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PhD thesis

Zebrafish (*Danio rerio*): a model to study *in-vivo* and *ex-vivo* cardiac regeneration

Scientific disciplinary sector: (BIO/06)

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Tesi di Dottorato di Ricerca

Zebrafish (*Danio rerio*): un modello per studiare la rigenerazione cardiaca *in-vivo* ed in *ex-vivo*

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Steps

As every blossom fades
and all youth sinks into old age,
so every life's design, each flower of wisdom,
attains its prime and cannot last forever.
The heart must submit itself courageously
to life's call without a hint of grief,
A magic dwells in each beginning,
protecting us, telling us how to live.
High purposed we shall traverse realm on realm,
cleaving to none as to a home,
the world of spirit wishes not to fetter us
but raise us higher, step by step.
Scarce in some safe accustomed sphere of life
have we establish a house, then we grow lax;
only he who is ready to journey forth
can throw old habits off.
Maybe death's hour too will send us out new-born
towards undreamed-lands,
maybe life's call to us will never find an end
Courage my heart, take leave and fare thee well.
(Hermann Hesse)

To my nephew Leonardo

Gradini

Come ogni fior languisce e
giovinezza cede a vecchiaia,
anche la vita in tutti i gradi suoi fiorisce,
insieme ad ogni senno e virtù, e può durare eterna.
Quando la vita chiama, il cuore
sia pronto a partire ed a ricominciare,
per offrirsi sereno e valoroso ad altri, nuovi vincoli e legami.
Ogni inizio contiene una magia
che ci protegge e a vivere ci aiuta.
Dobbiamo attraversare spazi e spazi,
senza fermare in alcun d'essi il piede,
lo spirto universal non vuol legarci,
ma su di grado in grado sollevarci.
Appena ci avvezziamo ad una sede
rischiamo d'infiacchire nell'ignavia:
sol chi e' disposto a muoversi e partire
vince la consuetudine inceppante.
Forse il momento stesso della morte
ci farà andare incontro a nuovi spazi:
della vita il richiamo non ha fine....
Su, cuore mio, congedati e guarisci.
(Hermann Hesse)

A mio nipote Leonardo

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ABSTRACT (English version)

Despite continuing progress in medical therapies and in revascularization strategies at coronaries level, heart diseases remains a leading cause of mortality throughout industrialized countries. Mammals have an extremely limited capacity to repair damaged heart tissue after a myocardial infarction. Regeneration, the replacement of damaged or lost body parts, is a primary goal of stem cell research. In fact, during this process, differentiated cells reenter the cell cycle and proliferate to generate a mass of undifferentiated cells. Several vertebrates display different ability to regenerate organs and tissues, thus encouraging biologists to seek out models for heart regeneration. The robust capacity of zebrafish (*Danio rerio*) to regenerate a variety of tissues, position it as an ideal genetic model system for understanding the molecular and cellular events governing regeneration. Numerous works have shown that adult zebrafish can effectively regenerate *in-vivo* injured hearts submitted to partial surgical amputation of the ventricle area. In fact, during this process, new cardiomyocytes limit the scar formation and form new muscular tissue. However the molecular mechanisms that regulate this process remains unclear and resolving the genes, the microRNAs and the proteomic alterations that control these changes can illuminate how heart regeneration is naturally optimized. Activation of the regenerative potential of human tissue implicates a novel therapeutic approach to supplement, or replace, conventional pharmacotherapy and mechanical intervention.

In this thesis, using qRT-PCR experiments, it was demonstrated that some microRNAs (miRNAs), as miR1 and miR-133, were down-regulated during the *in-vivo* regeneration of adult zebrafish hearts submitted to amputation of around 20% of the ventricle apex. Also, similar changing in microRNAs levels were observed during hypertrophic conditions induced *ex-vivo*. The adult zebrafish heart, containing numerous cardiac progenitors, resulted a good model to approach the general mechanisms of adult cardiac stem cell maintenance and cardiogenesis. Here, zebrafish was also used as model to test for a correlation between Fibroblast Growth factors (FGFs) signaling and cell proliferation in adult injured hearts. With *In Situ* Hybridization (ISH) experiments, here were reported the time and space expression of FGFs targets genes (*erm*, *etv5*, *pea3*, *dusp6*, *sef sprouty4,* and *raldh2*), involved in *in-vivo* heart regeneration. With the aim to optimize the media to reproduce the regeneration process, it was also tested the ability of zebrafish heart to survive in *ex-vivo* cultures after the amputation of ventricular apex. Regenerating hearts in *ex-vivo* conditions were able to survive

and make contractions, and surprisingly showed different degree of cell replication, incorporating BrdU, in dependence to the culture media.

So far, there is no study regarding the proteomic aspect the heart regeneration in zebrafish. Proteomic offers an innovative approach to integrate the genomic study that alone is not sufficient to understand the all the cellular mechanisms. Here, for the fist time, it is reported a proteomic study of adult zebrafish heart, that allowed the identification of more than one hundred proteins, belonging to different biological classes as, enzymes, signal transduction, growth factors, cytoskeletal components, globin, and structural proteins. The identification of the proteins naturally expressed in the adult zebrafish heart is not only important as basic biological knowledge, but also, because it constitute a reference point to compare proteins expression during cardiac regeneration. In fact, applying innovative and sensible two-dimensional fluorescence difference gel electrophoresis (DiGE), combined with MALDI-TOF/TOF tandem mass spectrometry, here it was possible to identify proteins differentially expressed at 3 days after amputation of around 20% of the ventricle area, compared with hearts not injured. Among more than one hundred proteins spots detected in 2D-gels, three (ATP synthase, hyaluronan mediated motility receptor, and desmuslin) were up-regulated in injured hearts. Information concerning the global alteration protein pattern during heart regeneration will be helpful for a better formulation of new diagnostic and therapeutic markers. Also, due to the similarities between the fish and mammalian genomes, this experimental system should also provide clues to understanding human pathophysiology and the new frontier of stem cell regeneration system.

ABSTRACT (Versione Italiana)

Nonostante i continui progressi nelle terapie mediche e nelle strategie di rivascolarizzazione a livello delle coronarie, le malattie cardiache rimangono la principale causa di morte nei paesi industrializzati. I mammiferi mostrano una limitata capacità di riparare il tessuto cardiaco danneggiato dopo un infarto al miocardio. La rigenerazione, il rimpiazzo di parti corporee danneggiate o amputate, è l'obiettivo primario della ricerca sulle cellule staminali. Infatti, durante questo processo, cellule differenziate rientrano nel ciclo cellulare e proliferano per generare una massa di cellule indifferenziate. La capacità di rigenerare organi e tessuti varia ampiamente tra i vertebrati, incoraggiando i biologi a cercare modelli alternativi di rigenerazione cardiaca. La peculiare caratteristica del pesce zebra (*Danio rerio*) di rigenerare vari tessuti, lo ha promosso sistema modello genetico ideale per capire i meccanismi molecolari e cellulari alla base del processo rigenerativo. Numerosi lavori hanno dimostrato che il pesce zebra (o zebrafish) adulto può efficacemente rigenerare *in-vivo* il cuore soggetto a parziale amputazione del ventricolo. Durante questo processo, infatti, i cardiomiociti, stimolati a replicare da numerosi fattori di crescita, rimpiazzano la cicatrice con nuovo tessuto muscolare. Tuttavia, i meccanismi molecolari che regolano la rigenerazione rimangono poco chiari e, caratterizzare i geni, i microRNA e le alterazioni proteomiche che controllano questi cambiamenti, può offrire dati importanti su come la rigenerazione cardiaca sia naturalmente ottimizzata. L'attivazione del potenziale rigenerativo del tessuto umano comporterebbe un approccio terapeutico innovativo per supplementare o, addirittura rimpiazzare la farmacoterapia convenzionale e l'intervento meccanico.

In questo lavoro di tesi, attraverso esperimenti di qRT-PCR, è stato possibile dimostrare che alcuni microRNA (miRNA), quali miR1 e miR133, vengono down-regolati durante la rigenerazione *in-vivo* del cuore adulto di zebrafish, sottoposto ad amputazione di circa il 20% dell'apice ventricolare. Inoltre, simili variazioni dei livelli dei microRNA sono state osservate durante condizioni d'ipertrofia indotte *ex-vivo*. Il cuore di zebrafish adulto, contenendo numerosi progenitori cardiaci, è risultato un modello vantaggioso per capire i meccanismi che regolano il mantenimento delle cellule staminali adulte e la cardiogenesi. In questo lavoro, il pesce zebra è stato utilizzato anche come modello per testare l'esistenza di una correlazione tra la segnalazione dei Fattori di Crescita dei Fibroblasti (FGF) e la proliferazione cellulare nei cuori adulti operati. Attraverso esperimenti di Ibridazione *In Situ* (ISH), sono state studiate le espressioni nel tempo e nello spazio di geni target degli FGF

(*erm*, *etv5*, *pea3*, *dusp6*, *sef sprouty4,* and *raldh2*), implicati nella rigenerazione cardiaca. Con lo scopo di ottimizzare il mezzo di coltura per riprodurre il processo rigenerativo, è stata inoltre testate la capacità dello zebrafish di sopravvivere in colture *ex-vivo*, dopo l'amputazioe dell'apice ventricolare. I cuori lasciati rigenerare in colture *ex-vivo* sono stati in grado di sopravvivere e di emettere contrazioni e, sorprendentemente, hanno mostrato diversi gradi di replicazione cellulare, incorporando BrdU, in dipendenza al terreno di coltura.

Ad oggi, non sono presenti studi riguardanti l'aspetto proteomico della rigenerazione cardiaca in zebrafish. La proteomica offre un approccio innovativo per integrare lo studio genomico che da solo non è sufficiente per comprendere tutti i meccanismi cellulari. In questo studio, per la prima volta, è stato applicato un approccio proteomico per lo studio del cuore di zebrafish adulto. Ciò ha permesso l'identificazione di oltre cento proteine, appartenenti a diverse classi biologiche quali, enzimi, trasduttori di segnale, fattori di crescita, componenti citoscheletrici, globine e proteine strutturali. L'identificazione di proteine naturalmente espresse nel cuore di zebrafish adulto, non solo rappresenta un'importante conoscenza biologica di base, ma costituisce inoltre un punto di riferimento per il confronto delle espressioni proteiche durante la rigenerazione cardiaca. Infatti, in questo studio, applicando l'innovativa e sensibile tecnologia di elettroforesi bidimensionale differenziale (DiGE), combinata con spettrometria di massa tandem MALDI-TOF/TOF, e' stato possibile identificare proteine diversamente espresse a 3 giorni dopo l'amputazione di circa il 20% dell'area ventricolare, rispetto a cuori di controllo non operati. Tra i più di cento spot proteici osservati con gel 2D, tre (ATP sintasi, recettore di motilità mediata da ialurone, e desmuslina), sono risultati up-regolati nei cuori in rigenerazione. Le informazioni riguardanti le alterazioni proteiche globali durante la rigenerazione cardiaca saranno utili per migliorare la formulazione di nuovi farmaci e marcatori terapeutici. Inoltre, data la forte somiglianza del genoma tra pesce e uomo, questo sistema sperimentale potrebbe offrire informazioni utili per capire le malattie umane e offrire nuove opportunità di rigenerazione mediante l'utilizzo di cellule staminali.

CHAPTER I: INTRODUCTION

CARDIAC DISEASES AND NEW INSIGHT FOR REGENERATIVE MEDICINE

In industrialized countries, cardiac diseases are the main causes of chronic heart failure, the leading cause of human mortality at present, and the expected largest cause of disease burden worldwide by 2020 (Gottlieb et al., 2007). Recent estimates indicate that end-stage heart failure with 2-year mortality rates of 70-80% affects over 60,000 patients in the United States each year. The vast majority are cardiomyopathies (with diminished contractility, lower cardiac output, and generally thin ventricular walls) and channelopathies, as arrhythmias (anomalies of electrical conduction and heart rhythm) (Leinwand, 2003). Myocardial infarction (MI) results in large-scale loss of cardiac muscle (often a billion or more myocytes) whereas other heart diseases, such as hypertension, valve diseases and genetic disorders (for examples, cardiomyopathies), cause more sporadic myocyte loss (Laflamme and Murry, 2005). MI most commonly results from coronary occlusions, due to a thrombus overlying an atherosclerotic plaque. Unfortunately, nowadays heart failure carries a poor prognosis with few treatment options and no effective therapy. In fact, once overt heart failure develops, about 30-45% of patients die within 1 year, unless they receive a heart transplant, the unique clinical procedure to reverse the decline in cardiac function and to restore damaged myocardium (Hosenpud et al., 1999). However, this option is available to very few patients due to a shortage of donor hearts and in addition it suffers from well known side effects arising from the accompanying medications as well as graft failure over the long term. Drug therapy is limited for all causes of heart failure and is palliative at best, with definitive cure for the most severe cases limited to those fortunate enough to receive heart transplantation. Repair is one of the major mechanisms needed for survival and proper functioning; however, defective repair with scar formation is an inevitable consequence of tissue damage, compromising the functions of the organ to a certain degree. The concept of stem cell-based therapies originated with the idea of replenishing damaged tissues with stem cells to increase the tissue's potential for repair (Scorsin et al., 1996; Oh et al., 2003; Cai et al., 2004; Wu et al., 2006a).

The discovery of stem cells capable of generating angiogenic or contractile cells and structures offers new horizons to patients suffering from myocardial diseases, points to the potential of cell therapy as a future treatment strategy for heart failure (Connold et al., 1997; Smith, 1998; Penn et al., 2002). Actually several research groups are working in the potential

for stem cells-based translational approach to explore the possibility to efficiently regenerate the heart (Gojo et al., 1997; Davani et al., 2005; Smits et al., 2005). Recent reports from different laboratories have shown that the heart of human adult as well as that of other species contains a population of resident stem cells that may differentiate into myoblasts and eventually into adult myocytes (Hierlihy et al., 2002; Abbott and Giordano, 2003; Tateishi et al., 2007). Today, various types of stem cells obtained from different tissues are commonly used for myocardial regeneration (Chiu et al., 1995; Taylor et al., 1998; Ooi et al., 2006; Wu et al., 2007; Piao et al., 2007. For reviews see Marín-García and Goldenthal, 2006; Segers and Lee, 2008). Stem cells are classified in totipotent, pluripotent and multipotent, according to their ability to generate other cell types (**Figure 1**). More precisely, totipotency refers to the ability of a cell to form cells of all lineages including the extra-embryonic tissue. In mammals, the only cells with this capacity are the zygote and early blastomeres. Pluripotency is the term applied to cells that have the ability to differentiate into all the cell types of the body, except the extra-embryonic tissue (placenta) (Pera, 2001). Multipotent stem cells are those that have the ability to differentiate into a limited number of different cell types (Reyes and Verfaillie, 2001). The stem cells reside in specific regions of tissues to ensure the organ development throughout embryonic, fetal and adult life and divide in case of tissue loss and injury (Nichols, 2001; Thisse and Zon, 2002). A stem cell is clonogenic, capable of unlimited self-renewal by symmetric division, while maintaining a stable diploid karyotype (Linke et al., 2005). Under the control of growth factors and the tissue environment, it is also capable of asymmetric division, one daughter resembling its mother, and one daughter giving rise to multiple types of differentiated cells (Fijnvandraat et al., 2003). In contrast, the progenitor cells that have been identified in adult organs thus far do not meet all of these criteria (Case et al., 2008).

