

# Molecular and cellular mechanisms of heart regeneration in zebrafish

Eduard Sleep Ronquillo

---

TESI DOCTORAL UPF / 2010

DIRECTOR DE LA TESI

Dr. Ángel Raya Chamorro (Institut de Bioenginyeria de  
Catalunya)



## Aknowledgements

This section is going to be long, I warn.

There is no other way to start this section than to thank the most important person in the development of this thesis' work, that is, Ángel Raya. He has been my thesis director for a bit more than four years now and I have learnt a lot from him, both actively by discussing results or data and passively by just watching him work. He is not one of the most privileged minds I have met so far in the scientific field but THE most. He translates his vast scientific knowledge, his capacity to ask the appropriate questions and his talent on resolving any kind of problems into everyday productivity. Not only at the scientific but also at the personal level he has been a constant help and time schedules have never been a problem for him. He has motivated me to widen my scientific vision and gave me the possibility to work on ample experimental models and techniques. I would write several other sentences about how beneficial has been his influence in my still short scientific career but he would probably suggest me to cut this off so I will move on.

I also want to thank very effusively Chris Jopling. The work presented here couldn't be achieved without his expertise and resolute mind. Even now that we don't work in the same place he happily offers himself to solve any problem I may have and answers every annoying call from my part.

Ignasi Rodríguez is the name of my inseparable scientific college, flat mate, and friend. We started to work in the same place at the same

time and we are both going to defend our respective thesis with only a five-day delay between one and the other. He is an especial case of a cultivated mind with high experimental skills and extreme kindness. He would do some of my work during the weekend or any other day if it were necessary. He also pushed me to follow correct directions and helped me in many other cases that are too numerous to be described or remembered.

Some of my best colleagues in the lab are Adriana Sánchez and Yvonne Richaud and I have to thank every minute I spent with them. Adriana is probably the person in the lab with whom I used and use to talk more to. We would go for a ten-minute coffee to explain our victories and failures of the day and go back to work fully charged from the positive energy she irradiates. Yvonne is probably the person who competes more fiercely with Ignasi in the aspect of kindness. If she can help in any way she doesn't say she will, she does it. I want to thank her for what I learnt from her in the cell culture room and in front of the computer while Photoshop was open, for her sense of humor and artistic skills.

I want to thank Senda and Alberto and all the other members of our new lab for helping me so much in many aspects in the laboratory and for the splendid after-hours "discussions" we spent holding a beer, in the case of Alberto just literally.

This splendid "discussions" occurred mostly when we were still placed next to the sea, where more than 30 researchers and scientific personnel composed the laboratory. I want to thank all the people

with whom I established contact with in the CMRB, especially at Bitacora's. They are all fantastic people and I miss them a lot.

Also especial thanks to the “histology” girls, Lola, Cristina 1 & 2 and Esther, for their cheerful personality, all them led by Mercè Martí, a wonderful person who was instrumental to the development of many aspects of my work.

Other people in the CMRB include Marina Raya and Stéphanie Boué, from whom I learnt several crucial things. The list of people I want to thank passes through Joaquín, Bea, Vaquero, Meritxell, Montse, Vlad and it follows for several more.

Thanks to Alex, Raquel, Erika, Borja, Cristina, Alba, Marko, Rudy and Clara for being PhD or Master students, and thus share many good moments with me in our lineage-restricted “sopars becaris”.

Thanks to Tat for her late but not less significant contribution to my thesis and personal life.

Finally, thanks to my family. Not because they knew much about what I was doing, ...well, mum did know something more..., but for their unconditional support all over these years and for growing me up as who I am.



## Abstract

In contrast to mammals, zebrafish do have the ability to regenerate their heart after injury. A better understanding of how regeneration-competent species do so should help developing strategies to enhance human cardiac regeneration. Here, by genetic lineage-tracing using an inducible Cre/lox system, we show that newly formed cardiomyocytes arise from the proliferation of differentiated heart muscle cells. These results argue against a significant contribution of stem or progenitor cells in this process. Our microarray and electron microscopy data provide evidence that cardiomyocyte proliferation is accomplished by limited cardiomyocyte dedifferentiation and increased expression of cell cycle regulators. One of these genes, *polo-like kinase 1* (*plk1*), is upregulated in the regenerating area of the zebrafish heart and, by specifically inhibiting plk1 activity, we show that it is essential for regeneration. We have also identified a series of additional transcripts differentially expressed during zebrafish heart regeneration that warrant further research. The data presented here offer new insights to understanding heart regeneration in zebrafish and should provide useful information for cardiac repair in humans.

## Resum

De manera oposada als mamífers, els peixos zebra sí tenen la capacitat de regenerar el cor després d'una lesió. Entenent millor com s'ho fan les espècies capaces de regenerar hauria d'ajudar-nos a desenvolupar estratègies per a augmentar la capacitat de regeneració en humans. Aquí, mitjançant un sistema Cre/lox de traçat genètic de llinatge, mostrem que la creació de nous cardiomiòcits prové de la proliferació de cèl·lules cardíques diferenciades. Aquests resultats discrepen amb una contribució significativa de cèl·lules mare o progenitores en aquest procés. Les dades obtingudes de *microarray* i de microscòpia electrònica evidencien que la proliferació de cardiomiòcits és deguda a una dediferenciació parcial i a un increment de l'expressió de gens que promouen el cicle cel·lular. Un d'aquests, el *polo-like kinase 1 (plk1)*, augmenta d'expressió a l'àrea regenerant del cor de peix zebra i, un cop inhibida la seva activitat, mostrem que és essencial per a la regeneració. També hem identificat una sèrie adicional de trànscrips que s'expressen de manera diferencial durant la regeneració cardíaca en el peix zebra i que mereixen més investigació. Els resultats aquí presents profunditzen en la comprensió de la regeneració cardíaca en el peix zebra i ofereixen informació rellevant per la teràpia cardíaca en humans.



## **Preface**

In 2010, ending a decade of extensive stem cell biology research, few people seem to be aware of how the regeneration field revolutionized the minds of those who lived more than two centuries ago. Some years before the onset of the French Revolution, thousands of snails were decapitated by naturalists and others to find out whether or not it was true that they would then equip themselves with new heads. This extraordinary phenomenon raised metaphysical questions among the philosophers of that time such as where was the residence of the soul.

Biologically, the observation that selected animals could regenerate many of their body parts opened a door to the thinking that one day, men would master the process of regeneration for their own benefit.

The present work results from the above mentioned, the need to master the understanding of how certain species deploy regenerative mechanisms in selected organs or structures to be able to recapitulate these mechanisms for human regeneration therapies.

Relatively recently, the amount of work produced in the stem cell field and the hope that it offered to regenerative medicine, has increased exponentially. However, when comparing different types of regeneration in vertebrates, one gets to the conclusion that, as a general rule, the deployment of the stem cell capacities are not the preferred mechanism for regeneration. Instead, it is generally more accepted to be relying on something called cellular plasticity, which involves the capacity of differentiated cells to undergo a series of phenotypical and molecular changes that allow them to restore a lost

population of cells and addressing the question of the cellular origin of regeneration can be technically challenging.

Here, we used some of the most modern techniques available for zebrafish to characterize molecularly the zebrafish heart regeneration response and have adapted one of the most relevant techniques used in mice to assess its cellular origin, that is, inducible genetic lineage-tracing.

Our results show for the first time, unambiguously, what is the cellular origin of zebrafish heart regeneration. Such knowledge, combined with data on the molecular events that take place in the zebrafish heart during regeneration, might have a substantial impact on how clinical strategies will be addressed to treat human cardiac disease.

# Index

	Page
Abstract .....	vii
Preface.....	ix
List of Figures.....	xii
1. INTRODUCTION.....	1
1.1 Regeneration.....	1
a) Origins .....	1
b) Mammals .....	2
1.2 Regeneration in vertebrates .....	4
a) Lessons from Amphibia .....	6
b) Heart regeneration in urodeles.....	9
1.3 The zebrafish as a model system.....	11
1.4 The zebrafish heart.....	16
1.5 Heart regeneration in zebrafish.....	18
a) Cellular bases .....	19
b) Molecular bases and non-myocardial cellular contribution .....	21
2. OBJECTIVES.....	29
3. RESULTS .....	31
3.1 Paper 1 .....	32
3.2 Paper 2 .....	53
4. DISCUSSION.....	67
4.1 Cellular bases of heart regeneration in zebrafish.....	67
4.2 Molecular mechanisms .....	71
4.3 Contribution to regenerative medicine .....	74
4.4 Open questions .....	78
5. CONCLUSIONS .....	81
References .....	83

## List of figures

	Page
Fig. 1. Epimorphic regeneration in the newt limb.....	5
Fig. 2. Establishment of signalling centers and initiation of outgrowth.....	15
Fig. 3. The adult zebrafish heart.....	17
Fig. 4. Morphology of the uninjured and amputated zebrafish hearts.....	18
Fig. 5. Model of the double developmental timer assay.....	22
Fig. 6. Gene expression profile of regenerating zebrafish heart.....	26
Fig. 7. Cardiomyocyte differentiation status and gene expression signatures during development and regeneration.....	79

# 1. INTRODUCTION

## 1.1 Regeneration

### a) Origins

Humans have long been fascinated about how other animals can regenerate some of their body parts after loss, injury or amputation. Indeed, studies on regeneration flourished as early as in the 18th century (Dinsmore, 1991), setting the origin of developmental biology as a research discipline (Okada, 1996). Réamur (1683-1757) and Trembley (1710-1784) initiated the first descriptions of regeneration in invertebrates such as crayfish and hydra, respectively. Soon after, Spallanzani (1729-1799) and others extended these studies to amphibian species, which became the preferred animal model for many years for the study of what was later defined as epimorphic regeneration (Morgan, 1901).

Regeneration is the process by which a lost body part is restored (Bely & Nyberg, 2010) and in the adult, it can be regarded as a striking example of postembryonic morphogenesis (Brookes & Kumar, 2008). Regeneration itself is a fascinating process; it involves the recognition of tissue loss or injury, followed by mechanisms that reconstruct the relevant structure. Mastering the knowledge of how such mechanisms are initiated, regulated, and finalized is expected to have a tremendous impact for regenerative medicine in humans.

Many invertebrate species have the capacity to regenerate entire body parts or even to generate two individuals as a result of amputations at any axis in their body plan. The most common examples are Hydra (cnidarian), Dugesia (planarian worm), Nereis (annelid worm) and Linckia (echinoderm) (Brockes, 1997). Regeneration research on invertebrate species has provided valuable insights into the biological bases of this process, and is still a very fertile and productive field of research, with Hydra and planarian (*Schmidtea mediterranea* in particular) being the most popular models (Cebria, 2007; Handberg-Thorsager et al., 2008; Pellettieri & Sanchez Alvarado, 2007; Salo & Baguna, 2002; Salo, 2006; Salo et al., 2009; Sanchez Alvarado, 2006). In spite of the conservation of some molecular mechanisms, the cellular bases by which invertebrates regenerate diverge from those used by vertebrates. For this reason, we will focus this introduction into the mechanisms of vertebrate regeneration, or the lack thereof.

## b) Mammals

Mammalian species have the capacity to replace lost cells from different tissues by several mechanisms. Cell duplication of beta cells (Dor et al., 2004) and hepatocytes (Michalopoulos & DeFrances, 1997), or stem cell proliferation followed by subsequent differentiation to replace intestinal epithelium, skin or blood cells, among others (Wagers & Weissman, 2004), are the two main mechanisms. Additionally, dedifferentiation and/or transdifferentiation capacity of some cell types has also been reported (Tosh & Slack, 2002). However, this type of cell renewal is mostly considered as tissue restoration and it should not be confused with epimorphic

regeneration (Raya et al., 2004; Tsonis, 2000). The mechanisms governing tissue restoration are maintained throughout life as cells from different tissues are continuously replaced. After minor damage, the same mechanisms used to establish tissue homeostasis are exacerbated in order to accelerate restoration. Such mechanisms, however, cannot compensate tissue loss after major injury and, in any case, do not provide the bases for rebuilding a complex structure. As a result, adult mammalian species have little or no regenerative capacity and they are not able to replace a damaged or missing structure.

Arguably the most striking regeneration event that occurs in mammals, and possibly the only exception to the rule discussed above, is that of digit tip regeneration first described in fetal and newborn mice (Borgens, 1982) and also reported in other species including humans (Illingworth, 1974).

Other vertebrate species, though, do have remarkable regenerative capacity. Whether regeneration represents an evolutionary adaptation acquired by regeneration-competent species, or a common ancestral trait lost during evolution of regeneration-incompetent species is still subject to hot debate (Brockes et al., 2001; Mescher & Neff, 2005; Tanaka & Ferretti, 2009; Bely & Nyberg, 2010). To address this issue, it will be important to establish molecular and cellular assays that can be comparatively applied to different representative taxons of evolution, taking into account their ability to regenerate.

