablation

In addition to mechanical and cryoinjury, in which several cardiac cell types are affected, myocardial-specific injury has also been achieved. Poss and colleagues generated an ablation system which induces expression of the diphtheria toxin A chain (DTA) in CreERT2-expressing CMs upon 4-hydroxytamoxifen (4-HT)-mediated recombination (Wang et al. 2011). This system caused massive cell death and depleted > 60 % of the ventricular CMs, resulting in lethargy, severe stress hypersensitivity, gasping phenotypes and reduced swimming performance, which are classical signs of heart failure and are not seen in other zebrafish heart injury models. Surprisingly, complete muscle regeneration occurred rapidly within 30 days and full functional recovery was observed at 45 dpi. Such rapid recovery could be explained by the fact that proliferation levels of spared CMs were found to be two to four times higher than those seen in other injury models. Intriguingly, robust cellular responses in the epicardium and the endocardium similar to other injury models were also observed, suggesting that CM cell death is sufficient to initiate an organ-wide regenerative program (Wang et al. 2011).

#### 10.2.3.2 Epicardial Ablation

Poss and colleagues also employed an inducible cell ablation system using *tcf21*-driven expression of bacterial nitroreductase, which converts the drug metronidazole into a cytotoxin, to inducibly kill epicardial cells in the adult zebrafish heart (Wang et al. 2015). While this technique revealed that the epicardium is required for myocardial regeneration (see Sect. 10.4.2), it also demonstrated that the epicardium itself can rapidly and efficiently regenerate from spared epicardial cells. Epicardial regeneration was found to involve directed migration of proliferating epicardial cells from the base of the ventricle towards its apex (Wang et al. 2015). The bulbus arteriosus, which is located at the base of the ventricle, appears to provide signals that direct this migration by repelling epicardial cells, as shown in a series of elegant tissue recombination experiments in cultures of explanted hearts (Wang et al. 2015).

Sonic hedgehog (Shh) is a strong candidate for such a bulbus arteriosus-derived signal, since Hedgehog signaling was shown to be required for epicardial regeneration and Shh-soaked beads could restore epicardial migration in hearts whose bulbus arteriosus had been removed (Wang et al. 2015). It will be interesting to test to which extent Shh-mediated directed cell migration is also involved in epicardial development.

### 10.3 Functional Recovery

Assays for assessing heart function, in particular quantification of cardiac hemodynamics using ultrasound, are difficult to perform in zebrafish due to the small size of the adult heart (Huang et al. 2015; Kang et al. 2015). Nevertheless, there is increasing evidence for functional recovery during zebrafish heart regeneration. Myocardium at the apex of the ventricle, which presumably has formed anew during regeneration in response to ventricular resection, was found to be electrically coupled to the rest of the ventricle, indicating functional recovery (Kikuchi et al. 2010). Likewise, coupling measured by optical mapping and injury-induced lengthening of action potential duration was found to be restored to pre-injury levels 45 days post genetic ablation of CMs (Wang et al. 2011). Electrocardiogram recordings showed prolonged QT intervals after cryoinjury, which recovered by 30 dpi (Chablais et al. 2011). However, another report found that regenerated hearts retained a prolonged QT interval after ventricular resection (Yu et al. 2010). Side-by-side comparison of different injury models as well as measurements of the electrophysiological properties of individual CMs (Nemtsas et al. 2010) will be instrumental in clarifying whether zebrafish can completely recover the electrophysiological properties of ventricular myocardium. Measurements of cardiac performance based on echocardiography indicated that heart function does recover; yet this might take longer than morphological regeneration. One study reported that heart function after cryoinjury recovered only within 180 days (Hein et al. 2015), while another showed that pumping function based on relative fractional volume shortening recovered within 60 days in cryoinjured hearts, while ventricular wall motion remained altered even after 140 days (Gonzalez-Rosa et al. 2014). Overall, these data indicate that zebrafish cannot only regenerate the architecture of the heart but also its function, albeit it remains unclear to what extent.

### 10.4 Regeneration of Non-myocardial Tissues

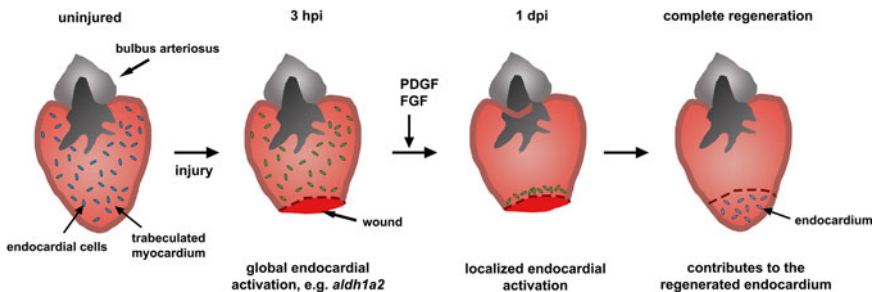
Heart injury in zebrafish triggers regenerative responses in all three primary layers of the heart, namely endocardium, epicardium and myocardium, plus regeneration of coronary vasculature. In this section, we summarize the current knowledge of cellular responses and their molecular regulation in the non-myocardial layers.



### 10.4.1 Endocardium

Endocardial cells were found to be the first to respond to heart injury after ventricular resection (Kikuchi et al. 2011b). In the uninjured ventricle, endocardial cells typically possess elongated nuclei and thin cell bodies that tightly adhere to myofibers, as revealed by transmission electron microscopy (TEM). As early as 1 h post ventricular resection (hpi), endocardial cells both near and distant from the amputation plane appear to round up and to detach from the underlying myofibers (Kikuchi et al. 2011b). Endocardial cells close to the amputation plane were reported to retain a disorganized appearance until 7 dpi, coinciding with rapid CM proliferation. There is also evidence that the endocardium is a source of retinoic acid, which promotes CM proliferation. As early as 3 and 6 hpi, expression of the retinoic acid-synthesizing enzyme *aldh1a2* (*raldh2*) was found to be upregulated in the entire endocardium, representing an intriguing organ-wide response. From 1 dpi onwards, *aldh1a2* expression became localized to endocardial cells at the injury site, where it persisted throughout the early stages of regeneration (until 14 dpi). Intriguingly, endocardial upregulation of *aldh1a2* occurred also in response to spontaneous infarcts observed in explanted adult zebrafish hearts and upon lipopolysaccharide (LPS) injection, which triggers a systemic inflammatory response. These data suggest that endocardial upregulation of *aldh1a2* is a fundamental response to cardiac injury and inflammation (Fig. 10.2) (Kikuchi et al. 2011b). To test the role of RA signaling in heart regeneration, Poss and colleagues inhibited the signal by systemic overexpression of dominant-negative retinoid acid receptors or the RA-degrading Cyp26 enzyme. Both treatments inhibited CM proliferation at 7 dpi, while RA injection was not sufficient to induce CM proliferation. These results suggest that

#### Endocardium



**Fig. 10.2** Regeneration of the endocardium following injury

In the uninjured heart, endocardial cells tightly adhere to myofibers of the trabeculated myocardium. Starting very early after injury, endocardial cells show signs of morphological and transcriptional alterations. In particular, at 3 h post injury (hpi), the retinoic acid-synthesizing enzyme *aldh1a2* is upregulated in endocardial cells in the entire heart, while expression is later restricted to endocardium at the wound border (1 dpi). PDGF and FGF signaling might be required for endocardial regeneration. Lineage tracing indicates that pre-existing endocardial cells contribute to the regenerated endocardium upon complete regeneration.