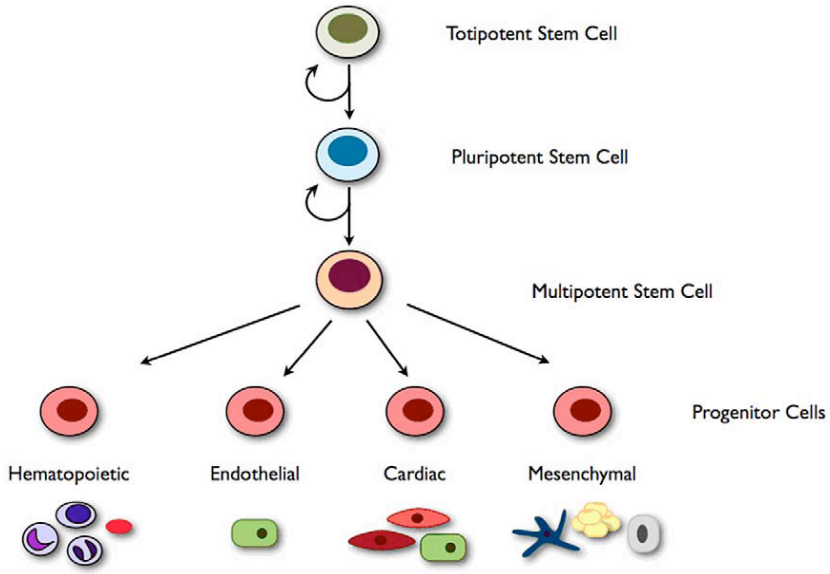

Figure 1 Stem cell hierarchy. Illustration of the stem cell hierarchy. Totipotency refers to the ability of a stem cell to form all cell types including extra embryonic tissue. Pluripotent stem cells are capable of unlimited self-renewal and can differentiate into any adult cell type (e.g. embryonic stem cells (ESCs) and induced pluripotent cells (iPS) cells). Multipotent stem cells have the ability to differentiate into a limited number of different cell types (e.g. hematopoietic stem cells (HSCs)). These multipotent stem cells can give rise to progenitor cells which can be multipotent (e.g. cardiac progenitor cells can differentiate into endothelial cells, smooth muscle cells or cardiomyocytes) or monopotent, giving rise to only one cell type (e.g. endothelial progenitor cells (EPCs)). From Sieveking and Martin, (2009).

Several alternatives approaches, objectives of the regenerative medicine, not mutually exclusive, may be employed to induce heart regeneration as preventing cell death or repopulating myocardium with new contractile cells (Foley and Mercola, 2004) (**Figure 2**). The most investigated strategy is implantation of stem cells into the heart, although this method is invasive, often clinically unsuitable, and can introduce harmful scar tissue, arrhythmia, calcification, or microinfarction in the heart (Mathur and Martin, 2004). Cells can be injected directly through the coronary arteries or using an intraventricular approach (Zhan-quan et al., 2007). Whit the aim of therapeutic neo-vascularization the cells need to be mobilized from their location with factors such as cytokines in response to an external stimulus, such as tissue ischemia (Wu et al, 2006b).

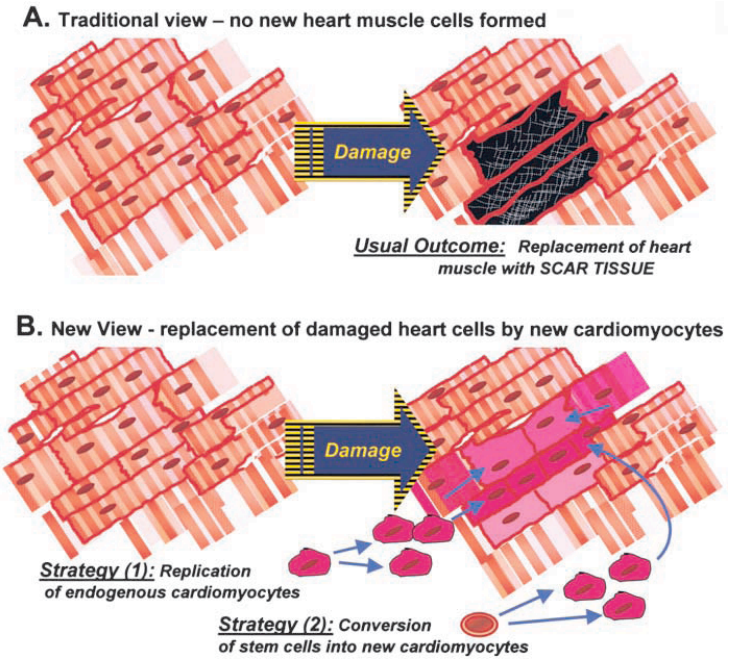

Figure 2 Repair of damaged heart muscle. From Grounds et al., (2002).

Then they need to successfully home to the site of ischemia and participate in neo-vessel formation. Stem cells treated groups shown improved left ventricular (L.V.) ejection fraction, reduced infarct scar size and decrease L.V. end-systolic volume. A recently developed "cell sheet engineering" technology has greatly improved the efficiency and efficacy of cell engraftment (Masuda et al., 2008). Cell-based cardiac repair offers the promise of rebuilding the injured heart from its component parts (Bai et al., 2007). Work began with the transplantation of authologous skeletal muscle satellite cells (commonly referred as myoblasts), progenitors committed cells that normally mediate regeneration of skeletal muscle (Baroffio et al., 1996; Leor et al., 1996), but recently the field has expanded to explore an array of cell types, including bone marrow cells, endothelian progenitors, mesenchymal stem cells, resident cardiac stem cells, and both mouse and human embryonic stem cells (Jia et al., 1997; Sakai et al., 1999; Rangappa et al., 2002; Sachinidis et al., 2003; Kang et al., 2007) (**Figure 3**). Recent clinical trials injecting bone marrow or skeletal myoblasts into the injured heart have yielded mixed results in term of cardiac regeneration (Murry et al., 1996; Dorfman et al., 1998; Gulbins et al., 2001; Xu et al., 2007b) and are not able to convert into true cardiomyocytes that could replace those irreversibly loss by heart attack (Atkins et al., 1999).

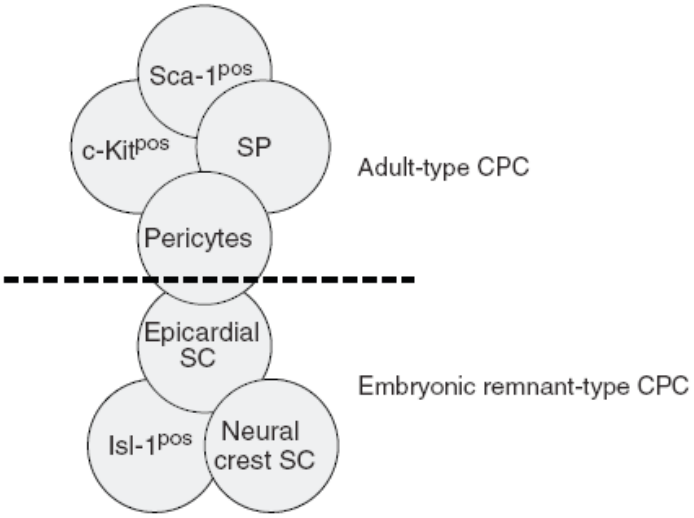

Figure 3 Schematic depiction of the resident cardiac stem cell pool. The majority of Cardiac Progenitors Cells (CPC) represent typical adult stem cells may originate from the bone marrow. They can express c-kit or Sca-1, have SP properties, or be found in the perivascular niche. Other CPC may be remnants from embryologic heart development and express isl-1, display neural crest cell characteristics, or be found within the epicardium. To what extent the two groups and their cell populations overlap is not known. From Stamm et al., (2009).

New insight in experimental cell therapy for heart failure has been given by Messina et al (2004) and Barile et al (2007): in these studies the Authors described the successful achievement of the engraftment and differentiation of the injected cells (adult, autologous, biopsies-derived human cardiac progenitors) in the infracted hearts of SCID mice, with their functional rescue in term of ejection fraction and echocardiogram. An alternative approach is therapeutically stimulating cardiomyocytes to re-enter the cell cycle and progress to mitosis and cytokinesis thought gene-mediated interventions targeting cell cycle regulators, or by injecting genes coding for mitotic cytokines (Bellafiore et al., 2006). In this way, the electromechanical syncytium is more likely to be preserved, since there would be no need for establishing new connections with implanted cells. In addition, the eventuality of rejection of allogenetic cells as well as the ethical problems derived from the use of human embryonic stem cells are avoided (Sugarman, 2007). Although adult mammalian cardiomyocytes show very little or no proliferation when cultured, FGF-1 treatment concomitant with p38 MPA kinase inhibition can stimulate their proliferation in culture. In other studies, the discovery of putative progenitors cells within the hearts of adult mammals has led to the suggestion that the

heart has the potential for homeostatic or regenerative renewal (Nemir et al., 2006). Experimental studies indicate that the delivery or mobilization of stem and progenitor cells may improve tissue perfusion and the contractile performance of the damage heart (Harding et al., 2007; Li et al., 2007b). Directional homing of stem cells is performed via the attraction of cells through the local delivery of various growth factors. Pre-clinical and clinical studies evaluating the therapeutic potential of various cell therapies have reported conflicting results, generating controversy (Min et al., 2002; Shizuru et al., 2005; Sieveking and Martin, 2009). The major barriers to the development of pharmacological agents or cell-based therapies for cardiac repair include obtaining not only an understanding of the identity of the resident cardiac stem/progenitors cells, but also the ability to generate a sufficient number of cells for therapeutic use (Goldenthal and García, 2003). Subsequent challenges involve the optimization of methods for the isolation and delivery of these cells, particularly concerning the timing of cell delivery. Thus, is important to understand how guide the differentiation of stem cells, control their survival and proliferation, identify factors that mediate their homing and modulate the heart's innate inflammatory and fibrotic responses (Hescheler et al., 1997). The discovery of several types of cardiovascular progenitor cells within the heart provides a unique opportunity to establish pharmacological agents capable of stimulating an increase in the low level of cardiac repair in mammalian hearts, reducing the scar formation and increasing cardiac function (Li et al., 1996; Solloway and Harvey, 2003).

MAMMALIAN HEART: OUTLINE OF STRUCTURE AND REGENERATIVE ABILITY

The mammalian heart is a vascular tube construct consisting of more than 20 cell types forming connective, contractile and vascular structures. Specific progenitor cells contribute to formation of the heart during fetal growth. The progenitor cells at the anterior plate mesoderm give rise to the heart. During fetal life, heart is formed from two different committed progenitor cell groups determined according to the expression of specific transcription molecules, and can be classified into primary and secondary heart fields. The primary heart field rises from the anterior splanchnic mesoderm and it gives rises to the heart crescent and later contributes to left ventricle and *atrium* formation. The secondary cardiac field is also termed the anterior heart field and it originates from the pharyngeal mesoderm located medially at the cardiac crescent. Subsequently it contributes to right ventricle and outflow tract formation (Dyer & Kirby, 2009). The development of a four-chambered heart is a multistep process and depends on unique genetic programs that are highly diverse and unique

for each species. In mice and rats, cardiomyocytes replicate actively during fetal life, but in the perinatal period proliferation ceases and myocytes undergo an additional round of DNA synthesis with nuclear division (karyokinesis), without subsequent cell division (cytokinesis). This leads to binucleation, a form of endoreduplication known as acytokinetic mitosis. The process starts about day four after birth and ends after three weeks, when over 85% of cardiomyocytes are binucleated. With the exception of humans and pigs, in the mammalian species studies so far most cardiomyocytes are binucleated (Klug et al., 1995). Pigs have a majority of tetranucleated myocytes, and cells containing up to 32 nuclei have been reported, but in the human heart most myocytes are mononucleated, and no myocytes containing more than two nuclei have been reported. Mammalian species have little or no ability to replace lost cardiac muscle. This poor regenerative capacity is due in part to the failure of adult cardiomyocytes, the beating cells in the heart, to undergo proliferation (Quaini et al., 2004). In fact, the normal response of mammalian hearts to injury or hypoxia is hypertrophy—the growth of cardiomyocytes without cell division. Although there is evidence that a stem cell-like population may exist in the adult mammalian heart, cell division in this organ is rare. For full-sized adult mammals, homeostasis maintains the *status quo*, calibrating organ size and function in response to changing physiological conditions, replacing damaged or senescent cells through direct structural cell proliferation, progenitor cell activity or hypertrophy of surrounding cells. Cardiac hypertrophy consists of hypertrophy of cardiomyocytes and hyperplasia of other cell types in the heart, such as fibroblasts (Poss et al., 2002). Indeed, although there is evidence that a stem cell–like population may exist in the adult mammalian heart, cell division in this organ is rare. Prolonged cardiac hypertrophy in humans can lead to dilation, poor contractility, and eventually heart failure and death. Hypertrophy initially results in response to load demands on the heart, and how it progress to cardiac failure is not understood at this time (Nicol et al., 2001). More recently, the discovery of cardiac stem cells and the finding that cardiomyocytes have certain capacities to proliferate challenged that concept and raised significant interest in investigating the molecular mechanisms of myocyte hyperplasia. According to a classical dogmas of mammalian heart regeneration, cardiomyocytes are terminally differentiated and become post-mitotic before or soon after birth and are generally considered to irreversibly withdraw from the cell cycle (Ahuja et al., 2007). Analysis of cardiac myocytes growth during early mammalian development indicates that cardiac myocyte DNA synthesis occurs primarily in uterus, with proliferating cells decreasing from 33% at midgestation to 2% at birth. Throughout life a mixture of young and

old cells is present in the normal myocardium. It was believed that human cardiac myocytes could not self-regenerate after an injury such as acute MI and that the main adaptive response to myocytes loss was hypertrophy, an increase in cardiomyocytes diameter without undergoing cell division. However, this dogma is now being replaced by the idea that there is some *in-vivo* proliferation of cardiomyocytes after damage. Using a mouse genetic fate-mapping strategy, Hsieh and colleagues (2007) showed strong evidence that stem or progenitor cells refreshed murine cardiomyocytes after heart injury, but not during up to one year of normal aging. Additionally, taking advantages of the incorporation of carbon-14 produced during the Cold War into DNA, Bergmann and colleagues (2009) established a way to determine the age of cardiomyocytes. Using this method, they demonstrated that human cardiomyocytes renew themselves, although at a low frequency. In mammals hearts, after an acute MI, the remaining viable myocardium undergoes a series of structural changes termed cardiac remodeling, consisting in not only in myocardial hypertrophy, but also in myocytes cell loss via apoptosis, coagulative necrosis of the myocardium (myofibrillar hypereosinophilia and loss of nuclei), nuclear ploidy, defective regeneration, infiltration of inflammatory cells such as neutrophils, progressive expansion of the initial infarct area, dilation of the left ventricular lumen and progressive replacement of contractile myocytes by a fibrin deposition at the injury site thought a process that begins around the second week and reaches its maximum about three months after infarction. Then, fibrin is replaced by scar tissue, thought to be permanent, altering left ventricular function. The development, progression and pathogenesis of heart failure is complex and multifactorial. Importantly, remodeling begins with hours of the MI and is initiated by migration of inflammatory cells, mainly macrophages and neutrophilis as well as fibroblasts, which produce tumor necrosis factor (TNF) alpha and transforming growth factor (TGF) (Janczewski et al., 2002). These cytokines stimulates mast cells and cardiac fibroblast proliferation. At the organ level, MI results in thinning of the injured wall and dilatation of the ventricular cavity, a process called ventricular remodeling. These structural changes markedly increase mechanical stress on the ventricular wall and promote progressive contractile dysfunctions. The extent of heart failure after MI is directly related to the amount of myocardium lost. When the heart is submitted to an increased workload, for example after acute MI, the cardiomyocytes enlarge and increase their ploidy status. Since polyploidization implies DNA replication, it can be assumed that adult cardiomyocytes retain the ability to enter into the cell cycle. There are evidences that under certain circumstances adult cardiomyocytes re-enter the cell cycle and advances to

mitosis but the knowledge of the triggering phenomena and the cascade of events leading to cardiomyocyte mitosis is still poor. The four phases of the mammalian cell cycle are tightly regulated at several checkpoints, ensuring that all activities are completed before initiation of the next phase, providing in this way a mechanism for the identification of defective cells. Positive cell cycle regulators (as for example, cyclins, the CDKs, and proto-oncogenes) are highly expressed in embryonic and newborn hearts, and are down-regulated in the adult heart. Various studies have used the approach of genetically targeting the cell cycle regulators to encourage the cardiomyocyte to re-enter the cell cycle and progress into mitosis and cytokinesis (Maltsev et al., 1994). However, whether these cells are derived from a resident pool of cardiomyocyte stem cells or from a renewable source of circulating bone marrow derived stem cells that home to the damaged myocardium is at present not known. Myocytes regeneration is evidenced by a ~2-fold increase in expression of cell cycle markers (Ki67 and phosphohistone H3) and ~13% reduction in mean myocytes diameter. Increased circulating levels of hepatocytes growth factor (HGF), leukemia inhibitory factor (LIF), and macrophage colony-stimulating factor (M-CSF) were associated with mobilization of c-kit$^+$, CD31$^+$, and CD133$^+$ progenitors cells and subsequent increase in myocardial c-kit$^+$ cells. It has been shown that for newt myotubes in tissues culture, the presence of thrombin and serum stimulates myonuclei to re-enter s-phase via phosporylation of the retinoblastoma (mass of undifferentiated/de-differentiated proliferating cells in the growth zone under the wound epidermis formed after damage) of amputated newt limbs (Tanaka et al., 1999). Another integral component of the remodeling process appears to be the development of neo-angiogenesis within the myocardial infarct scar, a process requiring activation of a latent collagenase and other proteinases. Loss of oxygenation to ventricular muscle, usually, because of occlusion of a coronary artery, will result in necrosis and stimulating the new angiogenesis. Moreover, recent observations suggest that a small number of human cardiomyocytes (15-25%) retain the capacity to proliferate and regenerate in response to ischemic injury (Anversa et al., 2006).