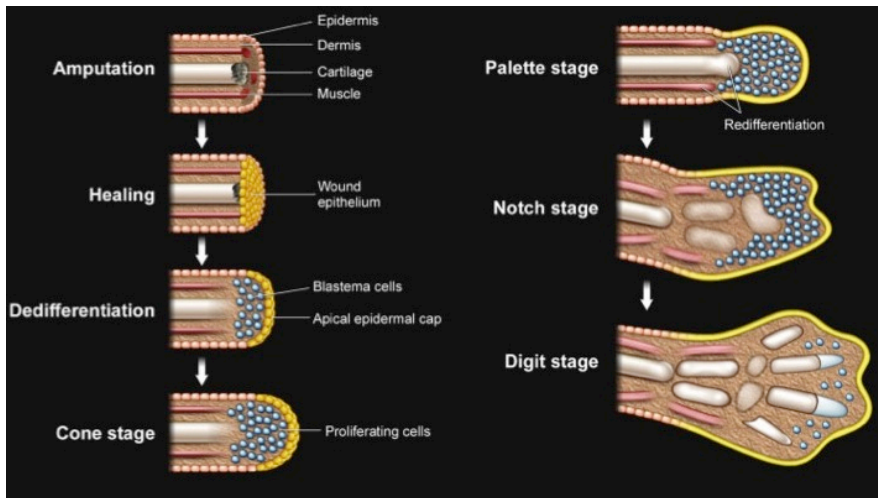
## 1.2 Regeneration in vertebrates

As for the purpose of epimorphic regeneration studies, some urodele amphibians (or salamanders) have been for long time the animal models by excellence (Odelberg, 2005; Tsonis, 2000). There are three families of salamanders that are commonly used to study regeneration: Salamandridae (known as newts), Ambystomatidae (known as mole salamanders), and Plethodontidae (known as lungless salamanders). They have been used to address many questions regarding regeneration in vertebrates, especially on limb regeneration, an aspect in which they are unique as adult vertebrates (Brockes, 1997). More recently, the zebrafish (a teleost fish) has also emerged as an ideal model system to study regeneration due to several reasons but mostly because of its suitability to be subject to the array of modern molecular tools available (see below, Point 1.3 The zebrafish as a model system).

The above-mentioned urodele amphibians and teleost fish, with some species-specific restrictions, can regenerate many complex structures and organs including their limbs/fins, tail, jaws, spinal cord, retinas, lenses, optic nerves, intestine, lateral line and heart. There are some differences on how regeneration is accomplished in different structures/organs and species (Tsonis, 2000), but there are also remarkable commonalities. For example, after limb or fin amputation the sequence of events that takes place (Fig. 1) is very similar, namely: 1) clot formation followed by a rapid migration of adjacent epidermal cells to form the wound epithelium or apical epithelial cap (AEC), 2) formation of a blastema –a mass of undifferentiated cells with high



proliferative capacity- underneath the wound epithelium, and 3) proliferation of blastema cells followed by their differentiation and pattern formation (Brockes, 1997; Odelberg, 2005; Tsonis, 2000).



**Figure 1. Epimorphic regeneration in the newt limb.** A diagram shows sequential steps of the regeneration process. Amputation of the limb leads to disruption of the epidermis, dermis, muscle and cartilage, and exposure to the outer environment. Epidermal cells then migrate to cover the injury site creating the wound epithelium, which delivers signals to the underlying mesenchyme. Cells from the wound epithelium start to proliferate creating what is known as the apical epidermal cap. At the same time cells in the mesenchyme start to dedifferentiate and become blastema cells. Proliferation of blastema cells is followed by a redifferentiation process to substitute for the lost elements after amputation. At the end of the regeneration process blastema cells stop proliferating and pattern is reestablished. From Stewart et al. (2007).

## a) Lessons from Amphibians

As these animals have the capacity to regenerate many of their body parts, the scientific literature is rich with fascinating data on the environmental, cellular and molecular mechanisms of regeneration obtained from amphibians.

Experiments performed on different tissues and cultured cells evidenced the differential dependence on local environmental cues and on intrinsic factors among regeneration-competent and non regeneration-competent species. Insights have been gained in two general aspects of regeneration: the formation of the wound epithelium and the early regeneration signals, and the cellular contribution to the blastema.

Wound epithelium and early signals. As mentioned above, the formation of an epithelial sheet covering the wound after injury in the limb is the first step of regeneration. It results from the migration of epithelial cells and is accomplished as soon as 2h post-amputation (Carlson et al., 1998). The role of the wound epithelium is crucial as it is absolutely required for regeneration to occur (Mescher, 1976). Indeed, it has been proposed that mammals' inability to regenerate might be due to the deployment of a dermal healing response, rather than a wound healing response (Goss, 1980). The signals that control or trigger the formation of the wound epithelium are not completely understood but it has been proposed that bioelectric currents could be one of these early signals (Nuccitelli, 2003). Once primed to respond, epithelial cells upregulate matrix degradation enzymes as early as 2h after amputation, thereby inhibiting basement membrane formation

between the wound epidermis and the underlying mesenchyme, thus allowing the direct interaction with one another (Yang et al., 1999). Once the epithelial cap is formed, it sends an array of signals to the mesenchyme underneath (reviewed in Campbell & Crews, 2008). This array of signals, together with other nerve-dependent signals (Kumar et al., 2007b; Singer, 1952) and the positional disparity of cells within the dermis (Gardiner et al., 1986; Kumar et al., 2007a) will instruct the blastema to be formed, grow and acquire pattern.

However, the formation of the wound epithelium is not necessary in the case of lens regeneration. Here, the first step of regeneration relies on a restricted systemic response, known as the anterior chamber-associated immune deviation (ACAID) (Streilein, 2003). It involves the trafficking of stimulated dendritic cells from the anterior chamber to the marginal zone of the spleen and the return of immune effector cells to the eye. If this path is blocked, regeneration of the lens cannot occur. Thus, regeneration is primed differently according to differential environmental conditions.

Cellular contribution to blastemas. The origin of the seemingly identical cells that form the mass of the blastema has been for long time an unanswered question, especially in the case of the limb, and specifically the muscle (Carlson, 2003). Early histological and electron microscopy studies observations described a process of dedifferentiation characterized by myonuclei breaking off from the damaged muscle fibers and migrating distally to reach the regeneration blastema (Carlson, 2003). However, opponents to this view considered that myonuclei in differentiated muscle fibers were incapable of re-entering mitosis. After satellite cells were discovered in frogs (Mauro

& Adams, 1961) and subsequently in other amphibian species (including the axolotl and the newt, in this case termed) postsatellite cells), they were found to contribute to the restoration of muscle (Carlson, 1970b). However, in the amphibian limb, epimorphic regeneration overrides tissue restoration (Carlson, 1970a). Dedifferentiation of myotubes and formation of mononucleated cells in the regeneration blastema was ultimately accepted in light of experiments using cell-labeling techniques and transplantation (Kintner & Brockes, 1984; Kumar et al., 2000; Lo et al., 1993). The contribution of each cell type in the regenerating limb has been recently addressed in the axolotl by using transgenic animals ubiquitously expressing EGFP and transplantation of adult or embryonic tissues to label specific cell types (Kragl et al., 2009). In this way, it was shown that each cell type conserved a memory of their tissue origin. Muscle gave rise to muscle, epidermis led to epidermis, dermis could make also tendons and cartilage, Schwann cells led to the same progeny and cartilage gave rise to tendons, perichondrium and perhaps dermis. Thus, the limb blastema cannot be considered as a homogeneous pool of cells, but rather as a heterogeneous population of cells that maintain memory of their tissue of origin and positional identity.

In the case of lens regeneration, pigmented epithelial cells (PECs) from the dorsal iris transdifferentiate to give rise to new lens cells (Reyer, 1954; Yamada, 1977).

## b) Heart regeneration in urodeles

The ability of newt adult cardiomyocytes to enter mitosis was first described by John Oberpriller and Jean C. Oberpriller (Oberpriller & Oberpriller, 1971). They amputated a portion of the newt's heart and allowed it to regenerate from 16 to 22 days. Taking advantage of the capacity of electron microscopy to readily detect the ultrastructural morphology of cardiomyocytes and mitotic chromosomes, they could establish a direct relationship between both. The observed mitotic cardiomyocytes were placed next to the wounded area of regenerating hearts but were not seen in uninjured hearts. These dividing cardiomyocytes retained some characteristics such as an associated basal lamina and glycogen granules within them. It was also noted that the bundles of myofilaments in mitotic cardiomyocytes sometimes had a disordered arrangement and they postulated that this was a physical requirement for the subsequent process of cytokinesis.

Not until 3 years later was the term "regeneration" used to describe what occurred to the salamander heart after injury (Becker et al., 1974). The same year, Oberpriller and Oberpriller extended their initial studies on the characteristics of mitotic cardiomyocytes by describing the full sequence of events after injury in the newt heart. After amputation, there was a formation of a blood clot, coagulation necrosis, macrophagic activity, regenerative activity of heart muscle, and connective tissue formation. Thus, newt hearts were capable of remarkable regeneration but failed to achieve it completely (Oberpriller & Oberpriller, 1974).

During the following years, electron microscopy and autoradiography were the two main techniques to characterize the cell type and DNA synthesis after delivery of tritiated thymidine in the injured heart. Both techniques were applied in a different experimental setting, that is, after grafting minced cardiac muscle to the injured heart to increase the reactive area of the wound (Bader & Oberpriller, 1978; Bader & Oberpriller, 1979), or in the different heart chambers after injury to the ventricle or the atrium (McDonnell & Oberpriller, 1983, 1984). These studies showed that the atrium of the newt heart is also capable of regeneration but that there is no increase in atrial DNA synthesis after ventricular injury. It was also confirmed the description of myofibrillar structure breakdown in tritium-labeled myocytes, which showed scattered myofilaments and no Z-bands in late stages of mitosis. During the course of regeneration different stages of myofibrillogenesis were observed and, by the end of regeneration, myocytes had numerous well-organized myofibrillae and intercellular junctions phenocopying those of uninjured cardiomyocytes.

Primary culture of newt cardiomyocytes was later established (Tate et al., 1987), and this allowed a number of different experiments to be performed, as these cardiomyocytes underwent DNA synthesis and mitosis under culture conditions (Tate & Oberpriller, 1989; Tate et al., 1989). It was shown that cardiomyocytes in culture responded positively with DNA synthesis to PDGF, aFGF, bFGF and O-tetradecanoylphorbol-13-acetate, and negatively to heparin and TGFbeta (Soonpaa et al., 1992, 1994). Time-lapse on phase-contrast light microscopy (Matz et al., 1998) and analysis of BrdU incorporation and phosphorylated-histone 3 staining (Bettencourt-

Dias et al., 2003) showed the heterogeneous capacity of mono and binucleated cardiomyocytes in terms of proliferation and ploidy of their progeny. Newt cardiomyocytes are about 98-100% mononucleated (Bettencourt-Dias et al., 2003; Tate & Oberpriller, 1989) and they predominantly gave rise to mononucleated progeny, which could undergo further rounds of cycling. However, a percentage of binucleated cardiomyocytes was also produced which had a lesser proliferative capacity.

In light of these results, it was proposed that comparing the factors that determine the proliferative capacity of newt mono and binucleated cardiomyocytes with those of mammalian cardiomyocytes, which are basically binucleated (Brodsky et al., 1991), could help understanding why mammalian cardiomyocytes are refractory to dividing.

### **1.3 The zebrafish as a model system**

The zebrafish, in contrast to the newt and other urodele amphibian species, benefits from a powerful set of genetic tools developed initially for research on developmental biology (Driever et al., 1994), a field in which the zebrafish has become a preferred animal model due to its short generation time, large clutch sizes, and transparency in the embryonic stage.

The zebrafish is amenable to be used in large screens to seek for defects in development and/or regeneration. Large numbers of

knockdown or knockout zebrafish can be obtained by mutagenizing the genome (Stainier, 2001) or by disrupting coding genes or their promoter/enhancer regions by viral insertion (Amsterdam et al., 1999; Amsterdam & Hopkins, 2006). There are several ways to mutagenize the zebrafish genome but the most common makes use of ethylnitrosourea (ENU), an alkylating mutagen inducing point mutations (Solnica-Krezel et al., 1994). This procedure allows for the screen of thousands of animals for developmental defects (Driever et al., 1996), or for the study of temperature-sensitive mutations affecting fin regeneration (Johnson & Weston, 1995). Other common way to knockdown gene function in zebrafish is the use of morpholinos, which are stable, modified oligonucleotides complementary to start or splice sites that block translation or splicing of the targeted genes (Nasevicius & Ekker, 2000). These can be injected into one cell-stage embryos to search for developmental defects or into regenerating tissues, such as the spinal cord (Becker et al., 2004). Coupling injection to electroporation was shown to increase the delivery of morpholinos into the tail fin and increase the effectiveness of the gene knockdown (Jazwinska et al., 2007; Thummel et al., 2006; Thummel et al., 2007). The zebrafish is also amenable to pharmacological inhibition and drugs can be typically applied in fish water (Iovine, 2007). The combination of the use of libraries of chemical compounds to screen for phenotypes related to development, disease and regeneration (Mathew et al., 2006; Murphey & Zon, 2006; Peterson et al., 2000), results in chemical genetics. This permits high-throughput analysis in the zebrafish embryo due to its small size and aqueous environment (Bowman & Zon, 2010).



Other advantages of the zebrafish include a sequenced genome and the existence of commercial gene expression arrays. For example, Affymetrix chips have been extensively used to study gene expression in many aspects including embryology, regeneration and development in mutants (Packham et al., 2009; Qian et al., 2005; Schebesta et al., 2006). The use of standardized gene chips allow for easy meta-analysis and comparisons of different experiments.