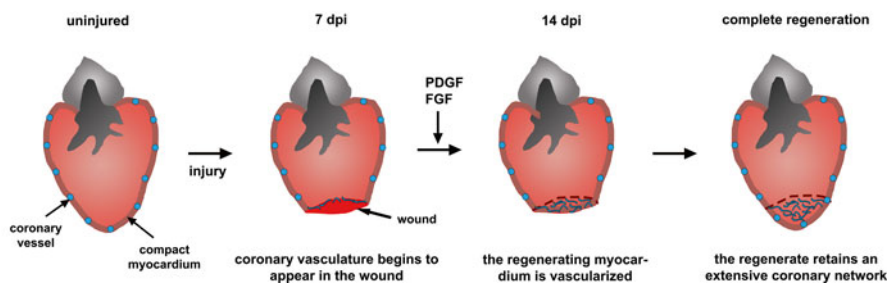


RA is a permissive but not instructive signal for CM proliferation and regeneration (Kikuchi et al. 2011b). Since the epicardium was also found to upregulate *aldh1a2* expression in response to heart injury (albeit later than the endocardium) (Kikuchi et al. 2011b), tissue-specific interference with RA production will be required to clarify whether endocardium or epicardium-derived RA regulates CM proliferation. However, injured mammalian hearts were found to display epicardial, but not endocardial *aldh1a2* expression, suggesting that endocardial RA production supports myocardial regeneration (Kikuchi et al. 2011b). Platelet derived growth factor (PDGF) and Fibroblast growth factor (FGF) signaling appear to be required for revascularization of the wound, and thus likely also for restoration of the endocardial layer.

### 10.4.2 Coronary Vasculature

Vascular supply of the myocardium varies widely among teleost fish, but the compact wall of the zebrafish ventricle is supported by coronary vessels which are located in the subepicardial space (Hu et al. 2001). No molecular marker that is expressed only in the coronary endothelium but not in endocardial cells has been described. Nevertheless, several studies have argued that in addition to endocardial cells, which express a transgenic endothelial marker, *fli:EGFP*, at low levels, a population of cells displaying stronger *fli:EGFP* could be identified in the wound tissue during heart regeneration, and these cells have been addressed as regenerating coronary vessels (Lepilina et al. 2006; Kim et al. 2010; Zhao et al. 2014). Genetic lineage-tracing experiments indicated that the endothelial cells of this presumptive regenerating coronary vasculature are derived from pre-existing endothelial cells, while perivascular cells are derived from the epicardium (Fig. 10.3) (Zhao et al.

#### Coronary vasculature



**Fig. 10.3** Regeneration of coronary vasculature

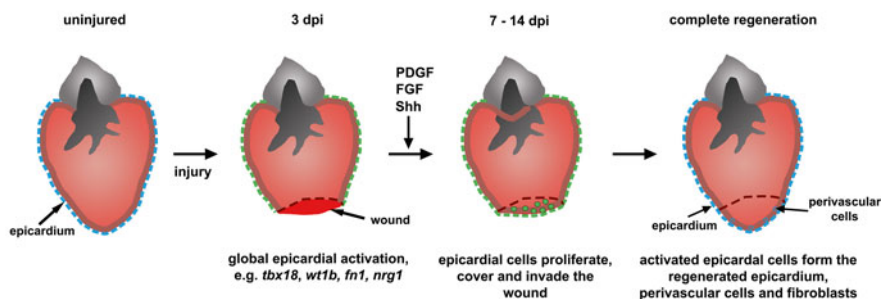
In the uninjured heart, coronary vasculature is observed in the compact layer myocardium. At 7 dpi, coronary vasculature begins to appear in the wound. At 14 dpi, the regenerating myocardium is heavily vascularized, which persists after complete regeneration. Regeneration of the coronary vasculature appears to be regulated by PDGF and FGF signaling.

2014; Kikuchi et al. 2011a). Systemic inhibition of FGF signaling via overexpression of a dominant-negative FGF receptor or pharmacological inhibition of PDGF signaling interfered with re-vascularization of the wound tissue (Lepilina et al. 2006; Kim et al. 2010). While FGF inhibition also resulted in defects in myocardial regeneration (Lepilina et al. 2006), neither FGF nor PDGF signaling are thought to regulate CM proliferation (Lepilina et al. 2006; Kim et al. 2010), indicating that re-vascularization is important for myocardial regeneration.

### 10.4.3 Epicardium

The epicardium, a mesothelial cell layer covering the outer surface of the ventricle, has been found to respond to heart injury by re-expression of genes associated with epicardial development, including *tbx18*, *wt1b* and *aldh1a2* and also by becoming proliferative at 3 dpi (Gonzalez-Rosa et al. 2011; Kikuchi et al. 2011b; Lepilina et al. 2006; Schnabel et al. 2011). By 7 dpi, epicardial cells were reported to enclose the wound and to integrate into the fibrous wound tissue. For instance, a large number of cells retaining the expression of *tbx18* was found in the wound by 14 dpi and in the regenerate at 30 dpi (Lepilina et al. 2006). During embryonic heart development epicardial cells are thought to undergo epithelial-to-mesenchymal transition (EMT) and to invade the subepicardial space and the myocardium to form smooth muscle cells of the coronary vasculature and myocardial fibroblasts (Dettman et al. 1998). In addition, some lineage tracing studies have found evidence for formation of CMs from epicardial cells during development and after heart injury, although the specificity of the Cre lines used for some of these studies has been questioned (Zhou et al. 2008; Cai et al. 2008; Smart et al. 2011; Rudat and Kispert 2012). To address the potential of epicardial cells during zebrafish heart regeneration, Poss and colleagues used inducible Cre lines driven by *tcf21* regulatory sequences and found that epicardial cells give rise to perivascular cells but not CMs after injury (Kikuchi et al. 2011a). This finding was substantiated by Mercader and colleagues using transplantation experiments, which in addition indicated that myofibroblasts are derived from epicardial cells during heart regeneration as well (Gonzalez-Rosa et al. 2012). Thus, during naturally occurring heart regeneration, the epicardium does not adopt CM fate (Fig. 10.4).

During embryonic development, the epicardium is thought to provide signals regulating myocardial development, including CM proliferation (Lavine and Ornitz 2008; Riley 2012). To test the requirement of the epicardium for zebrafish myocardial regeneration, Poss and colleagues ablated epicardial cells using *tc21*-driven expression of nitroreductase (Wang et al. 2015). Epicardial ablation induced early after ventricular resection (from 2 to 5 dpi) reduced CM proliferation at 7 dpi and resulted in defects in myocardial restoration, as evidenced by presence of scar tissue by 30 dpi. Thus, the epicardium is essential for myocardial regeneration in zebrafish. The molecular signals mediating its effects on CMs during regeneration are however unknown.

**Epicardium**

**Fig. 10.4** Regeneration of the epicardium

In the uninjured heart, the epicardium consists of a single layer of cells at the outline of the compact myocardium. At 3 dpi, epicardial activation, which is characterized by upregulation of *tbx18*, *wt1b*, *fn1* and *nrg1*, is observed in the entire heart. From 7 to 14 dpi, epicardial cells cover and invade the wound tissue. Lineage-tracing experiments demonstrate that epicardial cells give rise to the regenerated epicardium, perivascular cells of the coronary vasculature and myofibroblasts. Regeneration of the epicardium appears to be regulated by PDGF, FGF and Shh signaling.