ABILITY OF ORGAN REGENERATION IN AMPHIBIAN AND ZEBRAFISH AS COMPARISON WITH MAMMALS

Regeneration is a complex biological process by which animals can restore the shape, structure and function of body parts lost after injury, or after experimental amputation. This process is an evolutionary conserved feature of vertebrate species and requires the concerted action of mechanisms inducing and regulating dedifferentiation, pattern generation, and, in certain instances, trans-differentiation events (Ausoni and Sartore, 2009). Natural scientists have actively pursued the problem of regeneration since the 17th century, largely by utilizing invertebrate and lower vertebrate species possessing exceptional regenerative capacities (Dinsmore, 1991). Newts are the primary experimental model used to study vertebrate regeneration, as they can re-grow a striking number of adult structures, including limbs, tail, spinal cord, jaws, tongue, lens and optic nerve (Brockes, 1997; Ferretti and Géraudie, 1998) (**Figure 4**). It has long been recognized that Urodele amphibians such as the newt, have a remarkable regenerative ability for many tissues. Consubstantial with survival, tissues and structures of vertebrates cope with everyday wear and tear by being continuously renewed with the progeny of resident or circulating cells, which exhibit a varying degree of plasticity (Wagers et al., 2002). The cellular mechanisms underlying the phenomenon of tissue turnover are highly dependent on the specific type of tissues, and differentiation, but not cell dedifferentiation. The restitution of tissues of structures by increased activity of normal turnover mechanisms is called “tissue restoration”. In contrast with tissues restoration, true regeneration in vertebrates is considered as a type of epimorphic regeneration. It involves the deployment of a complex set of *de novo* mechanisms including dedifferentiation of post-mitotic cells, cell proliferation, pattern generation and, in some cases, trans-differentiation of adult specialized cells to rebuild parts of the body plan after amputation or injury.

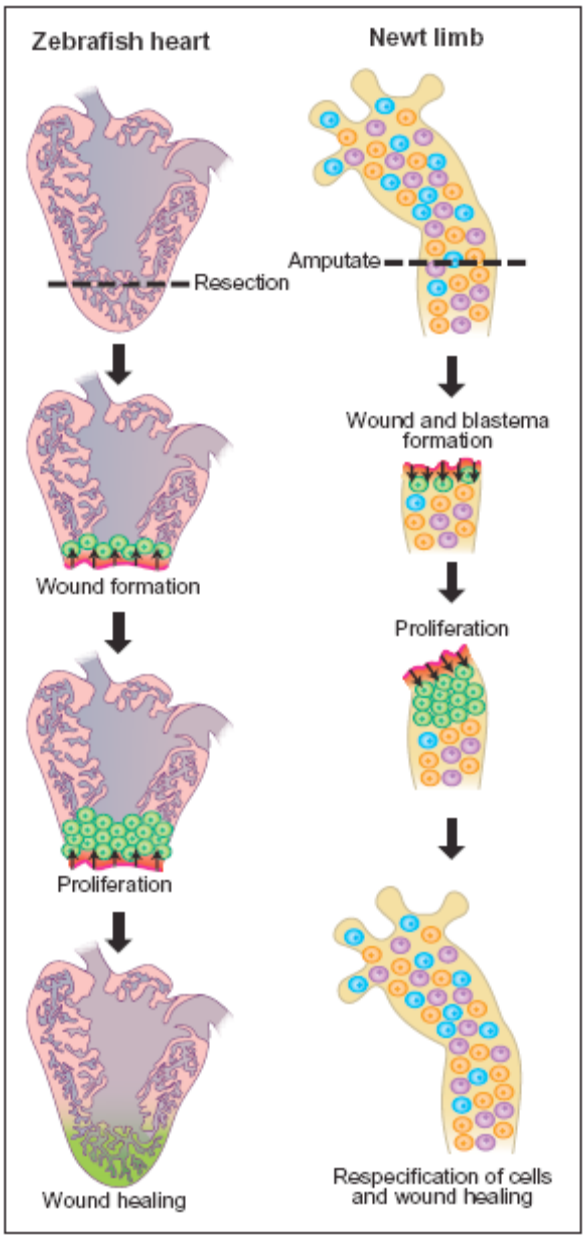

Figure 4. Regenerating hearts and limbs. (Left) Following surgical removal of a portion of the zebrafish ventricle, cardiomyocytes (green cells) adjacent to the wound site (bright red) undergo proliferation, presumably due to signals (arrows) emanating from the wound (Right). In contrast to the heart, the newt limbs is composed of various differentiated cell types (indicated by cells of different colors). Following injury, cells adjacent to the wound epithelium dedifferentiates to form a blastema (green). These cells proliferate and subsequently to re-form a properly patterned limb. From Scott and Stainier, (2002).

Different vertebrate species have different cardiac regeneration rates: high in Teleost fish, moderate in Urodele amphibians, and almost negligible in mammals (Scadding, 1977). Some organs are capable of rapid homeostatic adjustments to tissues loss or gain. Mammals, including humans, exhibit only a few examples of tissues and organs regeneration, most notably the ability of liver hepatocytes to regenerate damaged liver tissue. Progenitor cell populations have been identified within most mammalian organs, including skin, blood, bone, reproductive tissues, skeletal muscle, kidney, lung, liver, intestine, heart and brain. Unfortunately, not all of our organs are equally competent to regenerate, and it is known that heart and central nervous system are particularly resistant to regeneration after injury and that they form scar tissue (Poss et al., 2002). For example, the liver will increase mass through compensatory cell proliferation after partial hepatectomy, or reduce mass through apoptosis after experimentally induced hyperplasia, to maintain the appropriate size. Although robust replacement of structural cells has been discovered in mammalian tissues, such as the liver, blood and skin, most tissues do not share this remarkable ability, like the heart. In animal as salamander, hydra and flatworm, it has been described a huge regenerative capacities indeed, flatworms are able to generate an entirely new animal from a small piece of tissue (Salo and Baguna, 2002). The regeneration on limbs after amputation has been analyzed in detail in these organisms. This process is commonly divided in three sequential steps: (1) wound healing, in which the amputation site is covered by epithelial cells; (2) formation of a mass of undifferentiated progenitor cells, or blastema, by dedifferentiation of cells in the surrounding tissue; and (3) limb redevelopment, in which the correct pattern is generated in the blastema resulting in the regeneration of the amputated portions of the limb (trans-differentiation). The regeneration of limbs and lens in newts, as well as that of fins in zebrafish, relies on the formation, growth and patterning of a blastema. It is well established that, during limb regeneration in newts, multinucleated muscle cells dedifferentiate to adopt muscle or cartilage fates. In addition to limbs, tail, jaw and lens, newts also display the ability to regenerate large portions of their hearts after amputation. Although new heart regeneration does occur to some extent after partial resection of ventricular myocardium, there is only a modest level of tissue replacement that accompanies scarring. Despite the evident significance of understanding this process for the development of potential therapeutic target, heart regeneration in newts has not been addressed from molecular approaches, probably owing to the relative lack of genetic tools in this experimental model. While genetic approaches have been successfully applied to dissect many developmental, physiological and behavioral processes, and could conceivably

be applied to reveal factors required for regeneration, newts and other highly regenerative amphibians are not suitable for this analysis. This is because of their long generation times, enormous genomes, and the difficulty of maintaining large numbers of animals. Nonetheless, selected non-mammalian vertebrates, including Urodele amphibians and Teleost fish, display an elevated regenerative spectrum, with many more tissues capable of impressive regeneration. In particular, because of their amenability to genetic manipulation, zebrafish have proved to be a valuable laboratory model for understanding many aspects of vertebrate embryogenesis (Haffter and Nüsslein-Volhard, 1996). The robust capacity of zebrafish to regenerate a variety of tissues as fins, spinal cord, and retina, position it as an ideal genetic model system for understanding the molecular and cellular events governing regeneration (Wagner and Misof, 1992; Curado et al., 2007).

UTILITY OF USE OF ZEBRAFISH AS MODEL ORGANISM FOR BIOLOGICAL STUDIES AND CARDIAC REGENERATION

Several characteristics have made to use zebrafish as a model organism (Warren et al., 2000), as for instance, the relatively short generation time (3 months), the small size at maturation (3 to 4 cm long as an adult), the simple husbandry requirements, the ease of manipulation and maintenance, and the high fecundity (100-200 eggs per clutch) (Gerhard, 2003). Additionally, external fertilization coupled with optical clarity throughout embryogenesis allow the direct monitoring of developmental processes, and their manipulation using mechanical, chemical, or genetic technique (Nasevicius and Ekker, 2000; Bopp et al., 2006). Moreover, using zebrafish is further supported and facilitated by the sequencing of the zebrafish 1.7-Gb genome (Sanger Institute's *Danio rerio* sequencing project), which has facilitated the generation of microarrays for large scale expression profiling (Ton et al., 2002; Lo et al., 2003; Mathavan et al., 2005; Quian et al., 2005). Again, zebrafish can be used as model for pathogenesis study (Neely et al., 2002; Prouty et al., 2003), for hematopoietic and cardiovascular diseases (North and Zon, 2003; Zheng et al., 2008) and for discovering new therapeutic target (Peterson, 2004). Also, zebrafish mutations faithfully phenocopy many human disorders (Goldsmith and Harris, 2003; Meeker and Trede, 2008). Each mutation, once cloned, provides candidate genes and pathways for evaluation in the human (Anderson and Ingham, 2003). The collection of mutations in their entirely potentially provides a medical taxonomy, one based in developmental biology and genetics (Eisen, 1996).

Large-scale genetic screens have been performed in zebrafish after use of ethyl-nitrosourea (ENU) to generate point mutations. More than 7000 mutations in 600 genes have been so generated. Zebrafish orthologos exists for most human genes, and in fact, there are large regions of conserved synteny between chromosomes of fish and mammal (Barbazuk et al., 2000). There is believed to have been a genome duplication at the base of the Teleost radiation 450 million years ago (mya), as well as a second duplication 100 mya, with as many as 20% of genes having been duplicated in the fish as compared to the human. Although such defects are transient and not heritable, morpholinos can be used to assess the effects of diminishing function of orthologs of genes, including those known to be involved in disease, such as dystrophin, presenilin, and apolipoprotein E (For a review see Lieschke and Currie, 2007).

Zebrafish heart share common structures with mammalian hearts, serving as a model for vertebrate animal studies (Wang et al., 1998; Scott and Stainier, 2002; Sehnert and Stainier, 2002). The zebrafish is an ideal organism for the study of heart development because is capable to produce definitive hematopoietic cells and form a simple circulation two-chambered heart (Stainier et al., 1996). Furthermore the detection of perturbations in cardiogenesis and for regeneration because heart formation and function can be assessed visually in the embryo and because the fish is not dependent on blood circulation for survival during embryogenesis (Pelster and Burggren, 1996). Additionally, because the fish is not dependent on blood circulation for survival during embryogenesis (Pelster and Burggren, 1996), and the defects in heart development and/or function are more likely to be detected and recovered. Many zebrafish mutants with defects in cardiac development and function were identified in the large-scale mutagenesis screens and are described in detail elsewhere (Chen et al., 1996). Blood circulation in zebrafish begins by 24hpf and is clearly visible under the microscope. The process of blood development and the morphology of zebrafish blood closely parallels that of mammals (Wingert and Zon, 2003). There are both primitive and definitive waves of differentiation in the zebrafish, which produce primitive erythrocytes and macrophages, followed by definitive erythrocytes, B cells, T cells, monocytes, granulocytes, and thombocytes, respectively (Willet et al., 1999; Bennett et al., 2001; Lawson and Weinstein, 2002; Schoenebeck and Yelon, 2007). Many zebrafish orthologos of blood-specific genes demonstrated to be important in mouse and human development have been isolated, including *cmyb*, *gata1*, *gata2*, *globin*, *hhex*, *scl*, and *vegf* (Hansen et al., 1997; Gering

et al., 1998; Brownlie et al., 2003). Recently, Holtzinger and Evans (2007) have discovered that *gata5* and *gata6* in the zebrafish are functionally redundant for specification of cardiomyocytes. In the same year Jia et al., (2007) showed that vertebrate growth is regulated by functional antagonism between Gridlock and Gata5. Gata5 is also required for the development of the heart and the endoderm (Reiter et al., 1999). Although the genetic program appears to be highly conserved during hematopoietic development in the vertebrate embryo, the site of hematopoietic is not as consistent. Whereas primitive hematopoiesis in mice and humans takes place in the yolk sac, in the zebrafish, the primitive wave initiates in a region termed the intermediate cell mass (ICM) (Langeland and Kimmel, 1997; Orkin and Zon, 1997). Definitive hematopoiesis appears to initiate in the dorsal aorta of the zebrafish as it does in all the vertebrate studies to date; however, the site of adult hematopoiesis in the fish is the kidney, not the bone marrow as in mammals (Willet et al., 1997).

In vertebrates, the linear heart tube forms from the migration and fusion of bilateral cardiac progenitors fields at the midline, followed by cardiac looping to form an s-shaped heart. Distinct atrial and ventricular chambers with unique physiological and electrical properties arise, separated by a discrete domain known as the atrio-ventricular canal (AVC) (Stainier, 2001; Beis et al., 2005). The AVC give rise to the valves that ensure unidirectional flow of blood. In mammals, each chamber and valve-forming region becomes septated, resulting in a four-chambered heart. The embryonic heart of the zebrafish has a close anatomic resemblance to that of a human heart at 3 weeks of gestation (Hu et al., 2000; Auman and Yelon, 2004). The zebrafish heart manifests the same pattern of electrical excitation as does the human heart, with impulses generated in the sino-atrial node, propagated through the *atrium*, pausing in the atrio-ventricular (A-V) node, and thence to the ventricular (Hu et al., 2001; Ho et al, 2002; Beis et al., 2005). Our understanding of heart development has benefited greatly from zebrafish mutants that specifically disrupt cardiovascular form and function (Berdougo et al., 2003; Kinna et al., 2006). Mutations that mimic the most common arrhythmias, including pacemaker problems (*slow mo* and *reggae*) (Warren et al., 2001), A-V block (*hiphop* and *breakdance*), and atrial fibrillation (*island beat*, *isl*) have been described (Baker et al., 1997; Kopp et al., 2005). Indeed, it remains the only laboratory model system that is both amenable to genetic manipulation and capable of carrying out a robust regenerative response after the loss of complex tissue (Ho et al., 2007).