Transgenesis in zebrafish is also widely used by the scientific community in different fields and with different purposes. There are hundreds of zebrafish lines with reporters on specific cell types, including erythrocytes, cardiomyocytes, endothelial cells and many others. It has also been used to overexpress certain genes or their dominant-negative forms in a tissue- and/or time-specific manner (Poss et al., 2002a; Stoick-Cooper et al., 2007), and to express genetically encoded sensors of chemical compounds (Niethammer et al., 2009). Moreover, the production of transgenic animals has been significantly facilitated by the free distribution of the Tol2kit, a collection of plasmids containing different promoters, reporters and tags, which uses multisite gateway-based technology to generate transgenesis constructs in a short period of time (Kwan et al., 2007). More recent advances in transgenesis led to the establishment of a way to conditionally ablate cells in a tissue- and time-dependent manner so it could be used as a model to study regeneration (Curado et al., 2007). This was achieved by expressing the *E. coli* Nitroreductase (NTR) gene under the control of tissue-specific promoters. NTR is an enzyme that, upon the addition of the drug Metronidazole (Mtz), will

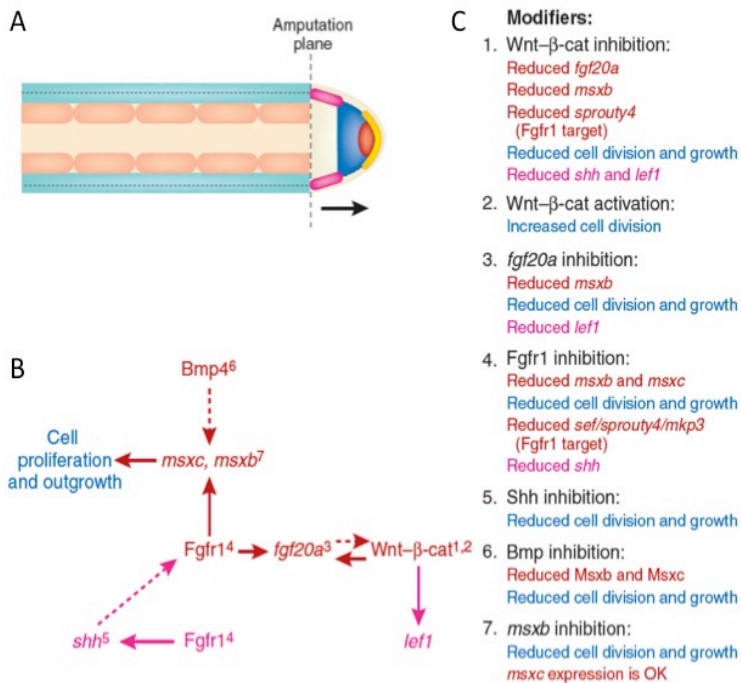
transform it into a potent DNA interstrand cross-linking agent, causing cell death.

Other tools to understand cell behavior include the existence of a transparent fish, *casper*. This fish can be used to track stem/progenitor cells or tumor cells by non-invasive techniques and they can also be crossed to numerous transgenic lines that label specific cell types to study distinct cell behaviors during angiogenesis, organ homeostasis and regeneration after injury (Pugach et al., 2009; White et al., 2008).

Perhaps the only molecular tool not yet available for zebrafish is the possibility to create specific knockouts by homologous recombination in embryonic stem (ES) cells. The derivation of zebrafish ES cells has been attempted by many laboratories, some having reported the generation of chimeras after injecting ES-like cells into blastula-stage embryos (Fan et al., 2004). However, those chimeras were not capable of germline transmission, and thus, cannot be used to create knockout animals. This limitation of the zebrafish model versus the mice, for example, has been partially overcome by the possibility to knockout genes using engineered zinc finger nucleases (Doyon et al., 2008; Meng et al., 2008). A very important step forward for the establishment of the zebrafish as a model system was the recent implementation of the conditional cell labeling technique (Hans et al., 2009), widely used in the mouse to study specific cell behaviors.

The zebrafish has also been widely used as a model for regeneration studies on fin regeneration (Akimenko et al., 2003; Poss et al., 2003). From the comparative histochemical analysis of three species of teleosts (Becerra et al., 1996), to the determination of signaling factors

by the use of mutants and transgenic lines (Makino et al., 2005; Nechiporuk & Keating, 2002; Poss et al., 2002a), the use of zebrafish in this field has arisen in popularity and complex regulatory networks have been described (for review, see Iovine, 2007) (Fig. 2).



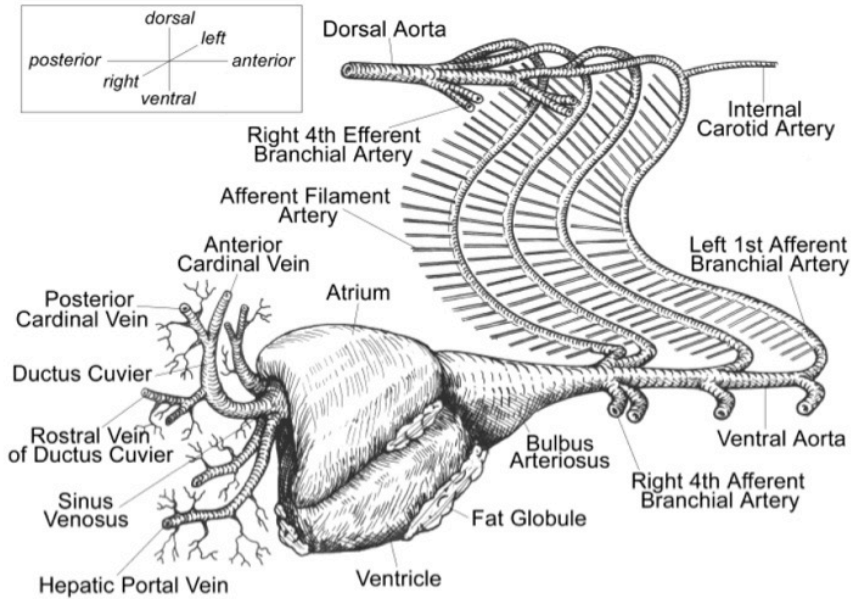
**Figure 2. Establishment of signaling centers and initiation of outgrowth.** (A) Cartoon of a longitudinal section of a single fin ray following blastemal reorganization. The basal layer of the epithelium is indicated by the dotted line, the apical epidermal cap in yellow. The distal blastema (red) is distal to the proximal blastema (dark blue). Proliferating cells from the proximal blastema will migrate laterally toward the basal epidermal layer (pink), and differentiate as bone forming cells. (B) Molecular pathways required for establishment of distal blastema, lateral basal epidermal layer and cell proliferation and outgrowth. Tissue of expression is color-coded to match that in (A); superscripts refer to list in (C). Dotted arrows indicate signaling events that seem to occur but are not clearly defined. (C) Genetic or chemical modifiers of the molecular mechanism shown in (B) and their effects on gene expression, outgrowth, or both. From Iovine (2007).

## 1.4 The zebrafish heart

The heart is the first definitive organ to develop and become functional, as embryo survival depends on its proper function (Clark, 1990). In the zebrafish, however, there is a period of several days when the heart is already functional, but not yet essential in the early developmental stages. This is because the early embryo can obtain enough oxygen from the medium by diffusion due to its small size and relatively low metabolism (Hu et al., 2000). This peculiarity has enabled analyzing mutants with compromised or no cardiac function for a considerable period of time (Stainier & Fishman, 1992; Stainier et al., 1996), in contrast to mammals. However, regarding the general aspects of cardiovascular development, zebrafish are comparable to avian and mammalian (Hu et al., 2000; Stainier, 2001).

The adult zebrafish heart consists of two chambers, the atrium and the ventricle, the sinus venosus, which collects blood from the animal body, and the outflow tract, which functions as a capacitor maintaining continuous blood flow into the gills arches (Hu et al., 2001) (Fig. 3). The ventricle consists of a vascularized compact myocardium of about 4 cell layers and the trabecular myocardium of about 2 cell layers. Ventricular cardiomyocytes are larger than those in the atrium (Hu et al., 2001). As a general trait, teleost cardiomyocytes are 2-10 times smaller than those of mammals, and are basically mononucleated; they have a greatly reduced sarcoplasmic reticulum, and lack the T-tubules system found in skeletal muscle and mammalian cardiac muscle (Farrell AP, 1992). The zebrafish epicardium is formed by a single layer of mesothelial cells supported by a basal lamina, and imbricated with collagen and vascular structures

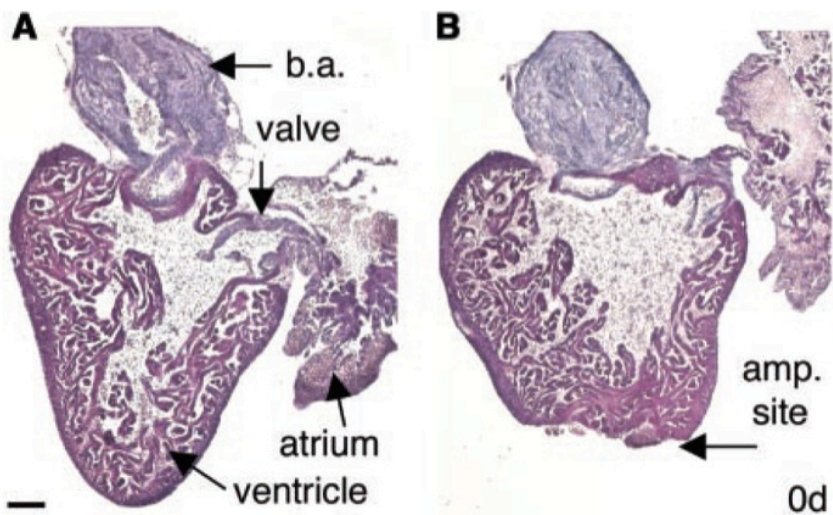
in the subepicardial space (Hu et al., 2001). These mesothelial cells are interdigitated to each other by desmosomes (Lemanski et al., 1975).



**Figure 3. The adult zebrafish heart.** An illustration of a posteroanterior view of an adult zebrafish heart and the major vasculature in the cardiac region. The atrium receives the venous return from the sinus venosus, which is connected to the ductus Cuvier and hepatic portal veins. The heart pumps the blood to the bulbus arteriosus along the definite chamber of atrium and ventricle. The ventricle forces the blood into the ventral aorta, which gives off paired vessels (afferent branchials) that arch upward between the successive gills to rejoin (efferent branchials) and form the dorsal aorta. Only the left branchials are shown in the illustration. The boxed area indicates the coordinates showing the orientation of the heart. From Hu et al. (2001).

## 1.5 Heart regeneration in zebrafish

In the turn of the 21st century two groups reported the ability of adult zebrafish to regenerate large portions of its heart (Poss et al., 2002b; Raya et al., 2003) (Fig. 4). Up to 20-30% of the ventricle was surgically removed at its apex with iridectomy scissors. This was easily accomplished in anesthetized adult fish, whose hearts were readily accessible after incision of the skin, muscle, and pericardial sac. After amputation, intense bleeding occurred but a blood clot was formed within less than one minute. Such an efficient process might be aided by the zebrafish low-pressure circulatory system (Raya et al., 2004), and probably because of this, amputated fish did not exhibit intense myocardium contraction at the site of resection or circulatory stasis, in



**Figure 4. Morphology of the uninjured and amputated zebrafish hearts.** Hematoxylin and eosin staining of an uninjured zebrafish heart (A) and after about 20% ventricular resection (B). Shown are the ventricle, the atrium, the atrio-ventricular valve and the bulbus arteriosus (b.a.). From Poss et al. (2002).

contrast to newts (Becker et al., 1974). The blood clot is then replaced first by fibrin, reaching maximum levels at 7 to 9 days, and later by collagen. Ultimately, due to increased cell proliferation next to the wound site, the lost myocardium is regenerated and hearts from 30-60 days post amputation (dpa) look histologically and functionally indistinguishable from uninjured hearts (Poss et al., 2002b; Raya et al., 2003).

#### a) Cellular bases

The cellular origin of the regenerated myocardium has been a controversial topic. The first experiments relied on BrdU injection into the fish and its incorporation into proliferative cells. By these means, Poss and colleagues (Poss et al., 2002b) identified by immunofluorescence, cells co-labeled with antibodies against BrdU and against myosin heavy chain, a marker for cardiomyocytes. This led to the conclusion that the proliferating cells were cardiomyocytes on its origin. Alternatively, Raya and colleagues (Raya et al., 2003) reached the same conclusion by analyzing the BrdU labeling under light microscopy, as they could observe BrdU-positive cells displaying morphological characteristics of cardiomyocytes. Both studies identified the vast majority of these cells in the region surrounding the wound and next to the epicardium. Pulse-chase experiments of BrdU labeling (Poss et al., 2002b) revealed that the leading edge of proliferation during regeneration was the new layer of outermost compact muscle, which was displaced inwards at later stages. By using an antibody against  $\beta$ -catenin to distinguish compact from trabecular

myocardium, it was shown that, after regeneration was completed, the compact layer of muscle increased to 10 to over 30 cell layers. However, this phenotype was not observed by others (Raya et al., 2003), who reported normal proportions of compact and trabecular zones (Raya et al., 2004).