Epicardial EMT during heart regeneration appears to be controlled by FGF and PDGF signaling (Lepilina et al. 2006; Kim et al. 2010). Inhibition of FGF signaling by systemic overexpression of a dominant-negative FGF receptor compromised integration of *tbx18*-positive cells into the regenerating myocardium in addition to its effects on re-vascularization (see Sect. 10.4.2) (Lepilina et al. 2006). Using an adult zebrafish epicardium explant culture, Lien and colleagues showed that PDGF signaling triggers epicardial cells to detach and form mesenchymal-like cell types. PDGF signaling was also required for epicardial cell proliferation both *in vitro* and *in vivo*, and treatment with a PDGF receptor inhibitor reduced the expression of the EMT marker *snail2* in regenerating hearts (Kim et al. 2010).

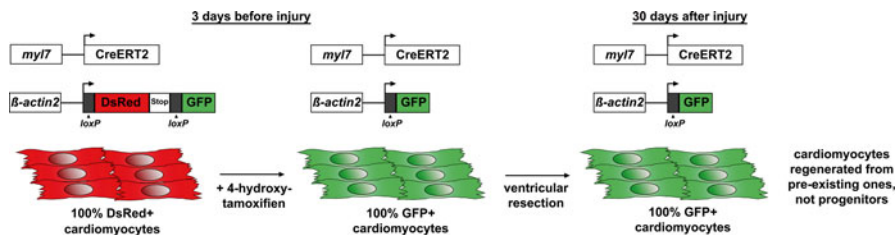
The epicardium also appears to be an important source of extracellular matrix (ECM), which influences the cellular behavior of regenerating CMs. Poss and colleagues showed that expression of fibronectin, a major ECM component, is strongly induced upon heart injury and mainly deposited by injury-activated epicardial cells (Wang et al. 2013). Myocardial regeneration was significantly inhibited in fish overexpressing a dominant-negative human fibronectin fragment as well as in fish homozygous for a *fibronectin1* mutation, resulting in severe fibrosis. Interestingly, such myocardial regeneration defects were not caused by defective CM proliferation. Instead, at 30 dpi, a larger number of CMs was present in the area flanking the injury in homozygous *fibronectin1* mutants, suggesting that fibronectin might be required for CM mobilization and integration into the injury site (Wang et al. 2013). Altogether, these data suggest that the epicardium regulates zebrafish heart regeneration in a number of ways.

## 10.5 Regeneration of the Myocardium

### 10.5.1 Cellular Sources of Regenerating Cardiomyocytes

Central to the ability of zebrafish to recover from heart injuries is the fact that they can replace lost CMs. So far, research into the cellular and molecular mechanisms of heart regeneration thus has largely focused on CM restoration. This is usually studied in the adult heart, where homeostatic myocardial cell cycle activity is very low, which allows for the identification of injury-induced regenerative mechanisms of CM production. Nevertheless, injuries to the rapidly developing embryonic zebrafish heart at stages where CMs are normally produced have also been proposed to represent a useful model of regeneration (Zhang et al. 2013). Using a nitroreductase-based CM-specific ablation system, Chi and colleagues reported that lost embryonic ventricular CMs are rapidly replaced (Zhang et al. 2013). Interestingly, they suggested that this is not only due to elevated proliferation of spared CMs, but also due to transdifferentiation of atrial into ventricular CMs as revealed by genetic lineage-tracing experiments. This process is at least partly mediated by Notch signaling since treatment of ablated hearts with the  $\gamma$ -secretase inhibitor DAPT reduced the appearance of atrially-derived CMs in the injured ventricle. Though intriguing, such atrial contribution to the regenerating ventricle was found to be minimal in adult CM-ablated hearts, suggesting that adult zebrafish rely on other sources of CMs for ventricular regeneration (Zhang et al. 2013).

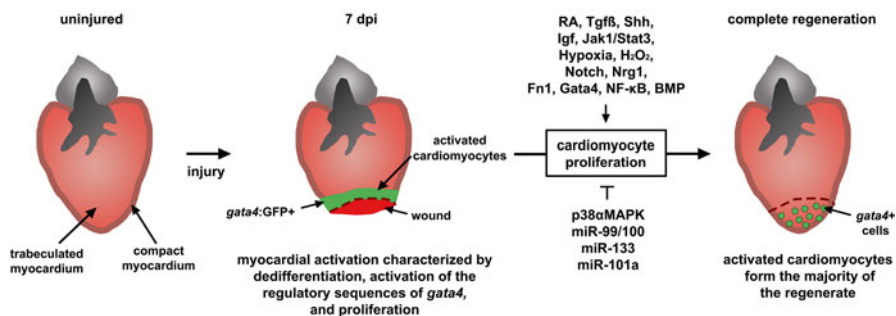
For a period of time, it remained unclear whether the newly forming CMs in adult regenerating hearts are derived from existing differentiated CMs or other sources like stem cells. Initial findings from Poss and colleagues suggested that CMs regenerate from progenitors, but not from differentiated spared CMs (Lepilina et al. 2006). However, subsequent genetic lineage-tracing experiments from two independent studies provided strong evidence for the latter mechanism (Jopling et al. 2010; Kikuchi et al. 2010), indicating that the earlier data which were based on differential maturation and stability of fluorescent proteins were artefacts (Kikuchi et al. 2010). Using an inducible CM-specific Cre line (*myl7:CreERT2*), Poss and colleagues demonstrated that differentiated CMs labeled 5 days before ventricular resection in adult fish contribute to the vast majority of the regenerated myocardium (Fig. 10.5) (Kikuchi et al. 2010). Izpisua-Belmonte and colleagues genetically lineage-traced embryonic CMs and similarly concluded that adult regenerating CMs are derived from cells that once had CM character (Jopling et al. 2010). Thus, the current consensus in the field is that zebrafish myocardial regeneration is achieved via cell cycle re-entry and proliferation of spared differentiated CMs (Figs. 10.6 and 10.7a). Similarly, neonatal mouse heart regeneration is thought to occur via spared CM proliferation as well (Porrello et al. 2011). Whether all CMs can equally contribute to regeneration or whether a particular subset of potentially less differentiated cells has higher regenerative potential is currently unknown. It is also worth noting that quantitative data showing that heart regeneration involves the



**Fig. 10.5** Cardiomyocyte lineage tracing experiments

To test whether differentiated cardiomyocytes are the precursors of regenerating cardiomyocytes, a dual transgenic system has been used. Tamoxifen-inducible CreERT2 recombinase is expressed specifically in differentiated cardiomyocytes under control of the *myl7* promoter. Three days before injury, Cre activity is induced by treating the fish with 4-hydroxytamoxifen. Consequently, CreERT2 removes a floxed DsRed-STOP cassette from a second transgene expressed under control of a  $\beta$ -*actin2* promoter, which is active in cardiomyocytes in the adult heart. Removal of the STOP cassette allows transcription of GFP, thus cardiomyocytes are genetically labeled with GFP. At 30 dpi, all of the regenerated cardiomyocytes are GFP positive, indicating that cardiomyocytes regenerate from pre-existing cardiomyocytes but not from stem or progenitor cells, which are GFP negative.