Zebrafish have a population of haemangioblasts, which is absent in chick and mammalian embryos, raising the possibility that these cells represent the evolutionary

ancestror of the second heart field. The genetic programs of these anterior haemangioblasts and the adjacent heart field are co-regulated, by transcriptional factors previously associated with heart but not blood or endothelial development (Chen and Fishman, 2000). The anterior lateral plate mesoderm (ALM) in zebrafish is a source of hematopoietic, endothelial and cardiogenic cells, with the blood and endothelium coming from the most rostral region and cardiac tissue deriving from the adjacent more posterior population. Differences between zebrafish and mouse/chick may in part reflect the different origins of the endoderm: whereas in chick and mouse, yolk sac haematopoiesis and vasculogenesis occur adjacent to the visceral endoderm, in zebrafish the ALM is adjacent to the definitive endoderm.

DEVELOPMENT AND STRUCTURE OF THE ZEBRAFISH HEART

The majority of biological research concerning the zebrafish has focused on its developmental stages because of its transparency of the embryo. The heart is enclosed in the pericardial cavity with paired pericardial muscles running in caudo-cranial direction and is covered by an epicardial membrane. Zebrafish heart develops from a simple tube, which bends and twists (loops) rightward to create the basic plan of the mature heart that consists of only one *atrium* and one ventricle (1 mm^3) composed of two concentric layers: an inner endothelial layer called the endocardium and an outer muscular layer termed the myocardium (Stainier and Fishman, 1992; Stainier et al., 1993; Glickman and Yelon, 2002). Two additional smaller compartments are the *sinus venosus* (SV) and the *bulbus arteriosus* (BA) (Forouhar et al., 2006). The embryonic zebrafish heart at 24 hpf is nearly identical to the two-chambered human heart at three weeks of gestation (Fishman and Chien, 1997). It is spontaneously contractile, emptying *atrium* and then ventricle sequentially to generate unidirectional blood flow (Bendig et al., 2006).

Heart formation involves the specification and differentiation of cardiac precursors, the integration of precursors into a tube, and the remodeling of the embryonic tube to create a fully functional organ (Glickman and Yelon, 2002). Cardiac progenitors can be identified at approximately 11 hours post fertilization (hpf) as marked by *nkx2.5* expression, termed the cardiac field (Stainier et al., 1993; Lee et al., 1994; Griffin et al., 2000). Angioblasts in zebrafish are first evident around 14 hpf and the formation of the zebrafish heart begins at approximately 16 hpf when zebrafish cardiac precursors begin to migrate to the midline from bilateral regions of the anterior lateral plate. Each bilateral cardiac sheet contains lateral atrial precursors that express Cardiac myosin light chain 2 (*Cmlc2*), and medial ventricle precursors

that express both Cmlc2 and Ventricular myosin heavy chain (*Vmhc*) (Yelon et al., 1999; Yelon, 2001; Huang et al., 2003; Rottbauer et al., 2006). For the embryonic heart development, it is also important the expression of NXT2, member of NXT proteins, involved in exporting nuclear RNA in eukaryotes, as demonstrated by Huang et al., (2005b). The timing of zebrafish myogenesis is regulated by *smacd3* (Ochi et al., 2008). For normal myocyte proliferation during early cardiac development is necessary the expression of *ndrg4* (Qu et al., 2008). Early cardiac connexin, *cx36,7/Ecx,* regulates myofibril orientation and heart morphogenesis by establishing *Nkx 2.5* expression (Sultana et al., 2008). *Hrt* is required for cardiovascular development in zebrafish (Szeto et al., 2002). The aorta begins to form in the posterior trunk at 17.5 hpf and complete its formation after the blood circulation starts (Thisse and Zon, 2002). The cone tilts and extends to produce the heart tube, which begins to beat as a peristaltic wave at 22 to 24 hpf (Stainier et al., 1993). Heart formation is completed through a series of remodeling steps that properly position the ventricle and *atrium*, and produce cardiac valves to ensure unidirectional blood flow through the heart (Stainier et al., 1992; Glickman and Yelon, 2002). By 36 hpf cardiac looping is completed and coordinated contractions of *atrium* and ventricle provide circulation to the head and trunk (Glickman and Yelon, 2002; Cha and Weinstein, 2007). Growth of a muscular *septum* late in human embryonic development, which divides the primitive *atrium* and ventricle into left and right parts, differentiates the adult human from the adult zebrafish heart, the latter retaining a single *atrium* and single ventricle. By 5 dpf, the larvae heart is essentially formed as noted in the adult Teleost configuration. The four compartments are now separated from another by three sets of fully functional valves (Weinstein and Fishman, 1996). In each compartment, the myocardium is about one cell layer thick except for the ventricle, which consists of two to three cell layers.

More than 35 mutations in the zebrafish prevent the normal acquisition of contractile function, without disturbing the generation of normal chamber cell fate or formation, and therefore functionally mimic dilated cardiomyopathies (Sehnert and Stainier, 2002; Basset and Currie, 2003; Huang et al., 2005a). *Pickwick*, a mutation in *titin*, the myofibrillar element around wich the actin-myosin arrays assemble and contributor of most of the elastic force to the muscle, has been cloned. It is interesting to note that a human dilated cardiomyopaty family has also recently been documented to have a *titin* mutation (Xu et al., 2002). These "heart failure" mutations in zebrafish will reveal not only candidate genes for disease

propensity in humans, but also potential factors and pathways that could be pharmacologically manipulated to improve contractile function (Chun and Chen, 2007).

Concern the contractile function, it is well known that vertebrates have evolved a nerve system to modulate cardiac activity in a sophisticated fashion (Incardona et al., 2004). These activities include heart rates, contractile force, action potential and conduction velocity that can be exposed to several anomalies (Ernest et al., 2000). Congenital anomalies of the heart affect 8 of 1000 live births. Three percent of these are due to inadequate chamber growth. Although there is a high sibling recurrence, suggesting a genetic component, these diseases in general are not transmitted in a Mendelian fashion. Several mutations in zebrafish disrupt generation of early heart form. For example, *heart and soul* causes the ventricle to form inside of the *atrium* and is due to a mutation in PKCλ. *Handsoff* and Pandora have diminutive ventricles. The former due to mutation in the bHLH transcription factor dHAND. *Jekyll* is due to a defect in the enzyme UDP-glucose dehydrogenase and lacks an A-V valve. *Casanova* is a sox-related gene that lacks endoderm and causes cardia bifida. The zebrafish homologue of caldesmon (CaD, an actin-binding protein implicated in the cytoskeletral organization and various signaling pathways) is similar to the mammalian low molecular weight caldesmon (l-CaD, associated with the development of tumor vasculature). It is essential for normal vertebrate cardiac looping, chamber formation, muscularization and proper cardiac function. The obvious contribution of CaD to cardiac muscularization offers clues for future therapeutic strategies in regeneration of cardiomyocytes.

Despite a two-chambered heart and lack of pulmonary vasculature the zebrafish heart parallels that of humans in terms of QT interval and heart rate (Arnaout et al., 2007). The *atrium* is medially dorsal and posterior to the ventricle. Similar to humans, the zebrafish heart is enchased by a pericardial sac in the thoracic cavity, and is situated below the pectoral bone of the pectoral fins. The *bulbous arteriosus* is analogous to the human aortic arch with thick contractile smooth muscle. Blood returns into the *sinus venosus*, which is analogous to the vena cava. The fundamental electrical properties are remarkably similar to those of humans, and the critical pathways in cardiovascular development parallel higher vertebrates (Kopp et al., 2007). In 2004, Forouhard at al., reported an electrocardiogram (ECG) of an embryonic zebrafish, revealing similar atrial and ventricular electrical signals as found in a human ECG. It has been demonstrated that zebrafish embryos are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia (Langheinrich et al., 2003). Ventricular trabeculae are more prominent in the zebrafish heart than in those of humans (Grimes et al.,

2006). Histological studies show that the ventricle of an adult zebrafish is composed of trabecular and compact myocardium, and surrounded by epicardium and endocardium. Using zebrafish mutant tr265/tr265, whose Band 3 mutation disrupts erythrocyte formation and result in anemia, Sun and colleagues (2009a) showed that cardiac hypertrophy involves both myocyte hypertrophy and hyperplasia in anemic zebrafish.

ZEBRAFISH HEART REGENERATION

The zebrafish has emerged as an excellent model for cardiovascular research (Curado and Stainier, 2006). Recently, it has been shown that adult zebrafish possess a unique yet poorly understood capacity for cardiac regeneration, restoring in 1-2 months, the ventricular muscle removed by surgical resection, offering new possibilities for experimentally approaching this fascinating biological phenomenon (Lepilina et al., 2006) (**Figure 5**). The regenerative ability of an adult zebrafish heart was uncovered by surgical resection of a large portion (20-30%) of the ventricular chamber including the apex (Poss et al, 2002). This was easily accomplished in anesthetized adult fish, whose hearts were readily accessible after incision of the skin, muscle, and pericardial sac. The ventricle was then gently pulled at the apex and cut at the desired position with iridectomy scissors. This surgical procedure was highly reproducible and relatively safe, with greater than 80% survival.

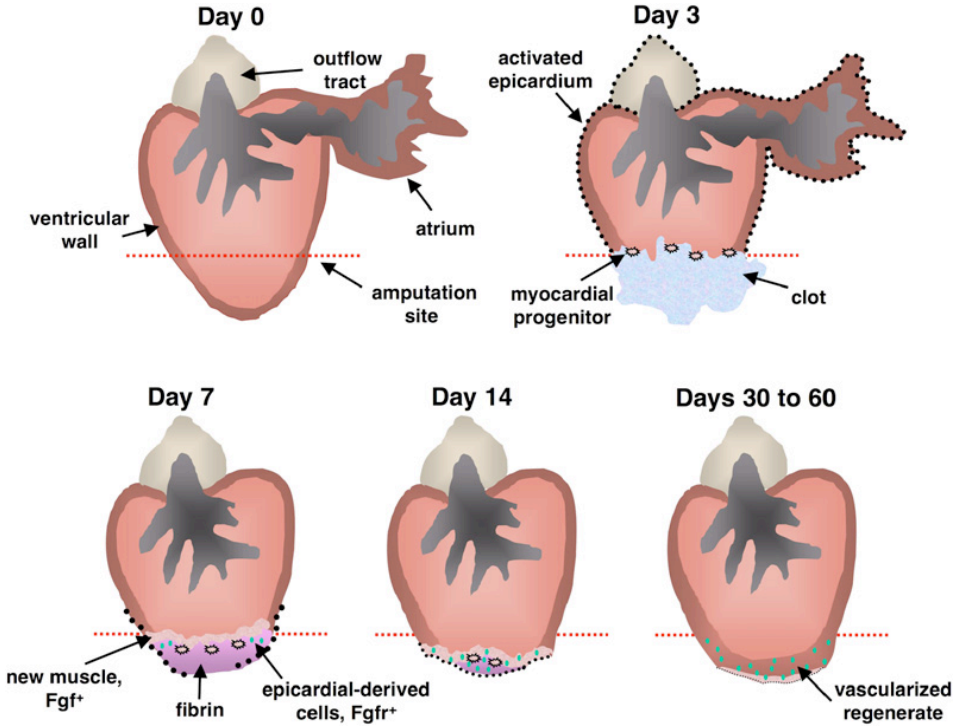

Figure 5. Model for Zebrafish Heart Regeneration Resection of the ventricular apex stimulates rapid expansion of the entire epicardium by 3 dpa (black dots), at which time myocardial progenitor cells first originate in the wound, express pre-cardiac markers, and associate with existing muscle. By 7 dpa, *raldh2/tbx18*-positive epicardial cells begin to surround and invade the wound. Meanwhile, there is continued seeding, maturation, and proliferation of myocardial progenitor cells, contributing the first layers of new muscle (stage 1). To coordinate these epicardial and myocardial events, regenerating myocardium synthesizes *Fgf17b* and possibly other factors with the potential to recruit *Fgfr2/Fgfr4*-presenting epicardial cells. Epicardial-derived cells undergo EMT in response and vascularize the regenerate (green dots). Presence of new coronary vasculature by 14 dpa extends progenitor cell activity and facilitates restoration and expansion of the ventricular wall (stage 2). From Lepilina et al., (2006).

In response to amputation, thrombosis immediately develops to achieve homeostasis in the ventricle. After a short initial phase of intense bleeding that stops within 1 minute, a blood clot formed that sealed the ventricle and kept fish from exsanguinations. The formation of the blood clot in the low-pressure fish circulatory system is remarkably efficient. Probably because of this, amputated fish did not, in contrast to newts, exhibit intense myocardium contraction at the site of resection, or circulatory stasis. First, the clot that seals the apex

matures within several days (1-4 day post-amputation -dpa), into fibrin, a complex milieu containing serum factors and degenerated erythrocytes. Functional cardiomyocytes and new contractile muscle infiltrated the injured area and sealed off the wound. Interestingly, the regenerated myocardium displayed a transiently hypertrophied compact zone, from 21-30 dpa, most likely reflecting a compensatory reaction to the hemodynamic overload subsequent to myocardial loss. Scarring and collagen deposition, characteristic of damaged mammalian hearts, did not occur. Remarkably, 60 days after surgery, the zebrafish heart appeared roughly normal both histologically and based on examination of heartbeat. In contrast, mouse hearts subjected to similar damage induced by freezing do not regenerate, but instead form scar tissue (Wills et al., 2008). In the infarcted mammalian ventricle, fibrin deposition attracts fibroblasts and inflammatory cells, and is a precursor to scarring. The normal response of mammalian hearts to injury or hypoxia is hypertrophy, the growth of cardiomyocytes without cell division. Cardiac hypertrophy refers to the cardiac remodeling process in response to a variety of intrinsic and extrinsic stimuli that stress the heart (Baohua et al., 2006). Initially, the heart compensates for the stress though increasing cardiac mass to normalize wall tension. However, if the underlying stress is untreated, cardiac hypertrophy can lead to sudden death or heart failure. The hallmarks of pathological hypertrophy include enlargement of individual cardiomyocytes, disarray or myofibrils, fibrosis in the extracellular matrix, reactivation of fetal transcriptional programs, and decreased cardiac function (Kitzmann et al., 2006).

The ventricular myocardium displays histological characteristics of hypertrophy at 21 and 31 dpa, most likely reflecting compensatory reaction to the hemodynamic overload subsequent to myocardial loss. Milan et al., (2006) obtained zebrafish ECG by inserting two needle electrodes through the ventral epidermis. They saw that the mean rate of the adult male zebrafish is 151±30 beats/min. Sun and colleagues (2009b) applied a minimally invasive methodology to monitor zebrafish heart function, electrical activities, and regeneration in real-time. They performed a micro-electrocardiograms to study post-ventricular amputation of the zebrafish heart and, unlike the human counterpart, they didn't observed ventricular tachycardia or fibrillation in response to ventricular amputation 2 and 4 dpa. Atrial arrhythmia was recorder after prolonged sedation. Ventricular amputation led to a shortened QTc interval without affecting the PR and QRS intervals from non-anesthetized and non-paralyzed adult zebrafish. It has been demonstrated that agents known to induce QT prolongation in humans led to QT prolongation in zebrafish embryos and drugs that induce repolarization abnomalies cause bradicardia in zebrafish (Milan et al., 2003). Understanding the cellular and molecular

mechanisms of this regenerative process can have exciting implications for human cardiac biology and diseases.

CELLULAR BASIS OF ZEBRAFISH HEART REGENERATION

Regeneration of the zebrafish heart appears to differ from that described in other tissues, including the newt limb and zebrafish fin, where differentiate cells (muscle, cartilage, and skin) adjacent to the wound site, first dedifferentiate to form a blastema or mass of pluripotent cells, which then give rise to a fully formed and patterned limb or fin (Biga and Goetz, 2006). The formation of the blastema that regenerates these tissues requires successive process of dedifferentiation, transdifferentiation, and pattern formation. The zebrafish myocardium is a relatively simple structure, composed of one major cell type, so the initial step of dedifferentiation observed during limb regeneration may not be required for cardiac regeneration. Using double transgenic animals with both nuclear-localized DsRed2 fluorescent reporter for cmlc2 (cmlc2:nRFP, where RFP is red fluorescent protein) and enhanced green fluorescent protein (EGFP), that can reveal temporal and spatial characteristics of promoter activation and inactivation, Lepilina and colleagues (2006) demonstrated that new myocardium arises from undifferentiated progenitors cells and maintained by a dynamic epicardial injury response (**Figure 6**).