In their 2003 paper, Raya et al. (2003) also addressed the question of whether developmental-like progenitor cells were activated during regeneration by analyzing the expression of *nkx2.5* and *tbx5*, two early markers of cardiac lineage. They could not detect any increase in expression by in situ hybridization upon amputation, and they addressed this question further by using a transgenic zebrafish line expressing EGFP under the control of the *CARP* (a direct target of *nkx2.5*) promoter. They could not see fluorescence from the EGFP at any time point analyzed beginning from 3dpa. The results from these different approaches lead to the conclusion that progenitor cells did not appear to be the ones driving the regenerative response, but the differentiated cardiomyocytes instead.

The notion that cardiomyocytes were the source of the newly formed muscular tissue was later supported by the observation that primary cultures of zebrafish cardiomyocytes were capable of responding to growth factors by increasing BrdU incorporation (Lien et al., 2006).

That same year though, immersed in the very raising popularity of the stem cell field, this view was shifted 180 degrees by evidence published by Lepilina et al. (2006). They used an elegant timing assay that takes advantage of the different kinetics in fluorescence and degradation of the EGFP versus DsRed2. The premises were that (1)

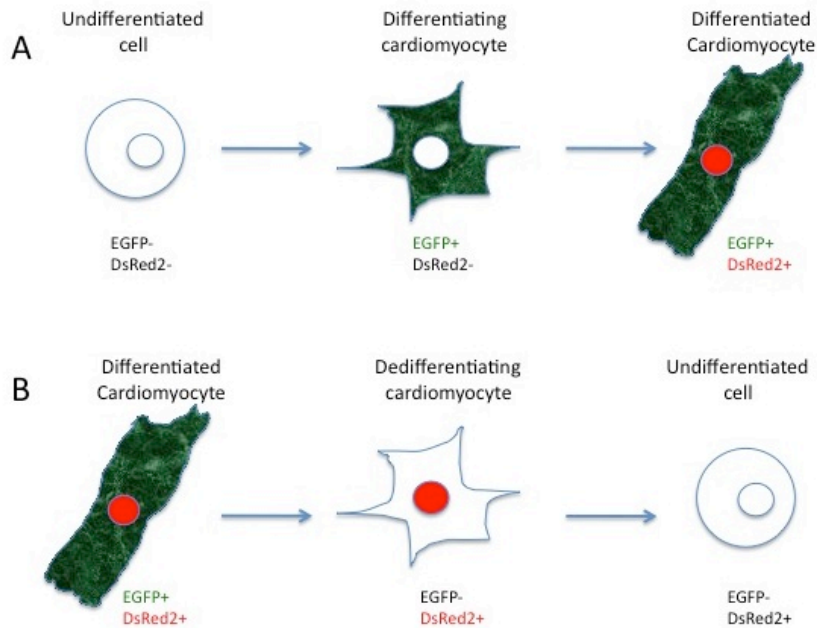


EGFP folds and fluoresces more rapidly than DsRed2 (they reported to be one day faster in embryonic zebrafish cardiomyocytes activating the *cmc2* promoter for the first time) and that (2) EGFP is also less stable than DsRed2 and degrades about twice as rapidly. This bidirectional developmental timer would have allowed them to (i) identify new cardiomyocytes arising from an undifferentiated state (EGFP-positive, DsRed2-negative) and (ii) identify once-expressing cardiomyocytes switching off the *cmc2* promoter in a presumable process of dedifferentiation (EGFP-negative, DsRed2-positive) (Fig. 5).

What they reported was that, in regenerating hearts, a front of EGFP-positive, DsRed2-negative appeared from 7dpa onwards and no EGFP-negative, DsRed2-positive cells were detected. They interpreted these results to indicate that newly formed cardiomyocytes arise from a pool of undifferentiated progenitor cells. They further supported their observations by in situ hybridization experiments detecting an increase of expression of early markers of cardiac progenitors such as *nkx2.5*, *hand2*, *tbx20* and *tbx5*, together with increased immunoreactivity to MEF2 protein (Lepilina et al., 2006).

## b) Molecular bases and non-myocardial cellular contribution

The first data about the molecular requirements for proper heart regeneration in zebrafish came as a byproduct of large mutagenesis screens primarily aimed to find genes required for zebrafish fin regeneration (Makino et al., 2005; Nechiporuk et al., 2003; Poss et al., 2002a; Whitehead et al., 2005).



**Figure 5. Model of the double developmental timer assay** used in Lepilina et al. (2006) study. (A) An unlabelled undifferentiated progenitor cell undergoing cardiomyocyte differentiation will activate its *cmlc2* promoter and thus the expression of EGFP and nuclear DsRed2. Because EGFP folds and fluoresces faster than DsRed2, it will appear earlier in differentiating cardiomyocytes. (B) A differentiated cardiomyocyte showing fluorescence of both EGFP and nuclear DsRed2 will turn down the activation of the *cmlc2* promoter as a result of dedifferentiation. Because EGFP is less stable than DsRed2, the dedifferentiating cardiomyocyte will lose EGFP fluorescence faster than DsRed2's.

In the first of this series of papers, Poss et al (Poss et al., 2002a) described the lack of fin regeneration in a temperature-sensitive mutant of the gene *monopolar spindle 1 (mps1)*, encoding a conserved, kinetocore-associated kinase, required for proper mitotic checkpoint (Abrieu et al., 2001; Weiss & Winey, 1996). In these mutants, the fin does not regenerate due to the failure of blastema cells to proliferate

(Poss et al., 2002a). Consistent with this, it was found that *mps1* mutants also failed to regenerate their hearts, and postulated that this gene was necessary for cardiomyocytes to proliferate, since wild-type cardiomyocytes next to the site of injury display increased expression of *msp1* (Poss et al., 2002b). A similar situation occurred in *nbl* (*no blastema*) mutants, which carried a point mutation in the gene coding for heat shock protein 60 (*hsp60*). The authors failed to detect BrdU incorporation in MEF2-positive cells (Makino et al., 2005).

More data came as part of an expression screen for candidate genes required for heart regeneration in zebrafish. Raya et al. (2003) found an overexpression of the genes encoding the muscle segment homeobox (*msx*) *msxB* and *msxC*, and of *notch1b* together with one of its ligands, *deltaC*. Although no loss-of-function was performed to confirm their requirement for proper regeneration, some hypotheses can be inferred from their expression patterns during heart regeneration and from data in the literature. *notch1b* and *deltaC* transcripts were upregulated in the endocardium of the whole ventricle from 1dpa to 7dpa, suggesting a role of the endocardium in aiding or instructing the proliferation of cardiomyocytes, as their expression is also seen during zebrafish heart development (Beis et al., 2005; Walsh & Stainier, 2001). Of special interest should be *msxB* and *msxC* for several reasons. They were found to be overexpressed in the cardiac tissue next to the wound, from 3dpa to 21dpa, but peaking at 14dpa. In zebrafish, the five *msx* genes (*msxA-E*) have been shown to be expressed during development in fins and other organs (Akimenko et al., 1995; Ekker et al., 1997; Zauner et al., 2003) and during fin regeneration, either in the adult (Akimenko et al., 1995) or in the

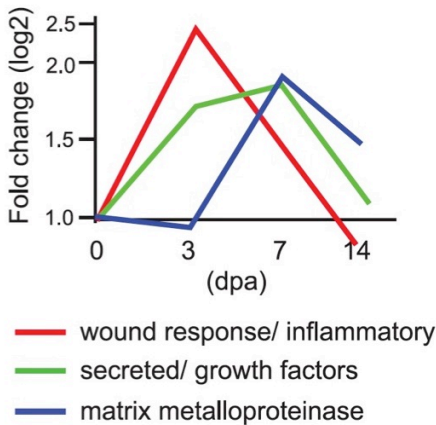
embryo (Mathew et al., 2007). However, neither *msxB* nor *msxC*, were found to be expressed during heart development in zebrafish (Raya et al., 2003), but are the only ones to be expressed in the blastema cells during adult fin regeneration (Akimenko et al., 1995). That meant that, at least in the heart, *msxB* and *msxC* overexpression during regeneration does not recapitulate a developmental program but instead they would presumably act as factors to induce dedifferentiation, a phenomenon observed in mammalian myotubes after ectopic expression of the related factor *msx1* (Odelberg et al., 2000).

Very interesting data was also revealed by work showing the dynamic action of genes expressed in the epicardium such as *raldh2* and *tbx18* (Lepilina et al., 2006), which were found to be upregulated in the whole heart at 3dpa, while their expression was restricted to the regenerating area of the ventricle at 14dpa. The penetration of cells expressing these genes into the wound, together with the increase of reporter expression in the wound from *fli1:EGFP* fish (in which EGFP is under the control of the endothelial-specific gene, *fli1*), prompted the authors to speculate about an epithelial-to-mesenchymal transition (EMT) process, homologous to what is seen during development (Timmerman et al., 2004). They further suggested an interaction between epicardial and myocardial cells involving fibroblast growth factor (fgf) signaling. They showed that the ligand *fgf17b* is upregulated in the myocardium, while the receptors *fgfr2* and *fgfr4* are upregulated in the epicardial cells that invade the wound at later stages of regeneration. Moreover, in the involvement of fgf signaling during heart regeneration was bolstered by using a transgenic line in which a

dominant-negative form of the *fgfr1* is expressed under the *hsp70* promoter (*hsp70:dn-fgfr1*). After heat-shocking the animals in the process of regeneration they observed that their regenerative capacity was reduced. This was accompanied with an increase of the scar size, a lack of infiltration of *tbx18*-positive epicardial cells and a lack of vascularisation as observed by the absence of EGFP expression in the *fli1:EGFP* background.

A more comprehensive approach to determine the molecular bases during heart regeneration came by means of a genome-wide expression analysis (Lien et al., 2006). Their samples consisted of one third of the ventricle containing the amputation plane obtained from 3dpa, 7dpa and 14dpa regenerating hearts and sham-operated hearts. 662 transcripts were found to be differentially expressed, and within those, they found a sequential expression profile of genes coding for wound response/inflammatory/anti-inflammatory factors, secreted molecules, and matrix metalloproteinases (MMPs) (Fig. 6). They also checked the expression pattern by in situ hybridization of some of these genes, namely *apoEb*, *midkine a*, *thymosin beta 4* and *pdgfb*. They obtained a clear signal for the first two genes. While *apoEb* peaked at 3dpa and appeared in a punctuate fashion suggesting expression from infiltrating macrophages, *midkine a* peaked at 7dpa and its expression could be detected in the compact layer of myocardium and the epicardium around the wound site. They also went one step further in their search for molecules that could stimulate cardiomyocytes to proliferate by adding some of the identified factors to primary cultures of cardiomyocytes, something that already proved valuable in the newt (Soonpaa et al., 1992, 1994). They tested mammalian ApoE4, Midkine

a, Progranulin, PDGF-AA, PDGF-AB, PDGF-BB and recombinant zebrafish PDGF-BB. They could only see a significant increase in BrdU incorporation in cardiomyocytes treated with both zebrafish and mammalian PDGF-BB (a 2,68-fold increase for the latter). The role of PDGF action was further addressed in vivo by the use of a chemical inhibitor. They observed a very mild decrease of BrdU incorporation into cardiomyocytes, although significant, in contrast to what they observed by the use of a MMPs inhibitor.



**Figure 6. Gene expression profile of regenerating zebrafish heart.** General trend and average fold change of expression of wound response/inflammatory genes, secreted molecules, and MMPs. The wound response/inflammatory genes are expressed early (peak at 3 dpa) during zebrafish heart regeneration. Genes coding for secreted molecules begin to express at 3 dpa and the expression level reach a peak at 7 dpa. The MMPs start to express at 7 dpa and last until 14 dpa. From Lien et al. (2006).

Notably, the transcriptional analysis of Lien et al. (2006) supported some, but not all previous data. For example, they found *mps1* (Poss et al., 2002b) to be upregulated and peaking at 3 dpa. On the other hand, they did not find upregulation of *notch1b* or *deltaC* (Raya et al., 2003) nor of *hsp60* (Makino et al., 2005). They argued that *notch1b* and *deltaC*

were probably not detected because of the different heart locations examined in the study of Raya et al. (2003), compared to theirs. They also postulated that the failure to detect an upregulation of *hsp60* might be explained by a post-translational control of hsp60 activity during regeneration.

Thus, the data obtained by several approaches may in some cases be complementary but in some others may have to be revisited.





## 2. OBJECTIVES

1. Characterize molecularly the initial steps of heart regeneration in zebrafish and identify general molecular signatures by the analysis of genome-wide transcriptional data.
2. Validate a list of candidate genes to have a role during heart regeneration in zebrafish by more sensitive techniques, both qualitatively (conventional RT-PCR, in situ hybridization), and quantitatively (quantitative RT-PCR).
3. Validate the functional role of selected candidate genes during heart regeneration in zebrafish in vivo.
4. Identify the cellular origin of the newly formed cardiomyocytes during the process of heart regeneration by inducible genetic labeling techniques.
5. Characterize the structural changes associated with the cells that give rise to new cardiomyocytes during heart regeneration in zebrafish.