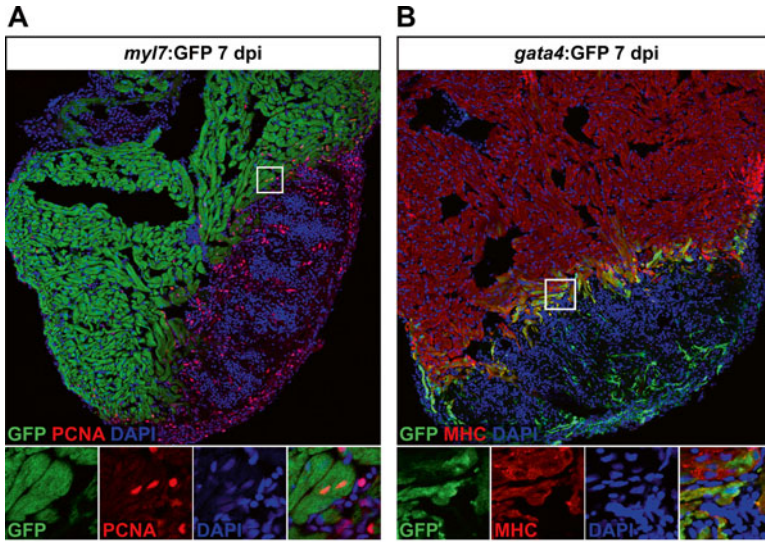
**Myocardium**



**Fig. 10.6** Regeneration of the myocardium

At 7 dpi, cardiomyocytes close to the wound are activated, as indicated by dedifferentiation, activation of the regulatory sequences of *gata4* and proliferation. Cardiomyocyte proliferation is regulated positively by RA, Tgfb, Shh, Igf, Jak1/Stat3, Hypoxia, H<sub>2</sub>O<sub>2</sub>, Notch, Nrg1, Fn1, Gata4, NF- $\kappa$ B and BMP and negatively by p38 $\alpha$ MAPK, miR-99/100, miR-133 and miR-101a. Lineage tracing indicates that regenerated cardiomyocytes are derived from spared, "activated" CMs at the wound border.

full restoration of CM numbers is missing. In addition, whether CM hypertrophy, which is prominent in injured mammalian hearts (Zebrowski and Engel 2013), also occurs in the zebrafish and if so, to which extent injury-induced CM cell cycle activity could be due to CM polyploidization has not been explored.



**Fig. 10.7** Cellular responses of cardiomyocytes to injury

(a) At 7 dpi, cardiomyocytes (marked by *myl7*:GFP) at the wound border re-enter the cell cycle and express the cell-cycle marker PCNA.

(b) At 7 dpi, cardiomyocytes (marked by myosin heavy chain (MHC) expression, red) at the wound border activate the regulatory sequence of *gata4* in *gata4*:GFP transgenic fish.

### 10.5.2 Cardiomyocyte Dedifferentiation

Cardiomyocytes do not only re-enter the cell cycle, but also appear to dedifferentiate during regeneration, which is both suggested by reported loss of characteristics of the differentiated state and by upregulation of embryonic genes. After injury, CMs close to the wound were found to be characterized by reduced expression of GFP driven by the *myl7* promoter, which is active in differentiated CMs, by partial loss of sarcomeric structures revealed by transmission electron microscopy and by alpha-actinin disorganization (Jopling et al. 2010; Kikuchi et al. 2010; Sallin et al. 2015; Schnabel et al. 2011; Wang et al. 2011; Wu et al. 2016). These changes, which are suggestive of CM dedifferentiation in response to injury, were found to be accompanied by reactivated expression of genes that are highly expressed in embryonic hearts, including *nppa*, *nppb*, embryonic myosin chains, and likely also cardiogenic transcription factors like *gata4* (Kikuchi et al. 2010; Sallin et al. 2015; Wang et al. 2013; Wu et al. 2016). Poss and colleagues found that a transgenic line driving GFP from regulatory sequences of *gata4*, a cardiac transcription factor expressed during embryonic heart development, is re-expressed in a subpopulation of CMs in the compact layer myocardium near the wound from three to seven dpi in injured hearts (Fig. 10.7b). By 14 dpi, GFP-positive cells were found in many cells around and within the injury site. A sub-set of these *gata4*:GFP-expressing CMs expressed markers of cell cycle activity, and their descendants, genetically marked by



inducible *gata4:CreERT2* from five to seven dpi, contributed significantly to the regenerated myocardium (Kikuchi et al. 2010). While strong evidence for the re-expression of endogenous *gata4* or other cardiogenic factors in CMs in injured hearts is still missing, overexpression of a dominant-negative form of Gata4 reduced CM proliferation in the compact layer myocardium, compromised myocardial regeneration at 30 dpi and resulted in extensive scarring (Gupta et al. 2013). Thus, reactivation of *gata4* in the compact layer myocardium appears to be crucial for successful heart regeneration (Gupta et al. 2013).

Izpisua-Belmonte and colleagues also suggested that dedifferentiation is of functional importance for CMs to proliferate since no mitotic CMs displayed highly organized sarcomeric structures (Jopling et al. 2010). However, mammalian CMs also reactivate expression of embryonic genes and show partial loss of sarcomeric structure after heart injury (D’Uva et al. 2015; Kubin et al. 2011). Therefore, CM dedifferentiation could be a shared injury response between the non-regenerating mammalian and the regenerating zebrafish heart (Szibor et al. 2014). Dedifferentiation thus might represent a prerequisite for proliferation, but likely is not sufficient or causal for CM proliferation, considering that mammalian CMs do not proliferate in response to heart injury. More detailed systematic analyses of molecular changes in CMs induced by injury in both models and the identification of experimental interventions that specifically block dedifferentiation will be essential to understand the functional role of CM dedifferentiation during heart regeneration.

Migration of CMs into the injured area, regulated by the Cxcl12-Cxcr4 system, has also been suggested to be an important event during regeneration (Itou et al. 2012). Pharmacological inhibition of Cxcr4 reduced the number of CMs in the injury site at 14 dpi and compromised myocardial regeneration at 60 dpi without affecting CM proliferation. While these data have been interpreted as indicative of the importance of CM migration during regeneration, more direct evidence for active CM migration is lacking.

### ***10.5.3 Molecular Regulation of Cardiomyocyte Regeneration***

Several studies have identified factors and signaling pathways regulating CM proliferation during zebrafish heart regeneration. Obvious candidates for such factors are those that also regulate embryonic CM proliferation during heart development. To screen for molecular regulators of embryonic CM proliferation, Poss and colleagues employed the fluorescent ubiquitination-based cell cycle indicator (FUCCI) system, which comprises two fusion proteins, mCherry-zCdt1 and Venus-hGeminin, which are expressed cyclically in the G1 and S/G2/M phases of the cell cycle, respectively (Choi et al. 2013). By combining transgenic expression of these proteins specifically in CMs (*myl7:FUCCI*) with a small-scale chemical screen targeting common developmental signaling pathways, Hedgehog (Hh), Insulin-like growth factor (Igf), and Transforming growth factor  $\beta$  (Tgf $\beta$ ) signaling were identified as positive regulators of CM proliferation during development (Choi et al. 2013).



### 10.5.3.1 Hedgehog Signaling

Hh signaling has been implicated in driving cardiac specification in the zebrafish embryo and reduction of Hh signaling causes cardiac morphogenesis defects in mice (Washington Smoak et al. 2005). Treatment of *myl7*:FUCCI embryos with the Smoothened agonist SAG, which activates Hh signaling, increased CM proliferation by 60 % as well as CM numbers by 10 % (Choi et al. 2013). On the other hand, treatment with cyclopamine (a Smoothened antagonist) reduced CM proliferation and numbers by 27 % and 19 %, respectively. These data suggest that Hh signaling is required for embryonic CM proliferation.