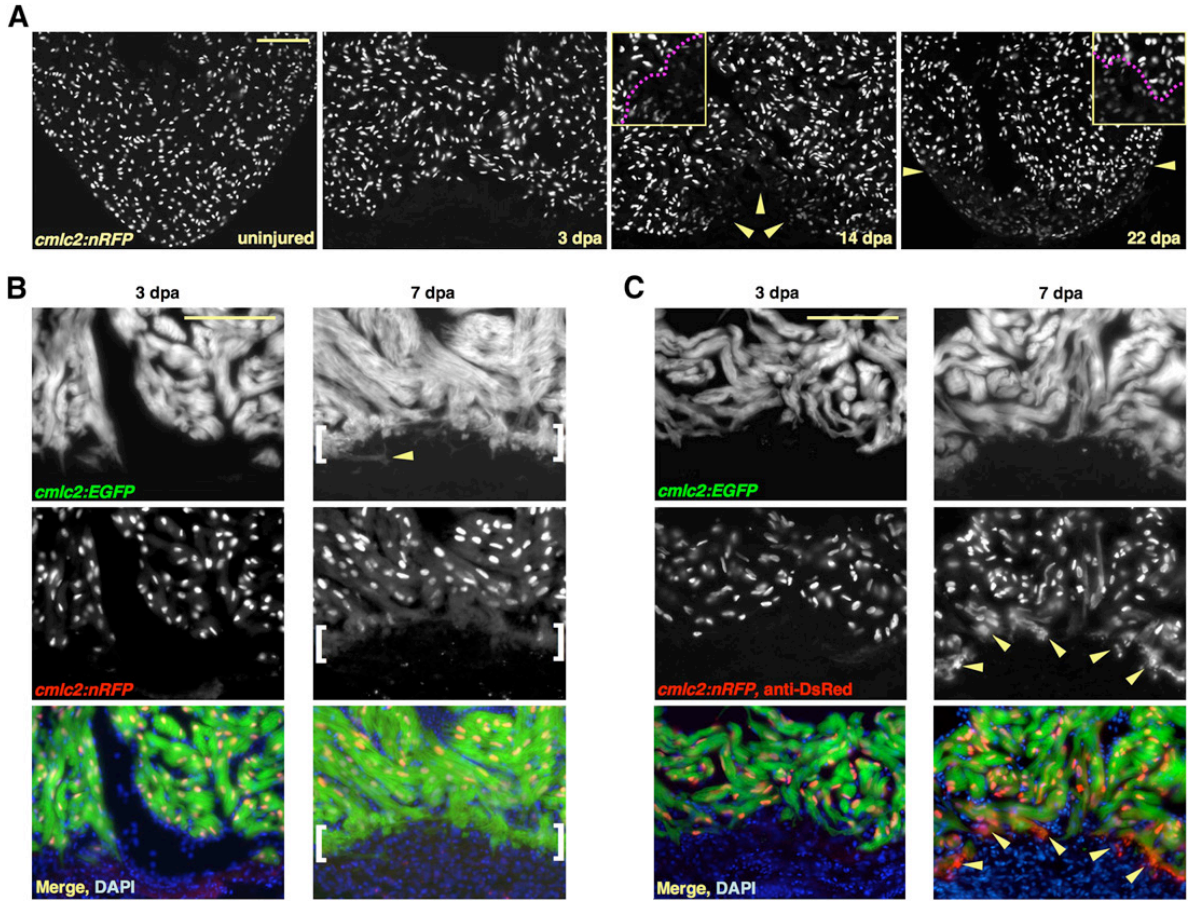

Figure 6. New Myocardium Arises from Undifferentiated Progenitor Cells. (A) Sections through uninjured and injured cmlc2:nRFP ventricles. A subpopulation of RFPlo nuclei manifests by 14 dpa (arrowheads), representing ostensibly the entire regenerate by 22 dpa. Magenta line in high-magnification insets delineates RFPhi CMs (above line) from RFPlo CMs. (B) cmlc2:nRFP; cmlc2:EGFP ventricles. EGFP and RFP expression from the cmlc2 promoter is reported at the same basoapical level at 3 dpa. By 7 dpa, an RFPneg front of newly differentiated muscle reporting the faster-fluorescing EGFP (brackets) appears apical to the EGFPposRFPpos portion. Arrowhead indicates an EGFPpos cell process extending into the clot. (C) cmlc2:nRFP; cmlc2:EGFP ventricles stained for DsRed immunoreactivity with an anti-DsRed antibody. At 3 dpa, there is no difference in appearance from the unstained 3 dpa ventricle in (B). By 7 dpa, a front of RFPcyto muscle (arrowheads), representing the most recently differentiated CMs, colabels EGFPpos tissue apical to natural RFPnuc fluorescence. Scale bar = 100 mm. From Lepilina et al., (2006).

To directly investigate the nature of cells that contribute to the regenerating tissue in the zebrafish heart, Poss and colleagues (2002) undertook an exhaustive series of bromodeoxyuridine (BrdU) pulse-chase analyses. Bromodeuxiridine (BrdU), a chemical that is only incorporated into cells undergoing *de novo* DNA synthesis (S phase), was injected into fish at various time point following the surgery. Cardiomyocytes from uninjured hearts rarely incorporate BrdU, even after prolonged exposure. However, there was a marked increase in BrdU incorporation following injury, which peaked at 14 days after surgery and was primarily localized to the outermost layer of the myocardium adjacent to the wound area. The regeneration resulted from proliferation of cardiomyocytes adjacent to the area of injury. Surprisingly, uninjured zebrafish hearts displayed a significant number of cardiomyocytes labeled with BrdU (Raya et al., 2003), indicating that normal cardiac cell turnover in this animal is much higher than in other organisms. Remarkably, a ten-fold increase in BrdU-labeled cardiomyocytes was detected in the area surrounding the lesion 2 weeks after amputation. These results were further confirmed by staining with anti-phosphorylated histone 3, a marker of condensed chromatin (Poss et al., 2002). The Authors then analyzed the source and fate of regenerating cardiomyocytes by administering BrdU at 7–14 dpa and analyzing labeled cells at 14, 30, and 60 dpa. These studies identified a leading edge of proliferation in the new layer of epicardial cardiomyocytes, which were continuously displaced inward to replenish the amputated region (Poss et al., 2002). The identification of a population of differentiated cardiomyocytes that re-enter the cell cycle and proliferate in response to heart amputation (Poss et al., 2002), together with the absence of molecular markers of *de novo* cardiomyocyte differentiation in the injured area (Raya et al., 2003), provide strong support for a scenario in which heart regeneration in zebrafish is carried out by resident cardiomyocytes. It has been recently reported that resident stem cells exist in the adult mammalian heart, which can participate in a regenerative response (Beltrami et al., 2003). Whether such cells exist in the zebrafish heart, and indeed whether they contribute to its regeneration after amputation, was not directly addressed in the studies by Poss and colleagues (2002) or by Raya and colleagues (2003). The ventricular resection induces developmental gene expression and proliferation within the epicardial layer, activated epicardial cells soon surround the wound with a portion of them penetrating several cell layers deep into the wound and regenerating muscle. Concomitantly, the new myocardium is substantially vascularized. Thus, it is assumed that the epicardial cells have similar roles as in the embryonic heart; that is, as a progenitor tissue that contributes smooth muscle and/or

endothelial cells during neovascularization. It will be very interesting to investigate the existence of resident stem cells and to analyze their behavior after heart amputation in the zebrafish, a vertebrate with regenerating capacity far exceeding that of mammals.

MOLECULAR BASES OF HEART REGENERATION IN ZEBRAFISH

The fact that adult zebrafish show a remarkable heart regenerating ability provides new and promising entry-points for advancing our knowledge of this complex phenomenon (Akimenko et al., 2003). Raya and colleagues (2003) analyzed the expression of *nkx2.5*, *tbx5*, and *CARP* in sections of regenerating hearts at different time points after amputation. *Nkx2.5* is the earliest known marker of cardiac lineage, and its expression, although at low levels, persists in the myocardium during adult life (Targoff et al., 2008). In zebrafish, comparable low levels of *nkx2.5* expression were detected in the myocardium of both control and regenerating hearts by in situ hybridization and RT-PCR. Similarly, *tbx5* expression did not change significantly during regeneration. To address the possibility that a transient expression of either *nkx2.5* or *tbx5* was missed by the in situ hybridization analyses, the authors made use of transgenic line expressing eGFP under the regulatory sequences of the *CARP* gene. *CARP* is a direct target of Nkx2.5, whose expression is limited to heart structures during cardiac development. Indeed, adult fish carrying this transgene do not display detectable eGFP fluorescence in their hearts. Consistent with the results of *nkx2.5* and *tbx5* expression, no upregulation of *CARP*-driven eGFP fluorescence was detected in regenerating transgenic hearts from this line.

Raya and colleagues (2003) demonstrated that adult zebrafish have a remarkable capacity to regenerate the heart in a process that involves up-regulation of *msxB* and *msxC* genes. Heart regeneration in zebrafish is accompanied by up-regulation of components of the Notch pathway, followed by members of the *Msx* family. These genes are not expressed during zebrafish heart development, indicating that regeneration involves the execution of a specific genetic program, rather than redeployment of a developmental program. Also, Raya and colleagues (2003) demonstrated that components of the Notch pathway are also up-regulated during zebrafish fin regeneration, suggesting that this pathway may play a general role in the activation of the regenerative process.

Some molecular mechanisms are likely to be shared by embryonic cardiogenesis and adult cardiac regeneration (e.g., myocardial differentiation programs) (Raya et al., 2004). However, heart regeneration invokes multiple distinct and definitive events, including (1)

iniziation by injury, (2) activation of quiescent tissue, (3) ostensibly local development as opposed to organ-and organism wide development, (4) simultaneously healing and growth, (5) morphogenesis on large scale to form adult tissue, and (6) possible long-term maintenance or de novo creation of progenitor cells. Not only is genome-wide information already available for sequence comparison or gene expression profiling analyses in this organism, but zebrafish also offer the possibility of conducting large-scale mutagenesis screens to identify genes required for heart regeneration (Wood and Schier, 2008). Proof-of-principle experiments have yielded successful results for both expectations. On the one hand, a temperature-sensitive mutation in *msp1*, a mitotic checkpoint kinase upregulated during cell proliferation and necessary for fin regeneration (Poss et al., 2002), was shown to result in scarring, rather than regeneration of the amputated heart (Poss et al., 2002). On the other hand, analysis of the expression pattern of *msxB* and *msxC*, encoding homeodomain-containing transcription factors upregulated in the regenerating fin blastema (Raya et al., 2003), revealed a strong expression of these transcripts in regenerating zebrafish hearts, starting as early as 3 dpa, in the myocardial areas surrounding the lesion area. Interestingly, neither gene is expressed in uninjured adult zebrafish heart, or during heart development in zebrafish embryos. Also, they saw that some of the pre-cardiac and early differentiation markers, as *hand2*, *nkx2.5*, *tbx20* and *tbx5* are expressed during heart regeneration. Retinaldehyde deydrogenase 2 gene (*raldh2*; also known as *aldh1a2*-Zfin) controls a rate-limiting enzyme for retinoic acid (RA) synthesis. it has been demonstrated that the reduction of RA signaling causes formation of an excess of cardiomyocytes. *Raldh2* is expressed throughout early zebrafish embryogenesis: in the blastula, *raldh2* is found at the embryonic margin; during gastrulation, *raldh2* is in involuting mesoderm and, after gastrulation, *raldh2* is in both lateral and paraxial mesoderm. Moreover, adult zebrafish submitted to rapid growth conditions show dramatic, hyperplastic cardiac growth and epicardial expression of *raldh2*. As early as 24 hr after partial ventricular amputation (hpa), *raldh2* was strongly induced in epicardium surrounding the ventricle, *atrium*, and outflow tract (**Figure 7**). Induced expression of raldh2 appeared sequentially, first in the outflow tract and atrial epicardium by 6–12 hpa and then joined by the ventricular epicardium (Lepilina et al., 2006). Ventricular and atrial epicardial cells also expressed *tbx18* by 1–2 dpa and consequently, began to proliferate. Thus, *nkx2.5*, *hand2* and *tbx20*, the earliest markers of the embryonic zebrafish heart field, were also the earliest detectable precardiac markers during heart regeneration (Yelon et al., 2000). Another factor, *tbx5*, is expressed very early during heart development and continues to be expressed at low levels in the adult

myocardium (Garrity et al., 2002). Recent studies illustrate that a proper balance of Wnt/-catenin signaling is also critical for the formation and proliferation of blastemal cells (Kawakami et al., 2006; Stoic-Cooper et al., 2007). This is consistent with result observed in the larval model by Mathew et al., (2009): when canonical Wnt signaling is blocked, the formation of wound epithelium and blastema are blocked. Normally is necessary to program the anterior limb formation since *tbx5* expression is related in embryogenesis to torax formation, in the adult zebrafish myocardium is not altered during heart regeneration. Recent results have shed light on the role of the outer non muscle layer of the heart, the epicardium, in zebrafish cardiac regeneration. During embryogenesis, the epicardium migrates out as a sheet from the proepicardium, a cluster or mesodermderived cells near the liver primordium and the septum transversum, to envelop the developing myocardial tube. Following this encasement, some epicardial-derived cells undergo an EMT into the subepicardial space and invade the myocardium to contribute endothelial and smooth muscle tissue of the coronary vasculature. Its developmental activation can be assayed by *raldh2* and *tbx18* expression, two genes known to be expressed within the embryonic epicardium. As early as 6 h post-ventricular resection, *raldh2* expression initiates within the epicardium of the outflow tract and *atrium* and then follows within the ventricle by 24 h. In addition, *tbx18* expression is observed within the atrial and ventricular epicardium by 1–2 days. Later on, cells positive for *tbx18* and/or *raldh2* appear at the wound site. This organ-wide response is intriguing for multiple reasons. First, the response is extremely rapid. The molecular details that connect the injury to this early response are unknown but are of interest, as their understanding will shed light on how heart regeneration is initiated. Second, the response is seen first distant from the wound site, within the *atrium*.