### 3. RESULTS

#### 3.1 Paper 1: Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation

Jopling C, Sleep E, Raya M, Martí M, Raya A, Izpisúa JC.  
[Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation.](#)  
Nature. 2010; 464(7288): 606-9.

### 3.2 Paper 2: Transcriptomics approach to investigate zebrafish heart regeneration

Sleep E, Boué S, Jopling C, Raya M, Raya A, Izpisua Belmonte JC. [Transcriptomics approach to investigate zebrafish heart regeneration.](#) J Cardiovasc Med (Hagerstown). 2010; 11(5): 369-80.

## **4. DISCUSSION**

The results presented here change the conception of how heart regeneration occurs in the zebrafish at the cellular level and is further supported at the molecular level. Overall, our data provide a new entry point for dissecting the mechanisms of heart regeneration in zebrafish with the hope that such knowledge will be ultimately used for the development of effective regenerative-based therapies for human cardiac disease.

### **4.1 Cellular bases of heart regeneration in zebrafish**

The identification of the cell type(s) that give rise to new cardiomyocytes is essential to understand how zebrafish heart regeneration takes place. Previous observations suggested that a set of cardiac progenitor or stem cells were the source of the newly formed cardiomyocytes (Lepilina et al., 2006). However, we consider that their experimental approaches had several caveats and some results appeared to be at odds with previous reports. These facts could have led the authors to misleading conclusions, as pointed out shortly after their publication (Borchardt & Braun, 2007). Specifically, our concerns include the following: First, DsRed2, similar to its former relatives, is an obligate tetramer and has been shown to aggregate under typical conditions of use, including the reducing environment of the cytosol and the presence of native proteins (Baird et al., 2000). This might have toxic effects and impede proper targeting to cellular compartments (Strack et al., 2008; Yanushevich et al., 2002). Second,

whereas EGFP was cytosolic, DsRed2 was targeted to the nucleus, thus providing another variable that was not taken into account for the decay kinetics. Third, DsRed2 has only been shown to degrade slower than EGFP in *Drosophila* S2 cells grown at 25°C (Verkhusha et al., 2003) but not in the zebrafish. The authors failed to show this important control in their study, something that could have easily been checked by co-injecting EGFP and DsRed2 mRNA into zebrafish embryos and following the decay of fluorescence. Fourth, although both EGFP and DsRed2 were under the control of the *cmlc2* promoter, the double transgenic line was obtained from a cross of two independent lines, harboring transgene insertions on different genome locations, and therefore their reporter expression possibly being subject to different positional influences (Hans et al., 2009). Fifth, and related to the previous concern, when using the single transgenic line expressing nuclear DsRed2, Lepilina et al. (2006) observed that nuclei of the regenerated myocardium had a lower intensity than the non-regenerated myocardium at 14dpa and 22dpa. The authors claimed to expect this result in less differentiated or transitioning cardiomyocytes. This was striking, as Raya et al. (2003) did not report any decrease in fluorescence in the regenerated area of hearts from *cmlc2*-EGFP zebrafish. This difference could point to a regeneration-dependent differential behavior of the reporter transgenes. Sixth, while observing low-intensity DsRed2 fluorescence driven by the *cmlc2* promoter in the nuclei of cardiomyocytes in the regenerating area at later stages of regeneration (which the authors interpreted as less differentiated or transitioning cardiomyocytes), they also observed an increase of MEF2 immunoreactivity in the regenerating area as early as 4dpa (which was interpreted as an evidence of pre-cardiac or early differentiation

marker). However, MEF2 has been shown to directly bind the *cmhc2* promoter and activate its transcription in vertebrates and flies (Sandmann et al., 2006). Thus, these two observations by Lepilina et al. (2006) seem to contradict one another and are not satisfactorily addressed in their studies. Seventh, the increase of expression detected by in situ hybridization of markers of cardiac progenitors such as *nkx2.5* and *tbx5* described by Lepilina et al. (2006) was not observed in previous reports analyzing the same markers in a similar context (Raya et al., 2003). Moreover, neither *nkx2.5*, nor *tbx5*, *tbx20* or *hand2* were found to be upregulated in the genome-wide transcriptome analysis of Lien et al. (2006), or in ours (Paper 2).

Considering the concerns described above, we should note that we were not convinced by the data presented by Lepilina et al. (2006), nor by their conclusions.

To unambiguously address the question of whether newly formed cardiomyocytes are derived from pre-existing cardiomyocytes or from a progenitor or stem cell source, we genetically labeled differentiated cardiomyocytes and their progeny. Our lineage tracing experiments provide strong support to the notion that cardiac progenitor or stem cells are not the source for new cardiac muscle (Paper 1). Rather, our results show that the proliferation of differentiated cardiomyocytes is the primary source of the regenerated myocardium. This was already suggested by previous studies (Poss et al., 2002b; Raya et al., 2003), although firm conclusions could not be drawn in the absence of inducible genetic labeling techniques. Consistent with this view, regeneration of the new heart was also proposed to depend on the proliferation of already existing cardiomyocytes (McDonnell &

Oberpriller, 1984; Oberpriller & Oberpriller, 1971). As the data obtained for the newt could not discriminate between different forms of regeneration, it will be very valuable to address this question in future lineage tracing labeling experiments, once this technique becomes available for this animal model.

We show that cardiomyocytes, especially those closest to the wound, undergo a partial dedifferentiation process characterized by a disorganization of the contractile apparatus and detachment of adjacent cardiomyocytes and among cardiomyocytes and endothelial cells. Disarrangement of contractile filaments in mitotic cardiomyocytes and the breakdown of myofibrillar structure in cardiomyocytes labeled after DNA synthesis was also noted in the case of newt heart regeneration (Bader & Oberpriller, 1979; Oberpriller & Oberpriller, 1971). In that case, it was suggested that the disruption of the myofibrils might be a physical factor to allow the cell to proceed to cytokinesis (Oberpriller & Oberpriller, 1971). Importantly, this phenotype was also found in cell culture experiments. Time-lapse analyses of plated newt cardiomyocytes showed that the myofibrillar structure suffered changes in cells undergoing mitosis and striations could only be detected at the very periphery of dividing cardiomyocytes (Bettencourt-Dias et al., 2003; Matz et al., 1998). Consistent with this, we also showed that cardiomyocytes that stained positive for phosphorylated-histone 3 (pH3), a marker of mitosis, failed to display a properly organized sarcomeric structure. Thus, the zebrafish and the newt seem to share common mechanisms that allow the cardiomyocyte to enter the cell cycle. It will be very interesting to



investigate how these two mechanisms, myofibrillar breakdown and cell cycle re-entry, are coordinately induced, deployed, and terminated.

As shown for zebrafish cardiomyocytes, cellular dedifferentiation and reacquisition of the differentiated state has also been observed in the case of limb and lens regeneration in the newt and the axolotl (Tanaka & Galliot, 2009; Tsonis, 2000). This suggests that cellular plasticity from the differentiated state may be a conserved feature used for regeneration in adult vertebrate regeneration-competent species (Brookes & Kumar, 2002; Odelberg, 2005).

## **4.2 Molecular mechanisms**

A myriad of factors might be involved in instructing the different cell types present in the zebrafish heart to proliferate, migrate or to change their phenotype in order to rebuild it after amputation. To gain more insights into this, and with an especial interest in initiator signals of regeneration, we undertook a transcriptional analysis of the zebrafish heart at the early stages of regeneration by the use of Affymetrix technology (Paper 2). Even though the 14,900 transcripts analyzed the Affymetrix GeneChip Zebrafish Genome Array correspond to around 60% of total annotated transcripts (according to the current Vega Genome Browser release 38), our transcriptional profiling allowed us to describe some of the initial steps of heart regeneration in molecular terms, that is, a partial cardiomyocyte dedifferentiation process followed by the progressive return to the differentiated state. This was characterized by an early downregulation of cardiac-specific transcription factors and a later upregulation of contractile proteins

and it was accompanied by a downregulation of genes involved in structural integrity of the cell and cell-cell adhesion. Thus, our molecular data provided molecular bases, at the transcriptional level, to our transmission electron microscopy observations of regenerating hearts.

We suspected that dedifferentiation in cardiomyocytes would be followed by, or be contemporary to, the upregulation of genes involved in cell proliferation. To enrich our list of candidate genes involved in the acquisition of a proliferation-competent status, we compared our transcriptional data to those of three other available transcriptional datasets on zebrafish tissue regeneration. Among the 33 genes (35 probesets) found to have differential expression in all types of zebrafish regeneration analyzed, we found the gene encoding polo-like kinase 1 (plk1), a positive regulator of cell cycle progression (Petronczki et al., 2008). *plk1* was found to be upregulated during heart regeneration in cells next to the wound, where the highest proportion of proliferative cardiomyocytes are found, suggesting that cardiomyocytes upregulated this gene to enter mitosis. Indeed, we found that pharmacological inhibition of plk1 in vivo blocked regeneration due to reduced cardiomyocyte proliferation upon amputation. Thus, plk1 has an essential role in enabling cell cycle progression in cardiomyocytes and can be added to the list of required genes for heart regeneration together with *mps1*, *fgfr1* (Poss et al., 2002a), and *hsp60* (Makino et al., 2005).

We also used the transcriptional comparison approach to address the question of which genes in the ~2,000-gene (2,006 probesets) list we obtained were more prone to have a specific role in the regeneration

of the zebrafish heart. We did this by comparing our data to that of Lien et al. (2006) and found more than 300 genes (318 probesets) shared between the two datasets. Three of such genes were found to be upregulated next to the injury site, namely *cathepsin S b.1* (*ctssb.1*), *cathepsin Z* (*ctsz*) and *glia maturation factor gamma* (*gmfg*).

The genes *ctssb.1* and *ctsz* encode for proteolytic enzymes. Apart from a general role in remodeling the extracellular matrix and allowing cell migration, such proteolytic enzymes could also be responsible for disrupting cell contacts, facilitating the reversal from a differentiated state and for allowing responses to soluble mediators (Brockes, 1997). It is appealing to speculate that *ctssb.1* and *ctsz* could have a role in mediating the dedifferentiation of cardiomyocytes either triggered by the release of inhibitory cell contacts, as it is found to be the case in cultured newt myotubes (Tanaka et al., 1997), or by proteolyzing a serum factor that would have an effect to cardiomyocytes. An example of the latter could be thrombin, which was shown to generate a ligand from the cleavage of a substrate in vertebrate serum leading to induction of cell cycle re-entry of cultured newt myotubes (Tanaka et al., 1999).

The gene *gmfg* encodes for an actin-binding protein predominantly expressed in microvascular endothelial cells and inflammatory cells (Ikeda et al., 2006; Walker, 2003). It will also be interesting to determine whether it can have any role on cardiomyocyte performance during regeneration or on other cellular processes such as the epithelial-to-mesenchymal transition. Such process is very important during heart development in zebrafish (Timmerman et al., 2004) and

was also suggested to be an important aspect of zebrafish heart regeneration (Lepilina et al., 2006).

At the molecular level, both zebrafish and newts seem to use conserved mechanisms to induce regeneration. Recently, a report comparing transcriptional data of regenerating zebrafish heart to an EST database from 14dpa newt hearts led to the conclusion that newt and zebrafish activate similar genes in response to injury (Borchardt et al., 2010). This is another example of the consistency in the mechanisms deployed by both animal species during heart regeneration and provides support to the view that information acquired in one model may complement the information gathered in the other.

Finally, the determination of what factors are involved in or influence the acquisition of a proliferation-competent status should be of outstanding interest. We suspect that the study of other unexplored members of the Wnt, TGF-beta, BMP, or IGF signaling pathways, as well as other pathways identified in our transcriptome profiling, will lead to interesting data regarding the control of different cellular aspects of regeneration, as they already have in other regenerating systems (Chablais & Jazwinska, 2010; Ho & Whitman, 2008; Kawakami et al., 2006; Smith et al., 2006; Stoick-Cooper et al., 2007).

### **4.3 Contribution to regenerative medicine**

Ischemic cardiomyopathy is the leading cause of death worldwide (Mathers et al., 2005). Myocardial infarction results from the

obstruction of a coronary artery, resulting in a reduced or absent blood supply with subsequent cellular hypoxia and necrosis. In mammals, after a myocardial infarction episode, the damaged myocardium is replaced by scar tissue featuring collagen deposition and tissue remodeling (Jugdutt et al., 1996; Lutgens et al., 1999; Nakatsuji et al., 1997). Although it has been reported that cardiomyocytes do proliferate in normal adult human hearts and that cell proliferation increases after myocardial infarction (Beltrami et al., 2003; Kajstura et al., 1998), the efficiency of this process appears to be too low to overcome the injury and cellular hypertrophy is the main compensatory response.