Interestingly, Hh signaling was also activated in CMs in the adult zebrafish heart 7 days after ventricular resection, as indicated by the expression of the Hh target gene *ptch2* in a transgenic reporter line (Choi et al. 2013). Treatment with cyclopamine 6 days after ventricular resection or genetic CM ablation reduced expression of the cell cycle maker PCNA in CMs. SAG treatment, on the other hand, increased CM cell cycle activity remarkably by 65 % after ventricular resection while it had no effect on adult uninjured zebrafish hearts. Together, these data suggest that zebrafish CM proliferation requires Hh signaling, which on its own is not sufficient to trigger regenerative proliferation (Choi et al. 2013). Hh ligands might be provided by the epicardium, which was reported to express a transgenic sonic hedgehog reporter line (Choi et al. 2013). Whether Hh signaling acts directly on CMs and is indeed activated by epicardium-derived ligands will have to be clarified using tissue-specific experimental manipulations.

Hh signaling was also found to be activated in the mouse myocardium after myocardial infarction (Kusano et al. 2005). Injection of Sonic Hedgehog (Shh) plasmid into infarcted hearts was sufficient to promote neovascularization, to protect CMs from apoptosis, to reduce post-MI remodeling and to improve functional recovery (Kusano et al. 2005). Therefore, although the beneficiary effects of Hh signaling on cardiac repair are conserved between zebrafish and mammals, the mode of actions seems to be different. It would be interesting to characterize the interaction partners and downstream targets of Hh signaling in both models to understand the molecular basis behind these differential responses to the activation of the same signaling pathway.

### 10.5.3.2 Insulin-Like Growth Factor Signaling

Igf signaling has been described to have positive effects on CM proliferation during mouse development (Li et al. 2011). In *myl7*:FUCCI zebrafish embryos, treatment with the Igf receptor antagonist NVP AEW541 reduced CM proliferation and number by 33 % and 14 %, respectively (Choi et al. 2013). This proliferation defect was verified by Lien and colleagues in an independent study (Huang et al. 2013). On the other hand, treatment with an Igf signaling agonist increased embryonic CM proliferation and number by 41 % and 17 %, respectively (Choi et al. 2013). These data suggest that Igf signaling is required for CM proliferation in the developing

mammalian and zebrafish heart. Components of the Igf signaling pathway were also found to be upregulated in the adult zebrafish heart in response to injury. RT-PCR analysis showed that expression of the ligand *igf2b* is upregulated by three dpi and peaks at seven dpi, in a temporal profile similar to that of CM proliferation, which peaks between seven and 14 dpi. *igf2b* expression appeared to be restricted to endocardial cells in the injury site as detected by *in situ* hybridization while Igf receptor 1 was expressed in CMs close to the amputation plane (Choi et al. 2013; Huang et al. 2013). Systemic Igf signaling inhibition in adult fish either by treatment with the NVP AEW541 antagonist or by overexpression of a dominant-negative form of Igf receptor 1 (*hspl70:dnigf1ra*) reduced PCNA expression in CMs after injury. Lien and colleagues found that activation of *gata4:EGFP* in a subset of CMs at the wound border was not affected by Igf receptor antagonist treatment, while Igf signaling inhibition blocked proliferation of this population and appeared to reduce their accumulation in the injured area by 14 dpi (Huang et al. 2013). Prolonged treatment with antagonist until 30 dpi inhibited regeneration, resulting in excessive fibrin and collagen deposition (Huang et al. 2013). On the other hand, overactivation of Igf signaling was sufficient to increase CM proliferation by 65 % and 36 % after ventricular resection and genetic CM ablation, respectively (Choi et al. 2013; Huang et al. 2013). Together, these data suggest that endocardium-derived Igf ligands regulate regenerative CM proliferation and successful regeneration. Again, whether proliferation is cell-autonomously regulated by Igf signaling activation in CMs and whether it is indeed activated by endocardially produced ligands will have to be clarified using tissue-specific manipulations.

### 10.5.3.3 Transforming Growth Factor $\beta$ Signaling

Treatment of *myl7:FUCCI* embryos with the Tgfb receptor inhibitor SB431542 reduced CM proliferation and number by 30 %, suggesting that Tgfb signaling promotes CM proliferation during development (Choi et al. 2013). The role of Tgfb signaling during zebrafish adult heart regeneration was further studied by Chablais and Jaźwińska (Chablais and Jazwinska 2012). Following cryoinjury, expression of three Tgfb isoforms (*tgfb1*, *tgfb2* and *tgfb3*) was upregulated in the wound at four and 14 dpi as detected by *in situ* hybridization. These Tgfb isoforms appeared to be expressed in several cell types including fibroblasts, epithelial cells of the wound, as well as some CMs at the wound border. Tgfb receptors, on the other hand, appeared to be expressed in the wound (both *alk4* and *alk5a*) and in the entire heart possibly in Vimentin-positive fibroblasts (*alk5a*), suggesting that both CMs and non-CMs can be responsive to Tgfb ligands. This idea was verified by immunostaining against phosphorylated Smad3, a readout for active Tgfb signaling, which was expressed in a large number of cells in the wound and in a sub-set of CMs at the wound border (Chablais and Jazwinska 2012). To understand the functional role of Tgfb signaling, cryoinjured fish were treated with the Tgfb receptor antagonist SB431542 and analyzed by histological staining at different time points. At four dpi, in both control and treated fish, fibrin deposition was evident in the wound. Interestingly, at 14 dpi,

while scar tissue containing fibrin and collagen was evident in control hearts (Chablais et al. 2011; Gonzalez-Rosa et al. 2011), Tgf $\beta$  signaling-inhibited fish failed to deposit collagen in the wound (Chablais and Jazwinska 2012). At 30 dpi, while little scar tissue could be detected in control hearts, implying that the fibrin and collagen depositions present at 14 dpi had been resolved and replaced by new muscle, Tgf $\beta$  inhibitor-treated fish still contained fibrin-rich wound tissue, which showed signs of mechanical deformation (Chablais and Jazwinska 2012). These data suggest an unexpected positive correlation between transient collagen deposition and successful heart regeneration in zebrafish. Based on drug-shift experiments, Chablais and Jazwińska suggested that Tgf $\beta$  signaling-mediated transient collagen-rich scar formation is required to stabilize the injured myocardium (Chablais and Jazwinska 2012). In addition to collagen deposition, Tgf $\beta$  also appears to be required for the production of other ECM proteins including the tissue remodeling protein Tenascin C and Fibronectin, which is crucial for zebrafish heart regeneration (Chablais and Jazwinska 2012; Wang et al. 2013). Lastly, consistent with its role during embryonic development, Tgf $\beta$  signaling inhibition reduced CM proliferation detected by either PCNA expression or BrdU incorporation (Chablais and Jazwinska 2012; Choi et al. 2013). Altogether, these studies suggest that Tgf $\beta$  signaling is required for both CM proliferation and collagen deposition in the wound after injury. Chablais and Jazwińska's study is the first to provide evidence for the functional importance of transient scarring during zebrafish heart regeneration (Chablais et al. 2011; Gonzalez-Rosa et al. 2011). This observation challenges the prevalent concept in the heart regeneration field that scarring and regeneration is mutually exclusive, and also opens up a new area of research into the molecular mechanisms of transient scar resolution. Similar to the pathways discussed above, future studies using cell-type specific manipulations will be necessary to clarify whether the effects of Tgf $\beta$  signaling on CM proliferation are direct.