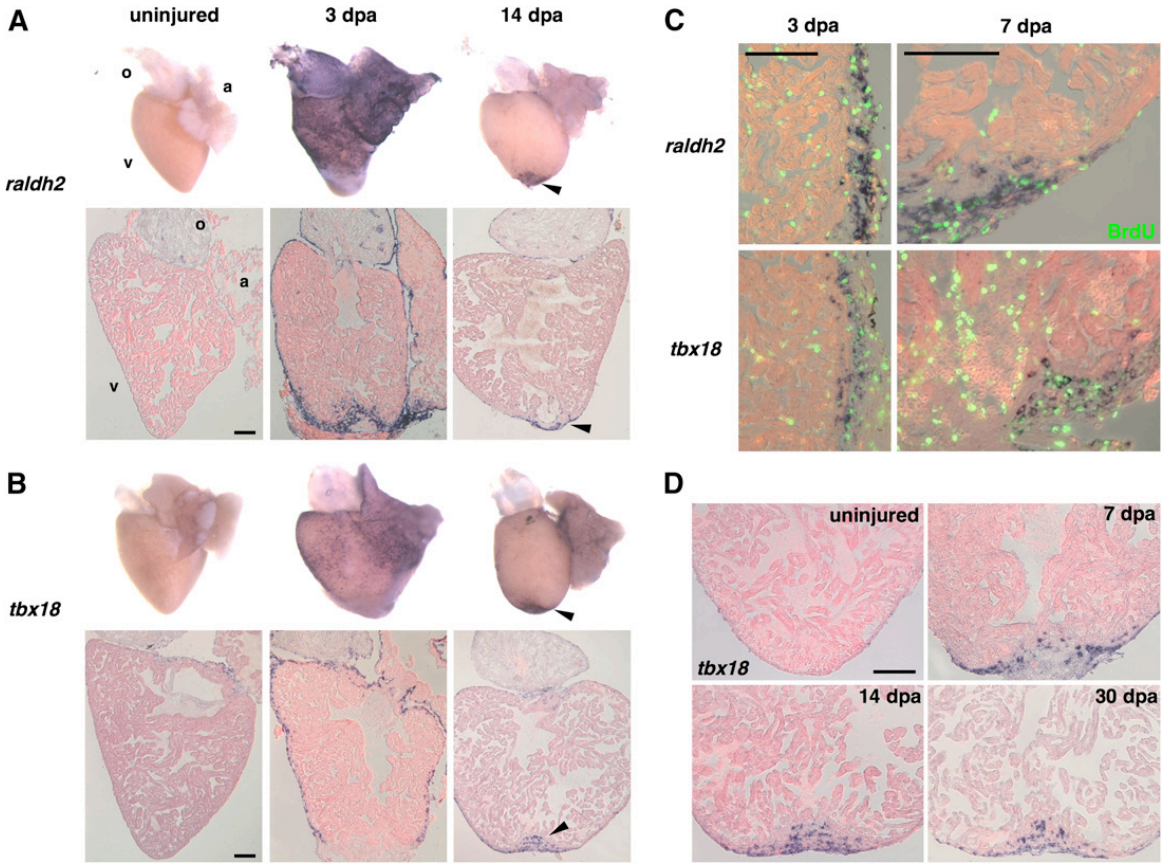

Figure 7 Organ-Wide Activation of Epicardial Cells and Invasion of the Regenerate (A and B) Whole-mount (top) and section (bottom) ISH of uninjured and 3 and 14 dpa hearts for *raldh2* (A) and *tbx18* (B). Expression of each marker is low or undetectable in the uninjured hearts but induced at 3 dpa in epicardial tissue surrounding the *atrium* (a) and ventricle (v) for *tbx18* and these tissues plus the outflow tract (o) for r*aldh2*. The endocardium surrounding wounded myofibers also expresses *raldh2*. By 14 dpa, expression of both markers is localized to the apical wound (arrowhead). (C) Colocalization of BrdU (green) with *raldh2* (top) and *tbx18* (bottom) in the ventricular epicardium at 3 and 7 dpa. The left images display a lateral edge of the 3 dpa ventricle away from the wound, while the right images display a lateral and apical portion of the 7 dpa ventricle including some of the wound. By 7 dpa, *raldh2/tbx18*/BrdU-positive cells begin to localize to the injury site. The majority of nonepicardial BrdU-positive cells are erythrocytes within the ventricular lumen. (D) Epicardial-derived cells positive for *tbx18* invade the regenerate by 7 dpa and remain by 30 dpa. Scale bar = 100 mm. From Lepilina et al., (2006).

Recent work has tried to address these questions through *in-vivo* developmental timing assays that employed double transgenic zebrafish strains carrying reporter constructs for the *cmlc2* (cardiac myosin light chain 2) promoter. In these experiments, transgenes for EGFP and nuclear-DsRed2, both simultaneously driven by separate *cmlc2* promoters, reported the contractile state of cardiac cells (Baird et al., 2000; Bevis and Glick, 2002). Because GFP folds and fluoresces more rapidly than DsRed2, any cardiac progenitors that begin to turn on the *cmlc2* promoter, and thus change their contractile status, will be transiently marked as GFP-positive/DsRed2- negative in nascent myocardium. Indeed, this was the case in the double-transgenic embryo, as GFP fluorescence is observed up to a day before DsRed2 fluorescence within developing cardiomyocytes. Within five days of ventricular resection, a front of GFP-positive/DsRed2-negative cardiomyocytes was found at the apical edge. This suggests that regenerating myocardium matures from undifferentiated, *Cmlc2*-negative, progenitor cells at the leading edge. In support of this mechanism, this leading edge was found to express a variety of molecular markers for the embryonic heart field, such as *hand2*, *tbx20* and *nkx2.5*, suggesting that these cells acquire cardiac fate reminiscent of cardiac progenitors that provide the first cardiomyocytes of the developing embryo. It is not known from what source(s) these apparent progenitor cells are derived. One possibility is that dedifferentiation of mature cardiac cells supply the regenerate with new progenitors.

A series of similarities between both regenerative and developmental processes argued for common mechanisms to some extent (Gardiner et al., 2002), although specific differences have also been reported (Carlson et al., 1998). In this respect, it is important to note that none of the genes members of the Notch signaling pathway, described to be up-regulated during heart regeneration in zebrafish (*msxB*, *msxC*, *notch1b*, and *deltaC*) appear to be expressed in the developing myocardium. Similarly, none of the heart development genes analyzed (*nkx2.5*, *tbx5*, and *CARP*) were up-regulated in the context of heart regeneration. These results indicate that heart regeneration in zebrafish involves the execution of a specific genetic program, rather than re-deployment of a developmental program. To address this issue further, Raya and colleagues (2003) attempted to induce regeneration in the developing heart of zebrafish embryos, by amputating a large portion of the prospective *atrium* of 24-h post-fertilization embryos. Notably, a strong upregulation of *msxB* and *msxC* transcripts was evident in the remnants of the cardiac tube from as early as 3 h post-amputation, until 24 h later (Raya et al., 2003). These results clearly indicate that regeneration and embryonic development do not rely on mutually exclusive genetic programs and that the expression of

molecular markers of regeneration is not an exclusive property of adult cells, but that embryonic cardiac cells are also competent to activate the genetic program of regeneration. In addition to the biological significance of this observation, it also raises the possibility that zebrafish embryos be used to investigate the molecular mechanisms underlying the heart regeneration ability displayed by adult individuals of this model organism. Taken together, these results indicate that *de novo* produced cardiomyocytes during heart regeneration in zebrafish are unlikely to be derived from undifferentiated stem cells or from dedifferentiated blastemal cells. Rather, the absence of upregulation of early cardiomyocytes differentiation markers after heart amputation suggests that the source of the regenerating tissue are differentiated cardiomyocytes that re-enter the cell cycle in response to heart injury. Comparative gene expression analysis revealed significant common gene expression changes in larval fin, adult caudal fin, and heart regenerating tissues, suggesting common molecular pathways choreographing the regeneration process. The physiological progression of fin regeneration in larvae and adults is similar, as both initiate with the formation of a wound epithelium, blastema formation, and the distal to proximal propagation of cell proliferation.

FGF SIGNALING IN CARDIAC REGENERATION

Fibroblast Growth Factors (FGFs) represent a large family of over 23 secreted glycosylated proteins that elicit a variety of important processes, like proper development and physiological homeostasis, and that are conserved throughout evolution. Mutations in mouse and zebrafish FGFs result in defect in ontogenic development and lethality (Ewton and Florini, 1990; Poss et al., 2000; Goishi et al., 2003; Molina et al., 2007). FGFs initiate signaling cascades through the extracellular interactions of ligands with a receptor tyrosine kinases (RTK) that, through intracellular cascade transduction, results in the activation of the RAS/MAPK, phopholipase-C-gamma (PLCγ) and phosphatidylinositol-3-kinase (PI3K) pathways that ultimately regulate gene transcription (Maciag and Friesel, 1995; Powers et al., 2000). During embryogenesis, FGFs are crucial in the specification of several tissues and for a multitude of developmental processes, tight regulation of its signal intensity and duration is crucial (Kardami and Fandrich, 1989; Marques et al, 2008) and further have a role in cell proliferation and survival (Itoh, 2007), Several modulators of the FGFs pathways have been identified, including the cytosolic protein sprouties and map kinase phosphatases. The generation of *in-vivo* reporters for FGF activity will provide a valuable tool to screen for genes or chemicals that modulate FGF signaling. For example, chemical screens with FGF-

reporter transgenic embryos have been used to identify specific compounds that can block FGFR signaling in the zebrafish. These compounds were counter screened against an indirect transgenic reporter for Vascular Endothelial Growth Factor (VEGF) signaling that plays an important role in formation of the aorta FGF proteins may act in a redundant manner so that the simultaneous inactivation of multiple FGF proteins has revealed overlapping functions for these secreted ligands in several vertebrate species. More in particular Lepilina et al. (2006) has demonstrated that zebrafish heart regeneration initiated predominantly by undifferentiated progenitors cells proceeds through two coordinated and FGF-dependent stages (**Figure 8**). First the formation of a blastema, comprised of progenitors cells that express pre-cardiac markers, undergo differentiation, and proliferate. Second, the induction of developmental markers that creates a new epithelial cover for the exposed myocardium. A subpopulation of these epicardial cells undergoes epithelial-to-mesenchymal transition (EMT), invades the wound, and provides new vasculature to regenerating muscle (Lepilina et al. 2006). During regeneration, the ligand *fgf17b* is induced in myocardium, while receptors *fgfr2* and *fgfr4* are induced in adjacent epicardial-derived cells. Lepilina and colleagues (2006) also demonstrated that when FGFs signaling is experimentally blocked by expression of dominant-negative FGF receptor, epicardial EMT and coronary neovascularization fail, prematurely arresting regeneration. FGFs are appealing candidates for signals that regulate the establishment of cardiomyocyte number. FGFs comprise a large family of secreted polypeptides thought to signal in a dose-dependent manner through receptor tyrosine kinases (Böttcher and Niehrs, 2005). *In-vitro* studies have even shown that FGFs promote epicardial EMT into collagen gels (Morabito et al., 2001). Lastly, the role of FGFs in the recruitment of epicardial cells into cardiac wounds and into the muscle regeneration, has been studied and expression of multiple FGF ligands and all 4 FGFr receptors (FGFrs) during regeneration has been evaluated (Cao et al., 2004): FGF17b, a member of the FGF8/17/18 subclass of FGF ligands, was the only ligand gene that showed detectable expression during the regeneration process (Reifers et al., 2000; Draper et al., 2001; Lepilina et al. 2006). Among zebrafish FGF ligands, FGF17b shows the highest homology to mammalian FGF17, which binds receptors FGFr1-3 (the ‘‘c’’ spliced forms) and FGFr4 (Olsen et al., 2006). In the uninjured adult zebrafish heart, smooth muscle cells in the outflow tract expressed FGFr1 and FGFr2, and valve mesenchyme expressed FGFr1, FGFr2, and FGFr4 (Lepilina et al., 2006).

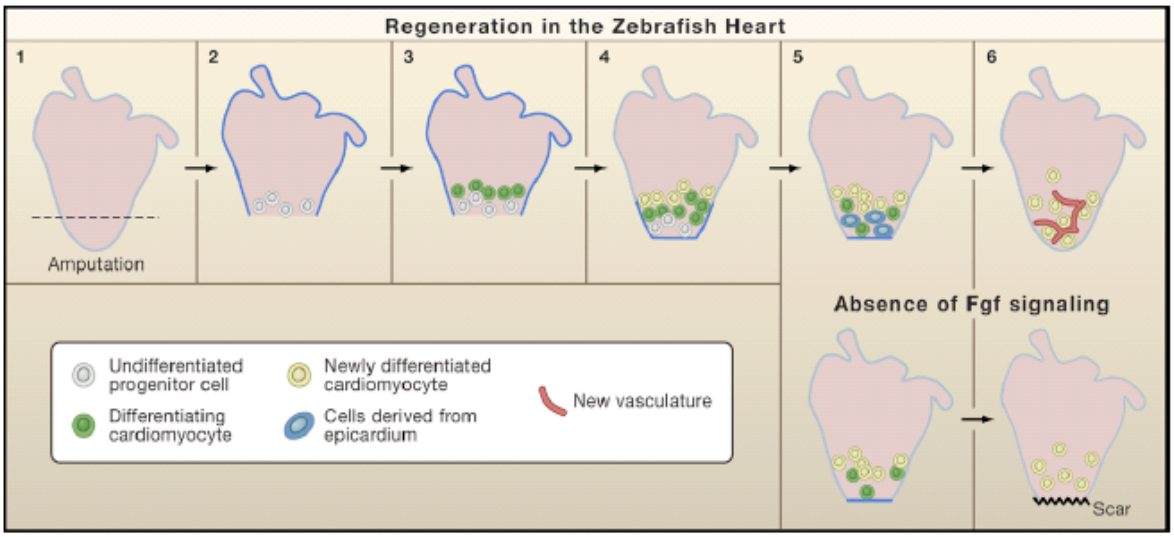

Figure 8 Heart Regeneration in Zebrafish. Following partial surgical removal of the ventricle, replacement cells appear to arise from undifferentiated progenitor cells that progressively differentiate into more mature cardiomyocytes (EGFP+RFP−, and then EGFP+RFP+). In addition, epicardial cells turn on expression of a number of genes, including *fgfr2*/*4* (activated epicardium, dark blue), and then subsequently appear to migrate toward the newly differentiated cardiomyocytes expressing *fgf17b*, leading to the establishment of new blood vessels in the regenerating tissue (steps 1–6). In the absence of Fgf signaling, the activated epicardial cells fail to invade the regenerating region, new blood vessels do not form, and a scar appears. From Curado and Stainier (2006).

Studies in a number of model organisms have implicated FGF signaling in cardiac specification (Zaffran and Frasch, 2002). FGF signaling has reiterative roles in regulating heart size and chamber proportionality: early in development, FGF signaling helps to establish properly sized and proportioned cardiac progenitor pools, and, at later stages, FGF signaling continues to contribute to the regulation of ventricular cell number, potentially by controlling population maintenance or growth. Various growth factors (e.g. FGF1, Neuregulin), small molecules (e.g. BIO, SB203580), viral oncoproteins (e.g. E1A, SV40 T antigen), an extracellular matrix protein (Periostin), and cell cycle activators (e.g. Cyclin A2, Cyclin D2, E2F1, E2F2, c-myc) were studied to induce mammalian cardiomyocyte proliferation in the hope that these molecules can be utilized for cardiac regeneration (Kühn et al., 2007). Recently, it has been demonstrated that a p38 inhibitor induced cardiomyocyte proliferation

(Brette et al., 2008). Treatment with a p38 inhibitor induced cell division in cultured adult cardiomyocytes demonstrated by transient dedifferentiation combined with cleavage furrow formation and separation of the cytoplasm studies to date have shown that cyclin D2, cyclin A2, FGF5, VEGF165, Periostin, and p38 inhibitor treatment promote cardiac regeneration due to induction of cardiomyocyte proliferation (van Amerongen and Engel, 2008). Unfortunately, improved cardiac function after treatment did not directly indicate that cardiomyocytes have proliferated; for example, growth factors could improve cardiac function by other mechanisms as they display pleiotropic actions. Inhibition of FGF signaling, a partway necessary for normal heart regeneration, disrupts epicardial cell supplementation and causes spontaneous ventricular scarring in uninjured adult fish. The significance of this distal activation and how this signal is transferred over these distances are unknown, but are of interest to the field. In the regenerating zebrafish heart, FGF receptors 2 and 4 are expressed in epicardial or epicardial-derived cells at or near the injury site, which was shown to express at least one FGF ligand. Furthermore, signaling by Fibroblast Growth Factors (FGFs) is necessary for epicardial cell activity during regeneration, as ectopic expression of a dominant negative transgene that inhibits signaling through FGF receptors disrupts this invasion of epicardial-derived cells, arresting regeneration. This stimulatory role for FGF signaling in adult zebrafish epicardial cells *in-vivo* appears to mirror its effects on cultured epicardial cells in *in-vitro* EMT assays. Together, these data indicate a specific role for FGF signaling indirecting the EMT of epicardial derived cells that ultimately vascularize the regenerate. Interestingly, treatment of injured rodent hearts by FGF supplementation along with p38 MAP kinase inhibition stimulated neovascularization, decreasing infarct size and the level of scarring These results suggest that both mammalian and non-mammalian vertebrates are responsive to FGFs after injury as a means to increase neovascularization, but only selected species like zebrafish naturally utilize FGFs to support myocardial regeneration. It is likely that additional factors will emerge that affects the production of coronary vessels in both zebrafish and mammalian models. Other recent studies support the idea that zebrafish have naturally optimized regenerative machinery that can function in both non-mammalian and mammalian injured hearts. The G-actin sequestering protein, Thymosin-b4, induces outgrowths from mammalian epicardial explants *in-vitro* (Bock-Marquette et al., 2004). Treatment of epicardial explants with Thymosin-b4 induces the differentiation of fibroblasts, endothelial and smooth muscle cells as assessed by gene expression and cellular morphology. In addition, *in-vivo* Thymosin-b4 treatment can partially restore cardiac survival and function following coronary

ligation in the mouse heart. Notably, during zebrafish heart regeneration, Thymosin-b4 expression is induced in the wound and compact myocardium, indicating that fish naturally release this epicardial stimulant upon injury. Using microarray technique Lien et al., (2006) demonstrated that both platelet-derived growth factor-a and -b (pdgf-a and pdgf-b) are upregulated during the heart regeneration in zebrafish. Also, it has been shown that PDGF-B homodimers induce DNA synthesis in adult zebrafish cardiomyocytes and that a chemichal inhibitor of PDGF receptor decreases DNA synthesis of cardiomyocytes both *in-vitro* and *in-vivo* during regeneration.