Therapies in use to prevent heart failure are limited. Prophylactic treatment is pharmacological in nature, aiming primarily to inhibit the neurohormonal axis that results in excessive cardiac activation through angiotensin- or norepinephrine-dependent pathways (Itescu et al., 2003). In recent years, some cell-based therapies including transplantation of skeletal myoblasts, hematopoietic stem cells, adipose-derived mesenchymal stem cells, and bone marrow-derived mesenchymal stem cells have been tested (reviewed in Sanchez et al., 2007). They rely on the differentiation capacity of these cells into cardiomyocytes and other cell types found in the heart. Although some cell-based therapies have proven to ameliorate cardiac function and decrease the risk of post-infarct heart failure, the long-term behaviour of those cells have not been well characterized in the *in vivo* context and their regenerative capacity remains uncertain. At this time, it is not clear whether the improvement in cardiac function is due to the ability of the transplanted progenitor cells to differentiate into the

relevant cell types or because of the secretion of pro-survival factors by these cells (Boudoulas & Hatzopoulos, 2009; Guan et al., 2007). Pre-clinical research has provided evidence on the differentiation capacity of progenitor cells from a variety of sources to give rise to different cardiac cell types, and on the identification of embryonic and adult cardiac stem cell populations (Baba et al., 2007; Beltrami et al., 2003; Garry & Olson, 2006; Guan & Hasenfuss, 2007; Laugwitz et al., 2005). Moreover, genetic fate-mapping studies showed that, in the mouse heart, stem cells contribute to new cardiomyocytes after injury (Hsieh et al., 2007). However, they do not do so in the normal aging period of 1 year. On the other hand it has been recently reported that the human heart replaces about 50% of its cardiomyocytes during a normal life span (Bergmann et al., 2009). Although in this study they could not rule out if new cardiomyocytes arose from cardiomyocyte duplication or from a stem/progenitor pool, the data obtained from genetic labeling in aging mice (Hsieh et al., 2007) could still be consistent with cardiomyocyte duplication.

The fact that zebrafish regenerates its heart by utilizing pre-existing cardiomyocytes as the primary source for new cardiac muscle opens a way to try to enhance mammalian cardiac regeneration by acting directly on cardiomyocytes analogously as the zebrafish does. Although the mammalian cardiomyocyte has mostly been regarded as a terminally differentiated cell with no proliferative potential, some have reported that this is not the case and that they can go all the way to cytokinesis (Bersell et al., 2009; Engel et al., 2005; Kuhn et al., 2007).

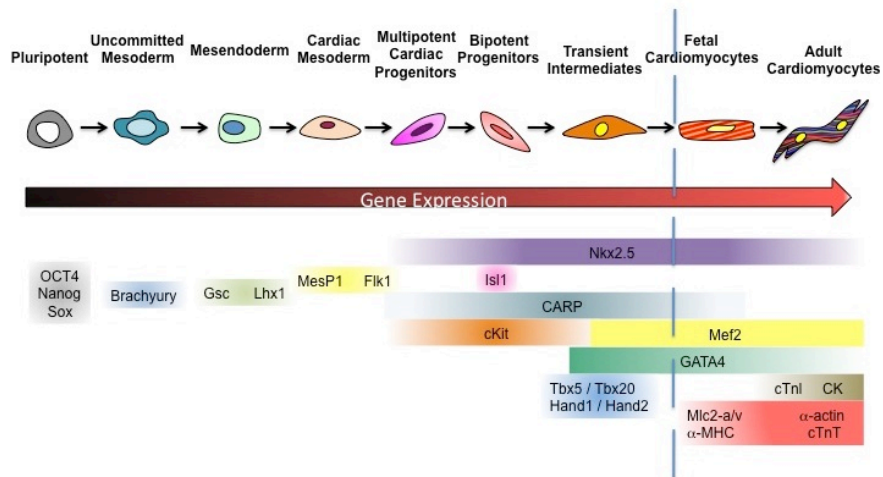
A parallelism to the comparison of skeletal muscle regeneration between mammals and amphibians can be drawn. Different behaviors of newt and mammalian myotubes in culture have been observed. The former respond to high concentrations of serum or thrombin by reentering the cell cycle, traversing S phase and arresting at G2 without cytopathology (Tanaka et al., 1997; Tanaka et al., 1999). The latter are refractory to both (Tanaka et al., 1997) unless they lack both copies of the retinoblastoma (Rb) gene or unless they are myotubes coming from the fusion of mouse C2 and newt A1 mononucleate cells (Velloso et al., 2001). Alternatively, mammalian myotubes can be induced to dedifferentiate by ectopically expressing a homeobox gene, *mx1* (Odelberg et al., 2000), which was shown to be upregulated during urodele regeneration (Simon et al., 1995). These results point to cell-autonomous differences regarding regeneration capacity, thus offering little hope for the successful development of non-genetic or xenobiotic-free regenerative therapies. However, mammalian myotubes can also be induced to dedifferentiate by culturing them with newt limb regeneration extract (McGann et al., 2001), or by treatment with certain chemical compounds such as myoseverin (Rosania et al., 2000). This suggests that, under the appropriate stimuli, mammalian myotubes can be forced to behave as regeneration-competent myotubes. Altogether, these observations point to local environment as a critical aspect in triggering the cellular plasticity seen in regeneration. This type of experiments could also be applied to research on heart regeneration to unveil crucial aspects of mammalian cardiomyocyte competence in terms of dedifferentiation and proliferation when compared to zebrafish.

## 4.4 Open questions

Some questions though, still need to be answered. One of the most important is how far does the cardiomyocyte need to dedifferentiate. Our data point to a mild dedifferentiation in which structural genes and myocyte-specific transcription factors are downregulated and characteristic cytoskeletal proteins are disassembled, presumably to facilitate cell division (Fig. 7). However, these observations come from snapshots at different times of regeneration. It could be possible that cardiomyocytes undergoing dedifferentiation go very transiently through a state close to that of embryonic cardiomyocytes and thus be overlooked by our transcriptional profiling approach. Moreover, our genetic lineage tracing experiments were designed to track differentiated cardiomyocytes and, as therefore, do not allow to follow cells that turn the *cmlc2* promoter off. This question was partially addressed in a previous study (Raya et al., 2003) by analyzing the reporter expression under the control of a developmentally expressed gene in the heart (*CARP*), but no evidences were found to take this hypothesis under consideration. A feasible way to directly address this possibility would be to genetically mark the cells that reactivate developmentally expressed genes using strategies similar to the one described here (Paper 1). A battery of candidates including those found in early progenitors such as *nkx2.5*, *CARP* or *ckit*, or more committed cardiomyoblasts such as *tbx5*, *tbx20* or *hand 1/2*, could be useful for this purpose (Fig. 7). In addition, it would be very interesting to analyze quantitatively the proliferating capacity of regenerating cardiomyocytes. In other words, do all cardiomyocytes



around the wound undergo the same number of cell divisions, or are there intrinsic differences between different cardiomyocytes, as seen in the case of mono- and binucleated newt cardiomyocytes (Matz et al., 1998)? Or even further, is it that just a few of them have the capacity or are instructed to divide continuously while others do not? Developing appropriate labeling techniques and culture conditions to track zebrafish cardiomyocytes for long time-periods will be instrumental to provide a definitive answer to these questions.



**Figure 7. Cardiomyocyte differentiation status and gene expression signatures during development and regeneration.** A schematic representation of the general steps in vertebrate heart development showing the progressive maturation from a pluripotent cell to an adult cardiomyocyte together with its changing expression signature is shown. During zebrafish heart regeneration, cardiomyocytes only dedifferentiate partially and do not go a long way back to the pluripotent state (indicated as a dashed line barrier). Instead, they conserve expression signatures and morphological features of adult or fetal cardiomyocytes.



## 5. CONCLUSIONS

1. Genome-wide transcriptional analysis identified about 2000 genes showing differential expression during the initial stage of heart regeneration in zebrafish and identified a molecular signature consistent with a partial dedifferentiation of cardiomyocytes.
2. Comparative analysis of different forms of regeneration in zebrafish identified a subset of about 318 genes showing differential expression specifically during heart regeneration and a subset of 33 genes with conserved differential expression in all the forms of regeneration analyzed.
3. Transcriptome data was validated for a number of genes by conventional and quantitative RT-PCR and the gene expression patterns were determined for four of the validated genes showing expression in putative cardiomyocytes, endothelial cells, epicardial cells, and infiltrated blood cells.
4. *plk1*, one of the candidate genes validated by RT-PCR and showing a suggestive expression pattern, is essential for proper heart regeneration in zebrafish, since pharmacological inhibition of *plk1* led to the failure of regeneration due to decreased cardiomyocyte proliferation.
5. We unambiguously identified the differentiated cardiomyocyte as the primary source of newly formed cardiomyocytes during heart regeneration in zebrafish by an inducible genetic labeling technique based on the Cre/loxP system.
6. Ultrastructural analysis of regenerating zebrafish hearts identified a series of changes consistent with dedifferentiating cardiomyocytes, including a disorganization of the sarcomeric structure and the cellular detachment from adjacent cardiomyocytes and endocardial cells.



## References

- Abrieu, A., Magnaghi-Jaulin, L., Kahana, J.A., Peter, M., Castro, A., Vigneron, S., Lorca, T., Cleveland, D.W., and Labbe, J.C. (2001). Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. *Cell* 106, 83-93.
- Akimenko, M.A., Johnson, S.L., Westerfield, M., and Ekker, M. (1995). Differential induction of four *msx* homeobox genes during fin development and regeneration in zebrafish. *Development* 121, 347-357.
- Akimenko, M.A., Mari-Beffa, M., Becerra, J., and Geraudie, J. (2003). Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev Dyn* 226, 190-201.
- Amsterdam, A., Burgess, S., Golling, G., Chen, W., Sun, Z., Townsend, K., Farrington, S., Haldi, M., and Hopkins, N. (1999). A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev* 13, 2713-2724.
- Amsterdam, A., and Hopkins, N. (2006). Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet* 22, 473-478.
- Baba, S., Heike, T., Umeda, K., Iwasa, T., Kaichi, S., Hiraumi, Y., Doi, H., Yoshimoto, M., Kanatsu-Shinohara, M., Shinohara, T., and Nakahata, T. (2007). Generation of cardiac and endothelial cells from neonatal mouse testis-derived multipotent germline stem cells. *Stem Cells* 25, 1375-1383.

- Bader, D., and Oberpriller, J.O. (1978). Repair and reorganization of minced cardiac muscle in the adult newt (*Notophthalmus viridescens*). *J Morphol* 155, 349-357.
- Bader, D., and Oberpriller, J. (1979). Autoradiographic and electron microscopic studies of minced cardiac muscle regeneration in the adult newt, *notophthalmus viridescens*. *J Exp Zool* 208, 177-193.
- Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (2000). Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc Natl Acad Sci U S A* 97, 11984-11989.
- Becerra, J., Junqueira, L.C., Bechara, I.J., and Montes, G.S. (1996). Regeneration of fin rays in teleosts: a histochemical, radioautographic, and ultrastructural study. *Arch Histol Cytol* 59, 15-35.
- Becker, C.G., Lieberoth, B.C., Morellini, F., Feldner, J., Becker, T., and Schachner, M. (2004). L1.1 is involved in spinal cord regeneration in adult zebrafish. *J Neurosci* 24, 7837-7842.
- Becker, R.O., Chapin, S., and Sherry, R. (1974). Regeneration of the ventricular myocardium in amphibians. *Nature* 248, 145-147.
- Beis, D., Bartman, T., Jin, S.W., Scott, I.C., D'Amico, L.A., Ober, E.A., Verkade, H., Frantsve, J., Field, H.A., Wehman, A., Baier, H., Tallafuss, A., Bally-Cuif, L., Chen, J.N., Stainier, D.Y., and Jungblut, B. (2005). Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. *Development* 132, 4193-4204.
- Beltrami, A.P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., Kasahara, H., Rota, M., Musso, E., Urbanek, K., Leri, A., Kajstura, J., Nadal-Ginard, B., and Anversa, P. (2003). Adult

- cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114, 763-776.
- Bely, A.E., and Nyberg, K.G. (2010). Evolution of animal regeneration: re-emergence of a field. *Trends Ecol Evol* 25, 161-170.
- Bergmann, O., Bhardwaj, R.D., Bernard, S., Zdunek, S., Barnabe-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B.A., Druid, H., Jovinge, S., and Frisen, J. (2009). Evidence for cardiomyocyte renewal in humans. *Science* 324, 98-102.
- Bersell, K., Arab, S., Haring, B., and Kuhn, B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 138, 257-270.
- Bettencourt-Dias, M., Mitnacht, S., and Brockes, J.P. (2003). Heterogeneous proliferative potential in regenerative adult newt cardiomyocytes. *J Cell Sci* 116, 4001-4009.
- Borchardt, T., and Braun, T. (2007). Cardiovascular regeneration in non-mammalian model systems: what are the differences between newts and man? *Thromb Haemost* 98, 311-318.
- Borchardt, T., Looso, M., Bruckskotten, M., Weis, P., Kruse, J., and Braun, T. (2010). Analysis of newly established EST databases reveals similarities between heart regeneration in newt and fish. *BMC Genomics* 11, 4.
- Borgens, R.B. (1982). Mice regrow the tips of their foretoes. *Science* 217, 747-750.
- Boudoulas, K.D., and Hatzopoulos, A.K. (2009). Cardiac repair and regeneration: the Rubik's cube of cell therapy for heart disease. *Dis Model Mech* 2, 344-358.