#### 10.5.3.4 Jak1/Stat3 Signaling

Several studies have performed transcriptional profiling of regenerating zebrafish hearts using whole ventricle samples (Lien et al. 2006; Sleep et al. 2010). Poss and colleagues performed profiling of RNAs that are specifically translated in zebrafish CMs using translating ribosome affinity purification (TRAP) technology (Fang et al. 2013). To do this, an EGFP reporter gene was fused to the N-terminus of the ribosomal protein L10a, whose expression was driven specifically in CMs under the regulatory sequence of *myl7* (*myl7*:TRAP). Translating mRNAs were isolated by immunoprecipitation with an antibody against EGFP, which were then processed for microarray analysis. Among the differentially regulated mRNAs, several members of the Janus kinases1/Signal transducer and activator of transcription 3 (Jak1/Stat3) pathway were found to be upregulated in 1 dpi samples, including *interleukin 6 signal transducer (il6st)*, *jak1*, *stat3* and the Jak1/Stat3 target gene *suppressor of cytokine signaling 3b (socs3b)* (Fang et al. 2013). *In situ* hybridization showed that these Jak1/Stat3 pathway genes are induced in an organ-wide manner at 1 dpi but

later expression appeared to localize to CMs at the amputation plane. To investigate the role of Jak1/Stat3 signaling during heart regeneration, Poss and colleagues used a Cre/lox-based system for inducible overexpression of a dominant-negative Stat3 (dnStat3) in CMs upon 4-hydroxytamoxifen induction, which was able to reduce expression of the target gene *socs3b* by 80 % following injury. Continuous dnStat3 expression from prior to heart injury compromised myocardial regeneration by 30 dpi and resulted in extensive scarring compared to control fish. In line with this, CM proliferation was also strongly reduced (~80 %) in dnStat3-expressing fish (Fang et al. 2013). Expression of *relaxin 3a* (*rln3a*), which codes for a peptide hormone, was found to be directly regulated by Stat3 in the regenerating heart. Daily retro-orbital injection of Rln3a protein in dnStat3-expressing fish partially rescued CM proliferation defects at seven dpi, suggesting that the effect of Jak1/Stat3 signaling on heart regeneration is at least partly mediated by Rln3a (Fang et al. 2013).

In contrast to the signaling pathways described above, the requirement of Jak1/Stat3 signaling for CM proliferation appears to be regeneration-specific. Larvae subjected to dnStat3 overexpression from 4 days post fertilization survived to adulthood, suggesting that Jak1/Stat3 signaling is not required for heart development (Fang et al. 2013). Furthermore, CM proliferation in juvenile and young adult zebrafish stimulated by low density growing conditions (Wills et al. 2008) was not affected by dnStat3 expression (Fang et al. 2013). Altogether, these data identify Jak1/Stat3 signaling as an injury-induced pathway that directly regulates regenerative CM proliferation.

### 10.5.3.5 Notch Signaling

Treatment of *myl7:FUCCI* embryos with a Notch signaling inhibitor ( $\gamma$ -secretase inhibitor) had no effect on CM proliferation, suggesting that Notch signaling is dispensable for CM proliferation in the embryonic heart (Choi et al. 2013). In contrast, Burns and colleagues found that Notch signaling is required for adult CM regeneration (Zhao et al. 2014). In adult zebrafish, three out of four Notch receptors (*notch1a*, *notch1b* and *notch2*) were upregulated in both endocardial and epicardial cells but not in CMs 7 days after ventricular resection as revealed by *in situ* hybridization (Zhao et al. 2014). To understand the role of Notch signaling during regeneration, Burns and colleagues inhibited Notch signaling by ubiquitously overexpressing a dominant negative isoform of the murine mastermind-like protein (dnMAML), which cannot recruit essential cofactors to the Notch transcriptional complex, rendering the complex inert. Continuous systemic Notch inhibition compromised myocardial regeneration in the resected ventricular apex by 30 dpi and resulted in extensive scarring with collagen deposition, suggesting that Notch signaling is required for heart regeneration (Zhao et al. 2014). Although Notch receptors were predominantly upregulated in endocardial and epicardial cells after injury, Notch signaling inhibition did not interfere with the early injury responses reported previously in both cell types (Kikuchi et al. 2011b; Lepilina et al. 2006), namely the formation of epicardium-derived cells and activation of *aldh1a2* expression in both

epicardium and endocardium at seven dpi (Zhao et al. 2014). Interestingly, while myocardial regeneration was compromised in Notch-inhibited hearts at 30 dpi, coronary endothelium regeneration as detected by *kdrl:mCherry* transgenics was not inhibited, indicating that Notch signaling is dispensable for neovascularization after injury. Cardiomyocyte proliferation, on the other hand, was found to be regulated by Notch signaling since continuous overexpression of dnMAML from one to seven dpi significantly reduced PCNA expression in CMs by 53 %. Intriguingly, Notch hyperactivation by systemic overexpression of the intracellular domain of the Notch receptor (NICD) also significantly reduced CM proliferation at seven dpi as well as myocardial regeneration at 30 dpi, suggesting that the right balance of Notch signaling activity is essential for successful heart regeneration (Zhao et al. 2014).

### 10.5.3.6 Neuregulin1 Signaling

As discussed above, while a number of pathways/factors have been identified to regulate CM regeneration, none of them are able to stimulate CM hyperplasia in the absence of injury. In contrast, a recent study by Poss and colleagues showed that Neuregulin1 (Nrg1) is a potent injury-induced mitogen for zebrafish heart regeneration and that its activation in the healthy adult heart is sufficient to induce rapid regenerative programs, resulting in excessive myocardial hyperplasia and cardiomegaly (Gemberling et al. 2015). Neuregulin1 is an extracellular factor of the EGF family that might act as a transmembrane protein, but can also give rise to secreted peptides due to ectodomain shedding (Mei and Nave 2014).

After genetic ablation of CMs, *nrg1* expression was rapidly induced in the heart at three and seven dpi, coinciding with induction of CM proliferation. *nrg1* was predominantly expressed in perivascular regions in the ventricular wall after genetic ablation and in the region surrounding the injury site after ventricular resection, as revealed by *in situ* hybridization. Co-localization studies with transgenic reporter lines marking different cardiac cell types showed that the dominant source of *nrg1* in the ventricular wall is *tcf21*-expressing epicardial cells. After ventricular resection, treatment with a small molecule inhibitor of ErbB receptors, which are required for Nrg1 signaling, reduced CM proliferation at seven dpi by 54 %. On the other hand, inducible overexpression of Nrg1 specifically in CMs using the Cre-Lox system increased proliferation at seven dpi by 84 %. These data suggest that Nrg1 is a potent mitogenic factor for CMs after injury (Gemberling et al. 2015).

Using the same CM-specific overexpression system, Poss and colleagues tested the effect of Nrg1 on the uninjured adult heart. Nrg1 overexpression for 7 days significantly induced CM proliferation, resulting in a remarkable thickening of the ventricular wall by 460 % after 30 days of overexpression (Gemberling et al. 2015). These changes are likely the result of CM hyperplasia instead of hypertrophy since CM cell size was not affected by continuous Nrg1 overexpression. However, prolonged Nrg1-induced hyperplasia compromised cardiac function as revealed by echocardiography and a swimming test which is an assay for cardiac function that is sensitive for heart injury and failure (Wang et al. 2011).