MicroRNAs: CHARACTERISTICS, FUNCTIONS AND THEIR INVOLVEMENT IN THE HEART REGENERATION PROCESS

MicroRNAs (miRNAs) are genomically encoded small RNAs used by organisms to regulate the expression of proteins generated from messenger RNA transcripts (Carrington and Ambros, 2003; Bartel, 2004) (**Figure 9**). miRNAs are non coding RNAs, highly conserved, abundant and predicted to regulate a large number of transcripts (Mishima et al., 2006). It has become clear that miRNAs are involved in many biological processes, including development, differentiation, proliferation and apoptosis (Reviewed by Callis and Wang, 2008). MicroRNA negatively regulate gene expression by binding to complementary target sequences at the 3'-untraslated regions (UTRs), trough translational repression and target mRNA degradation. (Pasquinelli et al., 2005; Ason et al., 2006; Flynt et al., 2007; Zhao and Srivastava, 2007). Nearly 500 mammalian miRNAs are transcribed in the nucleus and undergo successive processing events by the enzymes Drosha and Dicer to ultimately yield mature miRNAs of ~20–22 nucleotides (Berezikov et al., 2006). Mature miRNAs typically bind to target mRNAs by partial sequence matching after becoming incorporated into the RNA-induced silencing complex (RISC), resulting in degradation of the mRNA transcript and/or translational inhibition (Bagasra and Prilliman, 2004).

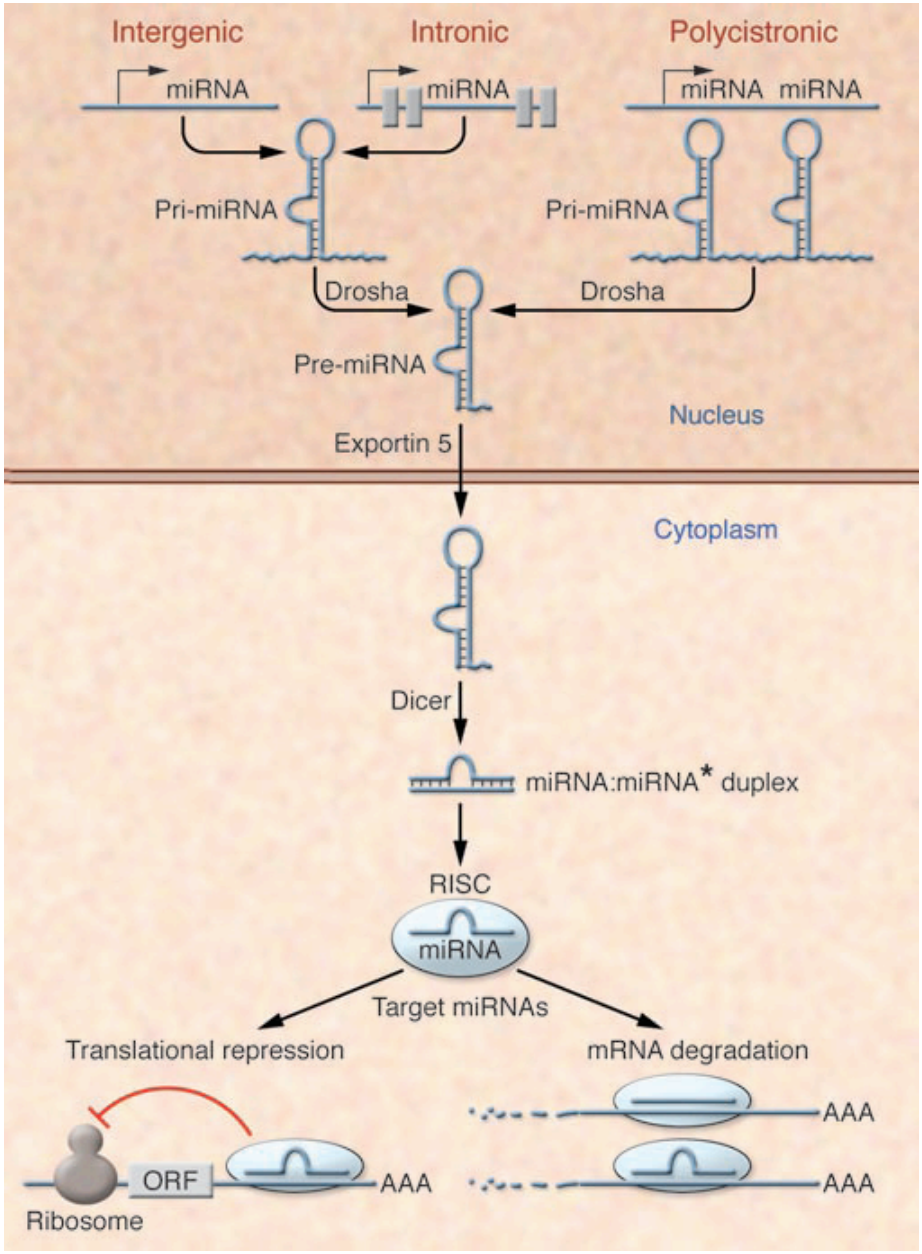

Figure 9 miRNA biogenesis and function. The primary transcripts of miRNAs, called pri-miRNAs, are transcribed as individual miRNA genes, from introns of protein-coding genes, or from polycistronic transcripts. The RNase Drosha further processes the pri-miRNA into 70–100 nucleotide, hairpin-shaped precursors, called pre-miRNA, which are exported from the nucleus by exportin 5. In the cytoplasm, the pre-miRNA is cleaved by Dicer into an miRNA:miRNA* duplex. Assembled into the RISC, the mature miRNA negatively regulates gene expression by either translational repression or mRNA degradation, which is dependent on sequence complementarity between the miRNA and the target mRNA. ORF, open reading frame. From van Rooij and Olson (2007).

The mechanism of action, in fact, appears to depend on the degree of complementarity between the miRNA and the target (McCarthy and Esser, 2007). Disruption of miRNAs in *Caenorhabditis elegans* and *Drosophila* suggest several ways by which miRNAs may control cellular events (Stark et al., 2003). In some cases, they function to "fine-tune" physiologic events, but in others they function as molecular "switches" (Brennecke et al., 2003; Johnston and Hobert, 2003; Sokol and Ambros, 2005). miRNAs can also function in a "fail-safe" mechanism to silence mRNAs that are unwanted in specific cell lineages (Cohen et al., 2006; Hornstein et al., 2005). In mice, interference with miRNA biogenesis by tissue-specific deletion of *Dicer* revealed a requirement of miRNA function during limb outgrowth (Bernstein et al., 2003) and in development of skin progenitors (Yi et al., 2006). However, the *in-vivo* requirement of specific miRNAs in mammals through targeted deletion remains unknown, and reliable prediction of mRNA targets is still problematic (Ason, 2006). Confronting the expression of some microRNAs between medaka, chicken, mouse and zebrafish, Ason et al., (2006) saw that the timing and location of miRNA expression is not strictly conserved in vertebrates and the variation of miRNA expression being more pronounced the greater the differences in physiology. Importantly, dysregulation of miRNAs has been linked to muscle-related diseases, such as cardiac hypertrophy (Rao et al., 2006; Chien, 2007). miRNAs therefore are a new class of regulators of muscle biology and they might become novel therapeutic targets in muscle-related human diseases (Callis et al., 2007). Also, the expression of microRNAs is dynamically regulated during cardiomyocytes hypertrophy (Tatsuguchi et al., 2007). Using microarray analysis Tsonis et al., (2007) discovered that miRNAs are involved in regeneration process; more in particular, they saw that *Let-7* members are potential regulators of dedifferentiation in lens and inner ear hair cell regeneration of the adult newt. Recent studies have revealed key roles of microRNAs as regulators of the growth, development, function, and stress responsiveness of the heart, providing glimpses of undiscovered regulatory mechanisms and potential therapeutic targets for the treatment of the heart disease. (van Rooij & Olson, 2007). In several recent studies, microarray analyses were performed to determine whether miRNAs are dysregulated in hypertrophic and failing hearts. These studies point to a collection of miRNAs that are up- and downregulated during pathological cardiac remodeling in rodents and humans (Latronico et al., 2008; Wang et al., 2009). *In-vitro* experiments using either over-expression or knockdown of miRNAs in cultured cardiomyocytes indicate that a subset of these miRNAs are indeed actively involved in cardiomyocyte hypertrophy (Tatsuguchi et al., 2007).

Many miRNAs are expressed in a tissue-specific manner and several miRNAs are specifically expressed in cardiac and skeletal muscle and play an important role in the regulation of muscle proliferation and differentiation processes (Callis and Wang, 2008). It has been demonstrated that miRNA biogenesis in the mouse heart is essential for cardiogenesis (Plasterk, 2006). A signature pattern of stress-responsive microRNA that can evoke cardiac hypertrophy and heart failure. van Rooij et al., (2006) described >12 miRNAs that are up- or down-regulated in cardiac tissue from mice in response to transverse aortic constriction or expression of activated calcineurin, stimuli that induce pathological cardiac remodeling. Many of these miRNAs were similarly regulated in failing human hearts. Similarly, cardiac over-expression of miR-195, which was up-regulated during cardiac hypertrophy, resulted in pathological cardiac growth and heart failure in transgenic mice. Northern blot analysis of the hypertrophy-regulated miRNAs in idiopathic end-stage failing human hearts showed increased expression of miR-24, miR-125b, miR-195, miR-199a, and miR-214, whereas the expression for miR-23 appeared to be variable within the non failing and failing groups. Thus, the altered pattern of miRNA expression in the failing human heart overlapped that of the hypertrophic mouse heart, suggesting that these miRNAs represent a molecular signature of adverse cardiac remodeling Over-expression of miRNAs evokes morphological changes in cardiomyocytes. miR-1 is among the most widely conserved miRNAs during evolution, and is found in the genomes of organisms as diverse as *C. elegans*, *Drosophyla*, zebrafish, chicken, mouse, and human. An ancient genomic duplication likely resulted in two distinct loci for the miR-1/miR-133 cluster in vertebrates, with identical mature sequences derived from the duplicated loci. In *Drosophila*, deletion of the single *miR-1* gene (*dmiR-1*), expressed specifically in cardiac and somatic muscle, results in a defect in muscle differentiation or maintenance (Kwon et al., 2005; Carè et al., 2007). *dmiR-1* targets the *Notch* ligand, *Delta*, a known regulator of cardiogenesis and myogenesis in flies (Kwon et al., 2005). In contrast, over-expression of miR-1 in mouse cardiac progenitors has a negative effect on proliferation, where it targets the transcription factor *Hand2*, which is involved in myocyte expansion (Zhao et al., 2005). More in particular it has been demonstrated that, miR-1-1 and miR-1-2 are specifically expressed in cardiac and skeletal muscle precursor cells. Similar to the heart, miR-1 over-expression in cultured skeletal myoblasts promotes skeletal muscle differentiation, as does the related but skeletal muscle-specific miR-206 (Chen et al., 2006; Kim et al., 2006). miR-133 over-expression curiously prevents skeletal muscle differentiation, suggesting that differential processing from the dicistronic transcript may

regulate cellular decisions of differentiation or proliferation (Chen and Lodish, 2005; Chen et al., 2006). Although significant dysregulation of miRNA expression has been reported in cardiac disease (Cheng et al., 2007; Sayed et al., 2007),it remains unknown if the heart requires miRNA function for normal development or maintenance. Furthermore, targeted deletion of the muscle-specific miRNA, miR-1-2, revealed numerous functions in the heart, including regulation of cardiac morphogenesis, electrical conduction, and cell-cycle control. (Kusenda et al., 2006; Zhao et al., 2007; Yang et al., 2007). The muscle specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting *GJA1* and *KCNJ2*. It was shown that miR-1 is overexpressed in individuals with coronary artery disease, and that when overexpressed in normal or infarcted rat hearts, it exacerbates arrhythmogenesis. Elimination of miR-1 by an antisense inhibitor in infarcted rat hearts relieved arrhythmogenesis. miR-1 overexpression slowed conduction and depolarized the cytoplasmic membrane by post-transcriptionally repressing KCNJ2 (which encodes the K+ channel subunit Kir2.1) and GJA1 (which encodes connexin 43), and this likely accounts at least in part for its arrhythmogenic potential. Thus, miR-1 may have important pathophysiological functions in the heart, and is a potential antiarrhythmic target. Neonatal hearts show increasing expression of miR-1 with age, and substantially higher levels are maintained in adult hearts as compared to neonatal hearts4–6, indicating that it might have cellular or pathophysiological functions other than myogenesis. It was found that miR-1 expression was elevated (B2.8-fold) in RNA samples from individuals with coronary artery disease (CAD; Supplementary Table 1 online) compared with those from humans with healthy hearts (HH). Some Authors have described muscle-specific miRNAs, such as the bicistronic miR-1 and miR-133 cluster and miR-206 (Callis et al., 2007). miR-1 and -133 are expressed in cardiac and skeletal muscle and are transcriptionally regulated by the myogenic differentiation factors MyoD, Mef2, and serum response factor (SRF) (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Kwon et al., 2005; Zhao et al., 2005; Chen et al., 2006; Adams et al., 2007). miR1 and miR-206 belong to the same miRNA gene family and share an identical seed sequence, which is defined by the 2-8 nucleic acids of the mature miRNA, whereas miR-133 is dissimilar to both miR-1 and miR-206. While miR-1 and miR-206 enhanced myogenesis, over-expression of miR-133 repressed myoblast differentiation and promoted myoblast proliferation. miR-1 and miR-133 play an important role in modulating muscle proliferation and differentiation (Callis et al., 2007). Carè et al., (2007) demonstrated that in mice and human miR-133 has a critical role in determining cardiomyocyte hypertrophy. In hypertrophyc hearts they observed a decreased expression of

both miR-133 and miR-1, which belong to the same transcriptional unit. *In-vitro* over-expression of miR-133 or miR-1 inhibited cardiac hypertrophy. In contrast, suppression of miR-133 by "decos" sequences induced hypertrophy. In mice miR-1 and miR-133 expressions are decreased during skeletal muscle hypertrophy in mice (McCarthy and Esser, 2007). Over-expression of miR-1 in the developing mouse heart resulted in reduced ventricular myocyte expansion and decreased the number of proliferating myocytes (Zhao et al., 2005). Using a sensitive and quantitative high-throughput technology, they profiled global miRNA expression in phenilephrine (PE)-treated mice neonatal cardiomyocytes Also they saw that specific inhibition of endogenous miR-21 or miR-18b with locked nucleic acid (LNA)-modified antisense oligonucleotides, augments hypertrophyc growth. It is interestling to note that miR-1/-206 and miR-133 have opposing effects although miR-1 and miR-133 derive from the same miRNA polycistron and are transcribed together (Callis et al., 2007). Inhibition of miR-133 was sufficient to induce cardiomegaly *in-vivo* (Carè et al., 2007); similarly, targeted deletion of miR-1-2 revealed numerous functions in the heart, including regulation of cardiac morphogenesis, electric conduction, and cell cycle control (Zhao et al., 2007). More recently, miR-1 and miR-133 were shown to produce opposing effects on oxidative stress-induced apoptosis in H9c2 cells, with miR-1 being proapoptotic and miR-133 being antiapoptotic. (Xu et al., 2007a; Yin et al., 2008). FGF-dependent depletion of microRNA-133 promotes appendage regeneration in zebrafish, defined by rapid changes in gene expression that achieve dramatic developmental effects. During zebrafish fin regeneration there is a dynamic regulation of many miRNAs from FGFs. In particular, miR-133 levels are high in uninjured fins but low during regeneration. When regeneration was blocked by FGF receptor inhibition, high miR-133 levels were quickly restored. Experimentally increasing amounts of miR-133 attenuated fin regeneration. Conversely, miR-133 antagonism during FGF receptor inhibition accelerated regeneration through increased proliferation within the regeneration blastema. The *Mps1* kinase, an established positive regulator of blastemal proliferation, is an *in-vivo* target of miR-133. In this way, miRNA depletion can be identified as a new regulatory mechanism for complex tissue regeneration. miR-133, has relatively high levels in the uninjured fin, but these levels drop sharply during regeneration. By a combination of gain of- function and loss-of-function experiments, our data indicate that miR-133 acts as a regenerative brake within a regulatory circuit for regeneration. Following amputation, FGF signaling reduces miR-133 expression as part of the regeneration program, facilitating normal expression of targets like the *mps1* kinase that direct blastemal proliferation and tissue renewal. miR-133 is depleted by

FGF signaling during fin regeneration and attenuates growth mechanisms. The expression of several miRNAs was identified as being regulated in response to cardiac muscle hypertrophy using a miRNA microarray screen (van Rooij et al., 2006). Further analysis of those miRNAs revealed that miR-195, whose expression is up-regulated during cardiac hypertrophy, is sufficient to induce hypertrophic growth in cultured cardiomyocytes as well as in transgenic mice. Dysregulation of miRNA expression was also found in patients with heart failure (van Rooij et al., 2006). These findings point out that miRNAs are likely associated with muscle-related diseases and are potential therapeutic targets. Cardiac overexpression of miR-195 is sufficient to drive cardiac hypertrophy (**Figure 10**).