- Bowman, T.V., and Zon, L.I. (2010). Swimming into the future of drug discovery: in vivo chemical screens in zebrafish. *ACS Chem Biol* 5, 159-161.
- Brockes, J.P. (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science* 276, 81-87.
- Brockes, J.P., and Kumar, A. (2002). Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat Rev Mol Cell Biol* 3, 566-574.
- Brockes, J.P., and Kumar, A. (2008). Comparative aspects of animal regeneration. *Annu Rev Cell Dev Biol* 24, 525-549.
- Brockes, J.P., Kumar, A., and Velloso, C.P. (2001). Regeneration as an evolutionary variable. *J Anat* 199, 3-11.
- Brodsky, V., Chernyaev, A.L., and Vasilyeva, I.A. (1991). Variability of the cardiomyocyte ploidy in normal human hearts. *Virchows Arch B Cell Pathol Incl Mol Pathol* 61, 289-294.
- Campbell, L.J., and Crews, C.M. (2008). Wound epidermis formation and function in urodele amphibian limb regeneration. *Cell Mol Life Sci* 65, 73-79.
- Carlson, B.M. (1970a). Relationship between the tissue and epimorphic regeneration of muscles. *Am Zool* 10, 175-186.
- Carlson, B.M. (1970b). Regeneration of the rat gastrocnemius muscle from sibling and non-sibling muscle fragments. *Am J Anat* 128, 21-31.
- Carlson, B.M. (2003). Muscle regeneration in amphibians and mammals: passing the torch. *Dev Dyn* 226, 167-181.
- Carlson, M.R., Bryant, S.V., and Gardiner, D.M. (1998). Expression of *Msx-2* during development, regeneration, and wound healing in axolotl limbs. *J Exp Zool* 282, 715-723.



- Cebria, F. (2007). Regenerating the central nervous system: how easy for planarians! *Dev Genes Evol* 217, 733-748.
- Chablais, F., and Jazwinska, A. (2010). IGF signaling between blastema and wound epidermis is required for fin regeneration. *Development* 137, 871-879.
- Clark, E.B. (1990). Growth, morphogenesis and function: The dynamics of cardiac development. In *Fetal, neonatal and infant heart disease*, N.W. Moller JH, Lock J, ed. (New York, Appleton-Century-Crofts), pp. 3-23.
- Curado, S., Anderson, R.M., Jungblut, B., Mumm, J., Schroeter, E., and Stainier, D.Y. (2007). Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. *Dev Dyn* 236, 1025-1035.
- Dinsmore, C.H. (1991). *A History of Regeneration Research* (New York, Cambridge Univ. Press).
- Dor, Y., Brown, J., Martinez, O.I., and Melton, D.A. (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 429, 41-46.
- Doyon, Y., McCammon, J.M., Miller, J.C., Faraji, F., Ngo, C., Katibah, G.E., Amora, R., Hocking, T.D., Zhang, L., Rebar, E.J., Gregory, P.D., Urnov, F.D., and Amacher, S.L. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 26, 702-708.
- Driever, W., Stemple, D., Schier, A., and Solnica-Krezel, L. (1994). Zebrafish: genetic tools for studying vertebrate development. *Trends Genet* 10, 152-159.
- Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini,

- Z., Belak, J., and Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123, 37-46.
- Ekker, M., Akimenko, M.A., Allende, M.L., Smith, R., Drouin, G., Langille, R.M., Weinberg, E.S., and Westerfield, M. (1997). Relationships among *msx* gene structure and function in zebrafish and other vertebrates. *Mol Biol Evol* 14, 1008-1022.
- Engel, F.B., Schebesta, M., Duong, M.T., Lu, G., Ren, S., Madwed, J.B., Jiang, H., Wang, Y., and Keating, M.T. (2005). p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev* 19, 1175-1187.
- Fan, L., Crodian, J., Liu, X., Alestrom, A., Alestrom, P., and Collodi, P. (2004). Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. *Zebrafish* 1, 21-26.
- Farrell AP, J.D. (1992). The heart. In *Fish physiology*, R.D. Hoar WS, Farrell AP, ed. (San Diego, Academic Press), pp. 1-87.
- Gardiner, D.M., Muneoka, K., and Bryant, S.V. (1986). The migration of dermal cells during blastema formation in axolotls. *Dev Biol* 118, 488-493.
- Garry, D.J., and Olson, E.N. (2006). A common progenitor at the heart of development. *Cell* 127, 1101-1104.
- Goss, R.J. (1980). Prospects of regeneration in man. *Clin Orthop Relat Res*, 270-282.
- Guan, K., and Hasenfuss, G. (2007). Do stem cells in the heart truly differentiate into cardiomyocytes? *J Mol Cell Cardiol* 43, 377-387.
- Guan, K., Wagner, S., Unsold, B., Maier, L.S., Kaiser, D., Hemmerlein, B., Nayernia, K., Engel, W., and Hasenfuss, G. (2007). Generation of

- functional cardiomyocytes from adult mouse spermatogonial stem cells. *Circ Res* 100, 1615-1625.
- Handberg-Thorsager, M., Fernandez, E., and Salo, E. (2008). Stem cells and regeneration in planarians. *Front Biosci* 13, 6374-6394.
- Hans, S., Kaslin, J., Freudenreich, D., and Brand, M. (2009). Temporally-controlled site-specific recombination in zebrafish. *PLoS One* 4, e4640.
- Ho, D.M., and Whitman, M. (2008). TGF-beta signaling is required for multiple processes during *Xenopus* tail regeneration. *Dev Biol* 315, 203-216.
- Hsieh, P.C., Segers, V.F., Davis, M.E., MacGillivray, C., Gannon, J., Molkentin, J.D., Robbins, J., and Lee, R.T. (2007). Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med* 13, 970-974.
- Hu, N., Sedmera, D., Yost, H.J., and Clark, E.B. (2000). Structure and function of the developing zebrafish heart. *Anat Rec* 260, 148-157.
- Hu, N., Yost, H.J., and Clark, E.B. (2001). Cardiac morphology and blood pressure in the adult zebrafish. *Anat Rec* 264, 1-12.
- Ikeda, K., Kundu, R.K., Ikeda, S., Kobara, M., Matsubara, H., and Quertermous, T. (2006). Glia maturation factor-gamma is preferentially expressed in microvascular endothelial and inflammatory cells and modulates actin cytoskeleton reorganization. *Circ Res* 99, 424-433.
- Illingworth, C.M. (1974). Trapped fingers and amputated finger tips in children. *J Pediatr Surg* 9, 853-858.
- Irvine, M.K. (2007). Conserved mechanisms regulate outgrowth in zebrafish fins. *Nat Chem Biol* 3, 613-618.

- Itescu, S., Schuster, M.D., and Kocher, A.A. (2003). New directions in strategies using cell therapy for heart disease. *J Mol Med* 81, 288-296.
- Jazwinska, A., Badakov, R., and Keating, M.T. (2007). Activin-betaA signaling is required for zebrafish fin regeneration. *Curr Biol* 17, 1390-1395.
- Johnson, S.L., and Weston, J.A. (1995). Temperature-sensitive mutations that cause stage-specific defects in Zebrafish fin regeneration. *Genetics* 141, 1583-1595.
- Jugdutt, B.I., Joljart, M.J., and Khan, M.I. (1996). Rate of collagen deposition during healing and ventricular remodeling after myocardial infarction in rat and dog models. *Circulation* 94, 94-101.
- Kajstura, J., Leri, A., Finato, N., Di Loreto, C., Beltrami, C.A., and Anversa, P. (1998). Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl Acad Sci U S A* 95, 8801-8805.
- Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Marti, M., Dubova, I., and Izpisua Belmonte, J.C. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. *Genes Dev* 20, 3232-3237.
- Kintner, C.R., and Brockes, J.P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating limb regeneration. *Nature* 308, 67-69.
- Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H.H., and Tanaka, E.M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature* 460, 60-65.
- Kuhn, B., del Monte, F., Hajjar, R.J., Chang, Y.S., Lebeche, D., Arab, S., and Keating, M.T. (2007). Periostin induces proliferation of

- differentiated cardiomyocytes and promotes cardiac repair. *Nat Med* 13, 962-969.
- Kumar, A., Velloso, C.P., Imokawa, Y., and Brockes, J.P. (2000). Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. *Dev Biol* 218, 125-136.
- Kumar, A., Gates, P.B., and Brockes, J.P. (2007a). Positional identity of adult stem cells in salamander limb regeneration. *C R Biol* 330, 485-490.
- Kumar, A., Godwin, J.W., Gates, P.B., Garza-Garcia, A.A., and Brockes, J.P. (2007b). Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. *Science* 318, 772-777.
- Kwan, K.M., Fujimoto, E., Grabher, C., Mangum, B.D., Hardy, M.E., Campbell, D.S., Parant, J.M., Yost, H.J., Kanki, J.P., and Chien, C.B. (2007). The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn* 236, 3088-3099.
- Laugwitz, K.L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L.Z., Cai, C.L., Lu, M.M., Reth, M., Platoshyn, O., Yuan, J.X., Evans, S., and Chien, K.R. (2005). Postnatal *Isl1*<sup>+</sup> cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* 433, 647-653.
- Lemanski, L.F., Fitts, E.P., and Marx, B.S. (1975). Fine structure of the heart in the Japanese Medaka, *Oryzias latipes*. *J Ultrastruct Res* 53, 37-65.
- Lepilina, A., Coon, A.N., Kikuchi, K., Holdway, J.E., Roberts, R.W., Burns, C.G., and Poss, K.D. (2006). A dynamic epicardial injury response

- supports progenitor cell activity during zebrafish heart regeneration. *Cell* 127, 607-619.
- Lien, C.L., Schebesta, M., Makino, S., Weber, G.J., and Keating, M.T. (2006). Gene expression analysis of zebrafish heart regeneration. *PLoS Biol* 4, e260.
- Lo, D.C., Allen, F., and Brockes, J.P. (1993). Reversal of muscle differentiation during urodele limb regeneration. *Proc Natl Acad Sci U S A* 90, 7230-7234.
- Lutgens, E., Daemen, M.J., de Muinck, E.D., Debets, J., Leenders, P., and Smits, J.F. (1999). Chronic myocardial infarction in the mouse: cardiac structural and functional changes. *Cardiovasc Res* 41, 586-593.
- Makino, S., Whitehead, G.G., Lien, C.L., Kim, S., Jhavar, P., Kono, A., Kawata, Y., and Keating, M.T. (2005). Heat-shock protein 60 is required for blastema formation and maintenance during regeneration. *Proc Natl Acad Sci U S A* 102, 14599-14604.
- Mathers, C.D., Fat, D.M., Inoue, M., Rao, C., and Lopez, A.D. (2005). Counting the dead and what they died from: an assessment of the global status of cause of death data. *Bull World Health Organ* 83, 171-177.
- Mathew, L.K., Andreasen, E.A., and Tanguay, R.L. (2006). Aryl hydrocarbon receptor activation inhibits regenerative growth. *Mol Pharmacol* 69, 257-265.
- Mathew, L.K., Sengupta, S., Kawakami, A., Andreasen, E.A., Lohr, C.V., Loynes, C.A., Renshaw, S.A., Peterson, R.T., and Tanguay, R.L. (2007). Unraveling tissue regeneration pathways using chemical genetics. *J Biol Chem* 282, 35202-35210.

- Matz, D.G., Oberpriller, J.O., and Oberpriller, J.C. (1998). Comparison of mitosis in binucleated and mononucleated newt cardiac myocytes. *Anat Rec* 251, 245-255.
- Mauro, A., and Adams, W.R. (1961). The structure of the sarcolemma of the frog skeletal muscle fiber. *J Biophys Biochem Cytol* 10(4)Suppl, 177-185.
- McDonnell, T.J., and Oberpriller, J.O. (1983). The atrial proliferative response following partial ventricular amputation in the heart of the adult newt. A light and electron microscopic autoradiographic study. *Tissue Cell* 15, 351-363.
- McDonnell, T.J., and Oberpriller, J.O. (1984). The response of the atrium to direct mechanical wounding in the adult heart of the newt, *Notophthalmus viridescens*. An electron-microscopic and autoradiographic study. *Cell Tissue Res* 235, 583-592.
- McGann, C.J., Odelberg, S.J., and Keating, M.T. (2001). Mammalian myotube dedifferentiation induced by newt regeneration extract. *Proc Natl Acad Sci U S A* 98, 13699-13704.
- Meng, X., Noyes, M.B., Zhu, L.J., Lawson, N.D., and Wolfe, S.A. (2008). Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol* 26, 695-701.
- Mescher, A.L. (1976). Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *J Exp Zool* 195, 117-128.
- Mescher, A.L., and Neff, A.W. (2005). Regenerative capacity and the developing immune system. *Adv Biochem Eng Biotechnol* 93, 39-66.