Nrg1 overexpression in the uninjured heart was sufficient to elicit responses similar to those seen during heart regeneration. During heart development, cortical muscle in the ventricular wall was reported to be typically formed from a small number of CMs, which expand laterally on the ventricular surface to form large clones, as revealed by CM clonal analysis (Gupta and Poss 2012). In contrast, after heart injury, CM regeneration appears to occur through proliferation near the injury site, which results in a number of small CM clones in the regenerated ventricular wall (Gupta et al. 2013). Overexpression of Nrg1 in juvenile fish 5 weeks post fertilization (wpf), a time at which cortical muscle first appears, resulted in ectopic ventricular wall thickening by 10 wpf and clonal analysis showed obvious clone mixing, which is more reminiscent of injury-induced regeneration (Gemberling et al. 2015). Furthermore, Nrg1 overexpression also activated the regulatory sequence of *gata4*, as well as the expression of *tgfb2*, *aldh1a2* and *fibronectin 1* which have all been functionally implicated in zebrafish heart regeneration (Gupta et al. 2013; Kikuchi et al. 2010, 2011b; Chablais and Jazwinska 2012; Wang et al. 2013). Finally, CM dedifferentiation, epicardial activation and myocardial vascularization were also rapidly induced by Nrg1 overexpression in uninjured hearts (Gemberling et al. 2015). Together, these data suggest that Nrg1 signaling is a potent inducer of regenerative programs that involve different cardiac cell types. Such a mitogenic and pro-regenerative role of Nrg1 on CMs appears to be conserved between zebrafish and mammals since both injection of Nrg1 or expression of a constitutively active ErbB2 has been reported to induce CM proliferation both *in vitro* and *in vivo* and to improve functional recovery after MI (Bersell et al. 2009; D’Uva et al. 2015).

### 10.5.3.7 Bone Morphogenetic Protein signaling

To identify regulators of zebrafish CM regeneration, Weidinger, Bakkers and colleagues employed Tomo-Seq, a recently developed method providing genome-wide transcriptional expression data with spatial resolution (Wu et al. 2016). This resulted in the identification of several hundred genes whose expression is enriched at the wound border, where CMs re-enter the cell cycle. Ligands, receptors and target genes of Bone Morphogenetic Protein (BMP) signaling were found to be expressed at the wound border, and nuclear accumulation of phosphorylated Smad1/5/8, a readout of active BMP signaling, was detected in dedifferentiating CMs at the wound border. Systemic interference with BMP signaling showed that the pathway is required for CM proliferation and muscle regeneration, while overexpression of the ligand Bmp2b was sufficient to augment CM proliferation and to speed up muscle regeneration (Wu et al. 2016). Weidinger, Bakkers and colleagues found that BMP signaling is not required for physiological CM proliferation in juvenile fish, indicating that it represents an injury-specific regulator of CM regeneration. In part, this might be due to BMP signaling being required for CM dedifferentiation (Wu et al. 2016). Interestingly, BMP signaling has also been reported to be upregulated in injured mammalian hearts, but there it appears to

play the opposite role than in zebrafish. Interference with BMP signaling protects mammalian CMs from apoptosis and improves the outcome after MI (Pachori et al. 2010). These results indicate that the differential ability of zebrafish and mammalian hearts to regenerate depends in part on opposing responses of CMs to BMP signaling.

### 10.5.3.8 NF- $\kappa$ B signaling

NF- $\kappa$ B signaling has also been identified as important regulator of zebrafish heart regeneration by Poss and colleagues (Karra et al. 2015). Using a transgenic reporter line, NF- $\kappa$ B activity was detected in CMs adjacent to the amputation plane throughout the first two weeks after injury. Inhibition of NF- $\kappa$ B activity specifically in CMs inhibited CM proliferation and myocardial regeneration at 7 and 30 dpi, respectively. In addition, NF- $\kappa$ B transcriptional complexes also appear to contribute to CM dedifferentiation by directly activating transcription of *gata4*. Interestingly, myocardial inhibition of NF- $\kappa$ B activity also resulted in defects in infiltration of epicardial cells into the wound at 14 dpi, suggesting a potential cross-talk of CMs and epicardial cells during regeneration (Karra et al. 2015).

### 10.5.3.9 p38 $\alpha$ MAPK

The activity of p38 $\alpha$  MAPK is inversely correlated with CM proliferation in mammals; inhibition of p38 $\alpha$  MAPK induces proliferation of cultured neonatal and adult CMs, which are normally non-proliferative (Engel et al. 2005). Moreover, co-treatment of adult rats with FGF1 and p38 $\alpha$  MAPK inhibitor after MI reduced scarring and markedly improved functional recovery (Engel et al. 2006). A study from Izpisua-Belmonte and colleagues revealed a similar role of p38 $\alpha$  MAPK on CM proliferation in zebrafish (Jopling et al. 2012b). To manipulate p38 $\alpha$  MAPK activity, Izpisua-Belmonte and colleagues targeted expression of either a dominant negative form of p38 $\alpha$  MAPK (dnp38 $\alpha$  MAPK) or a constitutive active (ca) MKK6, an upstream activator of p38 $\alpha$  MAPK, to CreERT2-expressing CMs upon 4-hydroxytamoxifen (4-HT)-mediated induction (Jopling et al. 2012b). While p38 $\alpha$  MAPK inhibition had no effect on myocardial regeneration after ventricular resection, caMKK6 overexpression significantly inhibited myocardial regeneration at 30 dpi. Moreover, mosaic expression of caMKK6 using a partially silenced CM-specific Cre line showed that caMKK6-expressing CMs were unable to incorporate BrdU, suggesting that p38 $\alpha$  MAPK activity inhibits CM proliferation and regeneration (Jopling et al. 2012b). These results suggest that p38 $\alpha$  MAPK activity in adult zebrafish CMs must be low for heart regeneration to occur.



### 10.5.3.10 Hypoxia

Hypoxia-inducible factors (HIFs) are the direct effectors of the hypoxic response. In mammals, it has been reported that HIF1 $\alpha$  overexpression in the myocardium reduces infarct size and improves functional recovery after MI (Kido et al. 2005). Izpisua-Belmonte and colleagues reported that at 7 days post ventricular resection in zebrafish, the wound tissue and a subset of CMs close to the amputation plane showed hypoxia induction as evidenced by Hypoxyprobe staining (Jopling et al. 2012a). Cardiomyocyte BrdU incorporation and regeneration in adult zebrafish preconditioned with phenylhydrazine, an anemia/hypoxia-inducing drug, was enhanced, while zebrafish overexpressing a dominant-negative form of HIF1 $\alpha$  in CMs showed inhibition of these processes. Together, these data suggest that hypoxia is required for myocardial regeneration in zebrafish (Jopling et al. 2012a). In cultured zebrafish CMs hypoxic treatment appeared to increase the number of mitotic and dedifferentiated CMs (Jopling et al. 2012a). Whether or not these effects could also be observed *in vivo* is, however, unclear.

### 10.5.3.11 Hydrogen Peroxide

Reactive oxygen species (ROS), despite their toxic potential, are crucial for regeneration of the tadpole tail in *Xenopus* and of the caudal fin in zebrafish (Gauron et al. 2013; Love et al. 2013). A study by Xiong and colleagues revealed a positive role of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in zebrafish heart regeneration as well (Han et al. 2014). After ventricular resection, *duox*, a member of the NADPH-oxidase and related dual oxidase family of proteins, which is responsible for the formation of superoxide anions and subsequently H<sub>2</sub>O<sub>2</sub>, was upregulated in epicardial cells at the site of injury from three to 14 dpi (Han et al. 2014). In line with the temporal expression profile of *duox*, the level of H<sub>2</sub>O<sub>2</sub> in CMs was also found to be elevated between three and 14 dpi, as detected by expression of Hyper, a fluorescent protein-based H<sub>2</sub>O<sub>2</sub> sensor (Belousov et al. 2006), in CMs in *myl7:Hyper* transgenics. Pharmacological inhibition of Duox activity by diphenyleiiodonium (DPI) or apocynin reduced H<sub>2</sub>O<sub>2</sub> production and CM proliferation at seven dpi. Prolonged treatment with DPI also compromised myocardial regeneration and resulted in scarring. These data suggest that H<sub>2</sub>O<sub>2</sub> is required for CM proliferation and regeneration (Han et al. 2014).