- Michalopoulos, G.K., and DeFrances, M.C. (1997). Liver regeneration. *Science* 276, 60-66.
- Morgan, T. (1901). *Regeneration* (New York, Macmillan).
- Murphey, R.D., and Zon, L.I. (2006). Small molecule screening in the zebrafish. *Methods* 39, 255-261.
- Nakatsuji, S., Yamate, J., Kuwamura, M., Kotani, T., and Sakuma, S. (1997). In vivo responses of macrophages and myofibroblasts in the healing following isoproterenol-induced myocardial injury in rats. *Virchows Arch* 430, 63-69.
- Nasevicius, A., and Ekker, S.C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 26, 216-220.
- Nechiporuk, A., and Keating, M.T. (2002). A proliferation gradient between proximal and *msxb*-expressing distal blastema directs zebrafish fin regeneration. *Development* 129, 2607-2617.
- Nechiporuk, A., Poss, K.D., Johnson, S.L., and Keating, M.T. (2003). Positional cloning of a temperature-sensitive mutant *emmental* reveals a role for *sly1* during cell proliferation in zebrafish fin regeneration. *Dev Biol* 258, 291-306.
- Niethammer, P., Grabher, C., Look, A.T., and Mitchison, T.J. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature* 459, 996-999.
- Nuccitelli, R. (2003). A role for endogenous electric fields in wound healing. *Curr Top Dev Biol* 58, 1-26.
- Oberpriller, J., and Oberpriller, J.C. (1971). Mitosis in Adult Newt Ventricle. *J Cell Biol* 49, 560-563.
- Oberpriller, J.O., and Oberpriller, J.C. (1974). Response of the adult newt ventricle to injury. *J Exp Zool* 187, 249-253.



- Odelberg, S.J., Kollhoff, A., and Keating, M.T. (2000). Dedifferentiation of mammalian myotubes induced by *msx1*. *Cell* 103, 1099-1109.
- Odelberg, S.J. (2005). Cellular plasticity in vertebrate regeneration. *Anat Rec B New Anat* 287, 25-35.
- Okada, T.S. (1996). A brief history of regeneration research-For admiring Professor Niazi's discovery of the effect of vitamin A on regeneration. *J Biosci* 21, 261-271.
- Packham, I.M., Gray, C., Heath, P.R., Hellewell, P.G., Ingham, P.W., Crossman, D.C., Milo, M., and Chico, T.J. (2009). Microarray profiling reveals CXCR4a is downregulated by blood flow in vivo and mediates collateral formation in zebrafish embryos. *Physiol Genomics* 38, 319-327.
- Pellettieri, J., and Sanchez Alvarado, A. (2007). Cell turnover and adult tissue homeostasis: from humans to planarians. *Annu Rev Genet* 41, 83-105.
- Peterson, R.T., Link, B.A., Dowling, J.E., and Schreiber, S.L. (2000). Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc Natl Acad Sci U S A* 97, 12965-12969.
- Petronczki, M., Lenart, P., and Peters, J.M. (2008). Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1. *Dev Cell* 14, 646-659.
- Poss, K.D., Nechiporuk, A., Hiram, A.M., Johnson, S.L., and Keating, M.T. (2002a). *Mps1* defines a proximal blastemal proliferative compartment essential for zebrafish fin regeneration. *Development* 129, 5141-5149.
- Poss, K.D., Wilson, L.G., and Keating, M.T. (2002b). Heart regeneration in zebrafish. *Science* 298, 2188-2190.

- Poss, K.D., Keating, M.T., and Nechiporuk, A. (2003). Tales of regeneration in zebrafish. *Dev Dyn* 226, 202-210.
- Pugach, E.K., Li, P., White, R., and Zon, L. (2009). Retro-orbital injection in adult zebrafish. *J Vis Exp*.
- Qian, F., Zhen, F., Ong, C., Jin, S.W., Meng Soo, H., Stainier, D.Y., Lin, S., Peng, J., and Wen, Z. (2005). Microarray analysis of zebrafish cloche mutant using amplified cDNA and identification of potential downstream target genes. *Dev Dyn* 233, 1163-1172.
- Raya, A., Koth, C.M., Buscher, D., Kawakami, Y., Itoh, T., Raya, R.M., Sternik, G., Tsai, H.J., Rodriguez-Esteban, C., and Izpisua-Belmonte, J.C. (2003). Activation of Notch signaling pathway precedes heart regeneration in zebrafish. *Proc Natl Acad Sci U S A* 100, 11889-11895.
- Raya, A., Consiglio, A., Kawakami, Y., Rodriguez-Esteban, C., and Izpisua-Belmonte, J.C. (2004). The zebrafish as a model of heart regeneration. *Cloning Stem Cells* 6, 345-351.
- Reyer, R.W. (1954). Regeneration of the lens in the amphibian eye. *Q Rev Biol* 29, 1-46.
- Rosania, G.R., Chang, Y.T., Perez, O., Sutherlin, D., Dong, H., Lockhart, D.J., and Schultz, P.G. (2000). Myoseverin, a microtubule-binding molecule with novel cellular effects. *Nat Biotechnol* 18, 304-308.
- Salo, E., and Baguna, J. (2002). Regeneration in planarians and other worms: New findings, new tools, and new perspectives. *J Exp Zool* 292, 528-539.
- Salo, E. (2006). The power of regeneration and the stem-cell kingdom: freshwater planarians (Platyhelminthes). *Bioessays* 28, 546-559.

- Salo, E., Abril, J.F., Adell, T., Cebria, F., Eckelt, K., Fernandez-Taboada, E., Handberg-Thorsager, M., Iglesias, M., Molina, M.D., and Rodriguez-Esteban, G. (2009). Planarian regeneration: achievements and future directions after 20 years of research. *Int J Dev Biol* 53, 1317-1327.
- Sanchez Alvarado, A. (2006). Planarian regeneration: its end is its beginning. *Cell* 124, 241-245.
- Sanchez, P.L., Villa, A., Sanz, R., Dominguez, M., Ludwig, I., Fernandez, M.E., and Fernandez-Aviles, F. (2007). Present and future of stem cells for cardiovascular therapy. *Ann Med* 39, 412-427.
- Sandmann, T., Jensen, L.J., Jakobsen, J.S., Karzynski, M.M., Eichenlaub, M.P., Bork, P., and Furlong, E.E. (2006). A temporal map of transcription factor activity: *mef2* directly regulates target genes at all stages of muscle development. *Dev Cell* 10, 797-807.
- Schebesta, M., Lien, C.L., Engel, F.B., and Keating, M.T. (2006). Transcriptional profiling of caudal fin regeneration in zebrafish. *ScientificWorldJournal* 6 *Suppl 1*, 38-54.
- Simon, H.G., Nelson, C., Goff, D., Laufer, E., Morgan, B.A., and Tabin, C. (1995). Differential expression of myogenic regulatory genes and *Msx-1* during dedifferentiation and redifferentiation of regenerating amphibian limbs. *Dev Dyn* 202, 1-12.
- Singer, M. (1952). The influence of the nerve in regeneration of the amphibian extremity. *Q Rev Biol* 27, 169-200.
- Smith, A., Avaron, F., Guay, D., Padhi, B.K., and Akimenko, M.A. (2006). Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblasts differentiation and function. *Dev Biol* 299, 438-454.

- Solnica-Krezel, L., Schier, A.F., and Driever, W. (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* 136, 1401-1420.
- Soonpaa, M.H., Oberpriller, J.O., and Oberpriller, J.C. (1992). Stimulation of DNA synthesis by PDGF in the newt cardiac myocyte. *J Mol Cell Cardiol* 24, 1039-1046.
- Soonpaa, M.H., Oberpriller, J.O., and Oberpriller, J.C. (1994). Factors altering DNA synthesis in the cardiac myocyte of the adult newt, *Notophthalmus viridescens*. *Cell Tissue Res* 275, 377-382.
- Stainier, D.Y., and Fishman, M.C. (1992). Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev Biol* 153, 91-101.
- Stainier, D.Y., Fouquet, B., Chen, J.N., Warren, K.S., Weinstein, B.M., Meiler, S.E., Mohideen, M.A., Neuhauss, S.C., Solnica-Krezel, L., Schier, A.F., Zwartkruis, F., Stemple, D.L., Malicki, J., Driever, W., and Fishman, M.C. (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* 123, 285-292.
- Stainier, D.Y. (2001). Zebrafish genetics and vertebrate heart formation. *Nat Rev Genet* 2, 39-48.
- Stewart, S., Rojas-Munoz, A., and Izpisua Belmonte, J.C. (2007). Bioelectricity and epimorphic regeneration. *Bioessays* 29, 1133-1137.
- Stoick-Cooper, C.L., Weidinger, G., Riehle, K.J., Hubbert, C., Major, M.B., Fausto, N., and Moon, R.T. (2007). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* 134, 479-489.

- Strack, R.L., Strongin, D.E., Bhattacharyya, D., Tao, W., Berman, A., Broxmeyer, H.E., Keenan, R.J., and Glick, B.S. (2008). A noncytotoxic DsRed variant for whole-cell labeling. *Nat Methods* 5, 955-957.
- Streilein, J.W. (2003). Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol* 3, 879-889.
- Tanaka, E.M., Gann, A.A., Gates, P.B., and Brockes, J.P. (1997). Newt myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. *J Cell Biol* 136, 155-165.
- Tanaka, E.M., Drechsel, D.N., and Brockes, J.P. (1999). Thrombin regulates S-phase re-entry by cultured newt myotubes. *Curr Biol* 9, 792-799.
- Tanaka, E.M., and Ferretti, P. (2009). Considering the evolution of regeneration in the central nervous system. *Nat Rev Neurosci* 10, 713-723.
- Tanaka, E.M., and Galliot, B. (2009). Triggering the regeneration and tissue repair programs. *Development* 136, 349-353.
- Tate, J.M., McDonnell, T.J., Oberpriller, J.C., and Oberpriller, J.O. (1987). Isolation of cardiac myocytes from the adult newt, *Notophthalmus viridescens*. an electron microscopic and quantitative light microscopic analysis. *Tissue Cell* 19, 577-585.
- Tate, J.M., and Oberpriller, J.O. (1989). Primary cell culture and morphological characterization of ventricular myocytes from the adult newt, *Notophthalmus viridescens*. *Anat Rec* 224, 29-42.
- Tate, J.M., Oberpriller, J.O., and Oberpriller, J.C. (1989). Analysis of DNA synthesis in cell cultures of the adult newt cardiac myocyte. *Tissue Cell* 21, 335-342.

- Thummel, R., Bai, S., Sarras, M.P., Jr., Song, P., McDermott, J., Brewer, J., Perry, M., Zhang, X., Hyde, D.R., and Godwin, A.R. (2006). Inhibition of zebrafish fin regeneration using in vivo electroporation of morpholinos against *fgfr1* and *msxb*. *Dev Dyn* 235, 336-346.
- Thummel, R., Ju, M., Sarras, M.P., Jr., and Godwin, A.R. (2007). Both *Hoxc13* orthologs are functionally important for zebrafish tail fin regeneration. *Dev Genes Evol* 217, 413-420.
- Timmerman, L.A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J.M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J.C., and de la Pompa, J.L. (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* 18, 99-115.
- Tosh, D., and Slack, J.M. (2002). How cells change their phenotype. *Nat Rev Mol Cell Biol* 3, 187-194.
- Tsonis, P.A. (2000). Regeneration in vertebrates. *Dev Biol* 221, 273-284.
- Velloso, C.P., Simon, A., and Brockes, J.P. (2001). Mammalian postmitotic nuclei reenter the cell cycle after serum stimulation in newt/mouse hybrid myotubes. *Curr Biol* 11, 855-858.
- Verkhusha, V.V., Kuznetsova, I.M., Stepanenko, O.V., Zarausky, A.G., Shavlovsky, M.M., Turoverov, K.K., and Uversky, V.N. (2003). High stability of Discosoma DsRed as compared to Aequorea EGFP. *Biochemistry* 42, 7879-7884.
- Wagers, A.J., and Weissman, I.L. (2004). Plasticity of adult stem cells. *Cell* 116, 639-648.
- Walker, M.G. (2003). Gene expression versus sequence for predicting function: Glia Maturation Factor gamma is not a glia maturation factor. *Genomics Proteomics Bioinformatics* 1, 52-57.

- Walsh, E.C., and Stainier, D.Y. (2001). UDP-glucose dehydrogenase required for cardiac valve formation in zebrafish. *Science* 293, 1670-1673.
- Weiss, E., and Winey, M. (1996). The *Saccharomyces cerevisiae* spindle pole body duplication gene *MPS1* is part of a mitotic checkpoint. *J Cell Biol* 132, 111-123.
- White, R.M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., Burns, C.E., and Zon, L.I. (2008). Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* 2, 183-189.
- Whitehead, G.G., Makino, S., Lien, C.L., and Keating, M.T. (2005). *fgf20* is essential for initiating zebrafish fin regeneration. *Science* 310, 1957-1960.
- Yamada, T. (1977). Control mechanisms in cell-type conversion in newt lens regeneration. *Monogr Dev Biol* 13, 1-126.
- Yang, E.V., Gardiner, D.M., Carlson, M.R., Nugas, C.A., and Bryant, S.V. (1999). Expression of *Mmp-9* and related matrix metalloproteinase genes during axolotl limb regeneration. *Dev Dyn* 216, 2-9.
- Yanushevich, Y.G., Staroverov, D.B., Savitsky, A.P., Fradkov, A.F., Gurskaya, N.G., Bulina, M.E., Lukyanov, K.A., and Lukyanov, S.A. (2002). A strategy for the generation of non-aggregating mutants of Anthozoa fluorescent proteins. *FEBS Lett* 511, 11-14.
- Zauner, H., Begemann, G., Mari-Beffa, M., and Meyer, A. (2003). Differential regulation of *msx* genes in the development of the gonopodium, an intromittent organ, and of the "sword," a sexually selected trait of swordtail fishes (*Xiphophorus*). *Evol Dev* 5, 466-477.