Intriguingly, H<sub>2</sub>O<sub>2</sub> was found to regulate CM proliferation and regeneration through its interaction with Erk1/2 signaling and its feedback regulator Dusp6 (Han et al. 2014). Dusp6 expression is stimulated by phosphorylated Erk1/2 (pErk1/2) but reciprocally deactivates pErk1/2 by dephosphorylation, hence forming a negative feedback loop that limits Erk1/2 activity (Nichols et al. 2000). H<sub>2</sub>O<sub>2</sub>, on the other hand, directly oxidizes Dusp6, which renders the protein prone for degradation, and hence leads to Erk1/2 overactivation (Chan et al. 2008). In zebrafish, both

phosphorylation of Erk1/2 and *dusp6* expression were found to be activated after ventricular resection (Han et al. 2014). DPI treatment in regenerating hearts induced Dusp6 stability and therefore reduced phosphorylation of Erk1/2. The effect of DPI on CM proliferation could be counteracted by co-treatment with BCI, a small molecule that inhibits phosphatase activity of Dusp6. In addition, systemic overexpression of Dusp6 in *hspl70:dusp6* transgenics reduced pErk1/2 expression, and compromised CM proliferation, *gata4* activation and myocardial regeneration. These data strongly suggest that endogenous H<sub>2</sub>O<sub>2</sub> regulates CM proliferation and regeneration by promoting Erk1/2 signaling through repressing Dusp6 activity (Han et al. 2014).

### 10.5.3.12 miRNAs

miRNAs have been implicated in various responses to MI in mammals, including cell death, proliferation, and metabolism (reviewed in Wang and Martin 2014). In zebrafish, several studies have demonstrated the importance of regulation of miRNAs for successful heart regeneration (Yin et al. 2012; Aguirre et al. 2014).

Using microarrays and real-time quantitative PCR (qPCR), Poss and colleagues found that a number of miRNAs are differentially regulated 7 days after ventricular resection, a large number of which have been shown to be modulated in injured mammalian heart (Yin et al. 2012). One of the identified miRNAs was the CM-specific miR-133, which had been found to negatively regulate zebrafish fin regeneration (Yin et al. 2008). *In situ* hybridization and qPCR analyses demonstrated that miR-133 levels decrease by seven and 14 dpi compared to uninjured hearts, and later return to baseline level at the completion of heart regeneration by 30 and 60 dpi, suggesting that downregulation of miR-133 levels could be important for heart regeneration (Yin et al. 2012). Indeed, loss- and gain-of-function experiments demonstrated that miR-133 negatively regulates CM proliferation and regeneration. Systemic inducible overexpression of the miR-133 precursor sequence increased the levels of mature miR-133 and reduced CM proliferation and regeneration at seven and 30 dpi, respectively (Yin et al. 2012). On the other hand, inducible overexpression of a miR-133 sponge RNA, which contained triplicate perfect binding sites for miR-133, reduced the levels of miR-133 and induced CM proliferation at seven and 30 dpi. Poss and colleagues further identified the gap junctional protein *connexin-43* (*cx43*) and the mitotic check point kinase *mip1* as targets of miR-133. While *Mip1* mutants showed heart regeneration defects (Poss et al. 2002), treatment with carbenoxolone (CBX), an inhibitor of Cx43, reduced CM proliferation at seven dpi after ventricular resection (Yin et al. 2012). Together, these data demonstrate the importance of downregulation of miR-133 levels during heart regeneration, whose effect is mediated through, at least partly, *Mip1* and Cx43 (Yin et al. 2012).

Yin and colleagues recently also described that downregulation of miR-101a after zebrafish heart injury is essential for CM proliferation (Beauchemin et al. 2015). In addition, this miR also seems to regulate transient scar removal. While its expression is downregulated shortly after injury, expression was found to actually

increase above levels in uninjured hearts between seven and 14 dpi. Depletion of miR-101a during this time using transgenic overexpression of a sponge construct resulted in increased scarring by 30 dpi (Beauchemin et al. 2015). Thus, miR-101a appears to play a dual role during heart regeneration, where its downregulation early allows CM proliferation, while increased levels later are required for scar removal.

The miRNAs miR-99/100 and *Let-7a/c* were also identified as important regulators of zebrafish heart regeneration and differential regulation of their expression was suggested as part of the reason why zebrafish can but adult mammals cannot regenerate their heart. Izpisua-Belmonte and colleagues showed that expression of these miRNAs is downregulated during zebrafish heart regeneration (Aguirre et al. 2014). Injection of miR-99/100 mimics into regenerating hearts reduced CM proliferation and compromised myocardial regeneration, which was suggested to be mediated through their targets beta subunit of farnesyl-transferase (Fnt) and SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily a, member 5 (Smarca5). Interestingly, downregulation of miR-99/100 and *Let-7a/c* and subsequent upregulation of Fnt and Smarca5 was not observed in mouse hearts after MI (Aguirre et al. 2014). Blockage of both miRNAs in mouse after MI resulted in CM dedifferentiation and improved functional recovery (Aguirre et al. 2014). These data suggest that downregulation of miR-99/100 and *Let-7a/c* is an essential regenerative response, which is conserved but dormant in the mammalian heart. This study demonstrates the importance of zebrafish as a model for deciphering molecular mechanisms underlying natural heart regeneration which could be applied to heal the non-regenerating mammalian heart.

## 10.6 Conclusions

The zebrafish has rapidly developed over the last decade as a model for studying cardiac regeneration. Recent discoveries have greatly improved our understanding of how natural heart regeneration is regulated. After injury, developmental gene programs are activated globally in the endocardium and epicardium, which proliferate and contribute to the regenerated endocardial (including new vasculature) and epicardial cells in a lineage-restricted manner, respectively. Activation of these two layers precedes that of CMs and is likely to provide endocardial scaffold and epicardial covering of the wound to support muscle regeneration. In addition, these two layers also act as a source of pro-regenerative factors which affect CM proliferation and regeneration in a paracrine manner, likely including ligands of FGF, RA, Igf, Tgf $\beta$  and Notch signaling, and Nrg1.

In response to injury, CMs have been shown to partially dedifferentiate, re-enter the cell cycle and proliferate to regenerate the lost muscle. Several signaling pathways have been identified as being required for regenerative CM proliferation, most of which appear to be also involved in regulating CM proliferation during embryonic development (RA, Hh, Igf, Tgf $\beta$ ), while injury-specific regulators have been identified as well (Jak1/Stat3, H<sub>2</sub>O<sub>2</sub>, Notch, BMP). Since most pathways have been

studied using systemic interference, the strongest evidence for a direct role on CMs exists for Jak1/Stat3 and BMP signaling; cell type-specific pathway manipulation will be required in the future to clarify cellular interactions during heart regeneration. Currently, heart regeneration studies in adult zebrafish are also limited by the lack of inducible genetic loss-of-function systems. Hopefully, in the near future, genome-editing tools like CRISPR/Cas9 will enable zebrafish researchers to induce tissue-specific genetic deletion of genes to further dissect the requirement of various factors or signaling pathway components during heart regeneration. Together with rapidly developing transcriptomic, proteomic and epigenomic tools, future studies promise to provide insights to important outstanding questions such as: (1) Why do zebrafish but not mammalian CMs retain the ability to proliferate throughout life? (2) How are regenerative responses initiated in the zebrafish heart? (3) What are the underlying mechanisms of scar removal in zebrafish? We believe the zebrafish will continue to thrive as an important model to understand natural heart regeneration, which might provide valuable insights to refine strategies for improving mammalian cardiac repair.

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