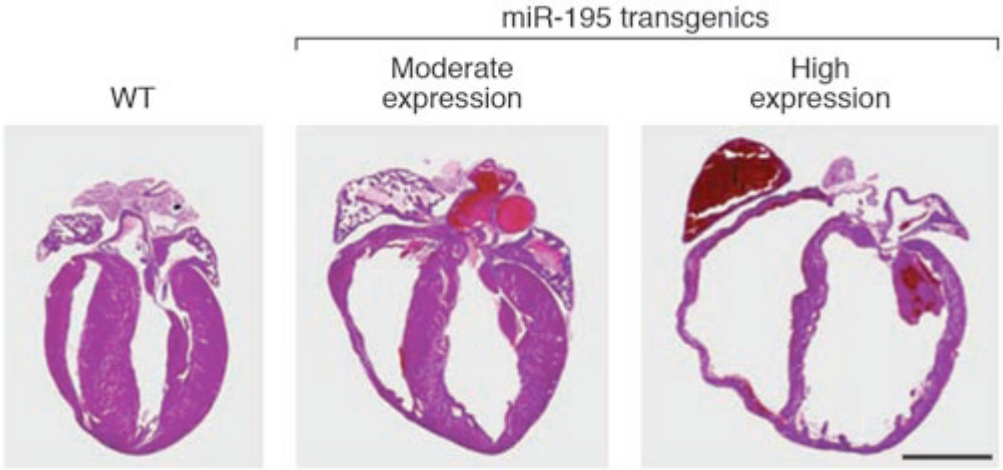

Figure 10 Induction of cardiac hypertrophy and heart failure by miR-195. H&E sections of 2-week-old wild-type and transgenic mice expressing miR-195 under control of the áMHC promoter. In transgenic as compared with wild-type mice, moderate levels of miR-195 expression (26-fold) cause cardiac hypertrophy, and higher levels of expression (29-fold) cause dilated cardiomyopathy with ventricular dilatation and wall thinning. Scale bar: 2 mm. From van Rooij and Olson (2007).

Authors overexpressed miR-24, miR-195, and miR-214 specifically in the heart under the control of the myosin heavy chain (MHC) promoter. Thus, down-regulation of miR-133 during cardiac hypertrophy would be predicted to result in an increase in SRF expression, which could contribute to the adverse remodeling response. It is also notable that miR-133a is encoded by the same pre-miRNA as miR-1, an inhibitor of cardiac growth. It will be interesting to determine whether forced overexpression of miR-133 and other miRNAs that

are down-regulated during cardiac hypertrophy suppress cardiac growth *in-vivo* Recently it has been demonstrated that microRNA-320 is involved in the regulation of cardiac ischemia/reperfusion (I/R) injury in murine hearts *in-vivo* and *ex-vivo*, by targeting heat-shock protein 20 (Ren et al., 2009). Overexpression of miR-320 enhanced cardiomyocyte death and apoptosis and *in-vivo* treatment with antagomir-320 reduced infarction size. Thus, miR-320 may constitute a new therapeutic target for ischemic heart diseases. van Rooij et al., (2007) demonstrated that mir-208 is an essential cardiac-specific regulator of βMHC expression and mediator of stress and T3 signaling in the mice heart. miR-208, encoded by an intron of the αMHC gene, is required for cardiomyocyte hypertrophy, fibrosis, and expression of βMHC in response to stress and hypothyroidism.

Current analysis indicates the zebrafish genome encodes 415 miRNAs, which can be grouped into 44 families (Thatcher et al., 2008). The largest of these families is the miR-430 family, containing 72 members largely clustered in two main locations along chromosome 4. Most zebrafish miRNAs exhibit tissue patterns of expression. By hight-throughput sequencing of small-RNA cDNA libraries from 5-day-old zebrafish larvae and adult zebrafish brain Kloosterman et al., (2006) found 139 known miRNAs and 66 new miRNAs. They analyzed the temporal and spatial expression patterns for35 new miRNAs and for 32 known miRNAs in the zebrafish by whole mount in situ hybridization and northen blotting, and they found that some of them were expressed ubiquitously, but many of the miRNAs were expressed in a tissue-specific manner. Wienholds et al., (2005) determined the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs in zebrafish embryos using microarray and in situ hybridization, using locked-nucleic acid-modified oligonucleotide probes. They saw that most miRNAs are expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development, which suggests that their role is not in tissue fate establishment but in differentiation or maintenance of tissue identity. Woltering and Durston, (2008) demonstrated that miR-10, conserved within the vertebrates, represses *HoxB1a* and *HoxB3a* and synergize with *HoxB4* in the repression of these target genes in zebrafish, genes involved in patterning the anterior-posterior axis. Using a target-selected inactivation of the *dicer1* gene in zebrafish, Wienholds et al., (2003) discovered that the microRNA-producing enzyme Dicer1, which is conserved from fungi to vertebrates, is essential for zebrafish development. Morton et al., (2008) demonstrated that the highly conserved miR-138 is expressed in specific domains in the zebrafish heart and is required to establish appropriate chamber-specific gene expression patterns. Also, disruption of miR-138

function led to ventricular expansion of gene expression normally restricted to the atrio-ventricular valve region and, ultimately, to disrupted ventricular cardiomyocyte morphology and cardiac function. They saw that miR-138 targets *aldh1a2*, encoding retinoic acid (RA) dehydrogenase (*Raldh2*). *Raldh2* is involved in RA synthesis, which is required for early chamber specification and anterior-posterior cardiac patterning in mouse, chick, and zebrafish, and is important for myocardial maturation (Stainier and Fishman, 1992).

Cardiac-specific overexpression of miR-195, which is consistently up-regulated in rodent and human hypertrophic hearts, for example, results in dilated cardiomyopathy and heart failure in mice as early as two weeks of age, implying that upregulation of miR-195 during cardiac hypertrophy actively contributes to the disease process (van Rooij et al., 2006). Based on target predictions for miR-195, Authors speculate that the cardiac phenotype of these transgenic mice results from the downregulation of multiple pro-survival proteins by this miRNA. Since miR-195 belongs to a small family of related miRNAs, it will be interesting to investigate the potential involvement of the other family members in cardiac disease.

AIM OF THE THESIS

Cardiac diseases are the major cause of death in developed countries, and heart failure is the most common cause of hospitalization in US citizens over 65. In particular, Myocardial Infarction (MI) is characterized by loss of functional cardiomyocytes that are not able to regenerate; for this reason, nowadays the only effective treatment is heart transplantation. Recent work demonstrates that the injection of adult bone marrow stem cells into infracted area can represent an effective alternative method for the treatment of MI. However, the routine application in the clinic requires further studies. Stem cells, which have the potential to form a wide variety of different cell types, are widely regarded as being essential to repair damaged organs. Another approach is the ability to manipulate the regeneration potential of the adult organ through the activation of adult stem cells in damaged tissue. Also, is not yet well clear the origin on new cardiomyocytes those repopulate the damaged area and which signals are involved. In this regard, it has been shown that the Teleost zebrafish (*Danio rerio*) efficiently regenerate the ventricle after a removal of about 20% of the ventricle apex *in-vivo*; for this reason and for all the vantages that this model organism offers for scientific studies, it can use as model to better understand the molecular differences with the human heart regeneration.

The present thesis has the aim to study the molecular mechanisms of heart regeneration in zebrafish; it has been demonstrated that microRNAs (miRNAs) are involved in many biological processes and also in cardiac regeneration and in hypertrophic conditions, but it isn't clear in which manner. In addition, the identification of the microRNA involved in heart regeneration is not complete and it is unknown if the same miRNAs are conserved in mammals. For this reason a purpose of the work is to understand the regulation of genetic translation by cardiac microRNAs, to identify with qRT-PCR, miRNAs naturally expressed in the adult zebrafish heart and those that are differentially expressed during the regeneration process *in-vivo* and in hypertrophic conditions induced *ex-vivo* with phenilephrine (PE) (**Chapter II**).

Other aim of the thesis is to characterize the time and space expression of genes during the regeneration process *in-vivo*, by whole-mount *In Situ* Hybridization experiments (ISH). Also, it is unknown if the zebrafish heart is able to survive in *ex-vivo* conditions and if injured hearts are able to regenerate under these conditions. For this reason, another aim of the thesis is to obtain an *ex-vivo* model of cardiac regeneration, optimize the culturing conditions to reproduce the *in-vivo* process and to understand the role of different Fibroblast Growth

Factors (FGFs) involved in this process. It is important to understand if the regeneration can occur under *ex-vivo* conditions and if this is as efficient as in *in-vivo* conditions. Further, we will determine if heart regeneration in *ex-vivo* involves proliferation of cardiac resident cells, through BrdU incorporation assays (**Chapter III**).

Despite the numerous genomic studies involving zebrafish, there are only a limited number of reports regarding the zebrafish proteome. Proteomic approaches are innovative and offer an understanding of all the proteins species within a tissue or embryo at a fixed stage. Little is known about which proteins are naturally present in the adult zebrafish heart and which are differentially synthesized (up or down regulated) during the *in-vivo* regeneration process. One aim of this study is to profile the proteome of zebrafish adult heart using two-dimensional polyacrylamide-gel electrophoresis (2DE), protein fractionation, and in-gel proteolysis, followed by MALDI-TOF MS analysis of proteolytic digests extracted from individual spots (**Chapter IV**). It is important to identify the proteins that are differently expressed during the heart regeneration and this will be performed using the Differential Gel Electroforesis (DiGE), an innovative and sensitive proteomic technique that allows the simultaneous comparison of two samples in the same 2D gel (**Chapter V**). Understanding the cellular and molecular mechanisms by which lower vertebrate model systems, as zebrafish, are able to faithfully regenerate complex organs, as the heart, after injury will help illuminate potential therapies for diseases of organ damage in humans and can yield a tremendous impact in modern translational medicine.

CHAPTER II

microRNAs expression in zebrafish (*Danio rerio*) hypertrophy-induced and regenerating hearts

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ABSTRACT

Despite being a well-recognized model to study cardiogenesis, zebrafish (*Danio rerio*) is an under utilized organism for studying cardiac remodeling, possibly since myocytes hypertrophy has not been reported in adult zebrafish. In this study, for the first time, the hypertrophy was induced in zebrafish hearts cultured *ex-vivo,* using phenilephrine. The effect of the treatment was dose and timing valued by histology and immunohistochemistry. Moreover, due to the similarities between fish and mammalian genomes, using qRT-PCR experiments, it was analyzed the expression of some microRNAs (miRNAs or miRs) (miR-1, miR-133a, miR-133b), already known to be involved in hypertrophic hearts in mammals. Vertebrates like Urodele amphibians and Teleost fish, as zebrafish, are able to effectively restore organs, including the heart, differently from mammals. In this study, 20% ventricle-deprived hearts were left regenerate *in-vivo* and analyzed from 1 to 30 days for the expression of the above-mentioned miRNAs. Both experimental designs showed down-regulation of miRNAs, especially miR-133a and miR-133b, demonstrating the importance of miRs in the hyperthrophy conditions and during the regeneration process. This experimental system, using different and easier model of study, should provide clues to understand human pathophysiology and the new frontier of stem cell regeneration system.

INTRODUCTION

Human heart disease can involve abnormalities in morphogenesis, cardiac rhythm, muscle maintenance, and function. Damage to the heart muscle is typically irreversible as cardiomyocytes terminally exit the cell cycle postnatally and have little or no regenerative capacity, despite the niches of cardiac progenitors that may contribute to basal turnover of myocytes (Soonpaa and Fiel, 1998; Torella et al., 2006). Networks of transcription factors regulate heart development and maintenance in a dose-dependent manner (Olson and Srivastava, 1996), but the effects of translational regulation on the titration of these pathways are still not completely understood. Among the factors involved in the heart development, maintenance, and regeneration, microRNAs (miRNAs or miRs) could play an important role. MiRNAs are genome encoded small RNAs that undergo successive processing events by the enzymes Drosha and Dicer to ultimately yield mature miRNAs of 20–22 nucleotides, used by organisms to regulate the expression of proteins generated from messenger RNA transcripts (Berezikov et al., 2006). This regulation is exerted by the down-regulation of protein

production, either by inhibiting the translation of protein from messenger RNA or by promoting the degradation of mRNA, primarily through base paring to the 3' untranslated region (UTR) of target mRNAs; this leads to mRNA cleavage and/or translation repression (Bartel, 2004). Growing evidence indicates that miRNAs are involved in basic cell functions and differentiation, including cardiac myocytes (Zhao et al., 2007). The complex cellular processes where myocytes undergo depend on precise spatiotemporal regulation of protein levels, some of which function as "rheostats" to execute programs in a quantitative fashion (Ambros, 2004; Kloosterman and Plasterk, 2006; Zhao and Srivastava, 2007). A few miRNAs, particularly miR-133 and miR-1, which are included in the same bicistronic unit, are specifically expressed in skeletal muscle and cardiac myocytes (Chen et al., 2006; Carè et al., 2007). Notably, miR-133 and miR-1 play key roles in skeletal myoblast proliferation and differentiation, respectively. For example, miR-133 overexpression prevents skeletal muscle differentiation, suggesting that differential processing from the dicistronic transcript may regulate cellular decisions of differentiation or proliferation (Chen et al., 2006; Carè et al., 2007). Although significant dysregulation of miRNA expression has been reported in cardiac disease (van Rooij et al., 2006; Sayed et al., 2007), recently it was demonstrated the role of miR-1 and miR-133, are involved in cardiac hyperthrophy (Carè et al., 2007; Elia et al., 2009).

Mammalian species have little or no ability to replace lost cardiac muscle. This poor regenerative capacity is due in part to the failure of adult cardiomyocytes, the beating cells in the heart, to undergo proliferation (Quaini et al., 2004). In fact, the normal response of mammalian hearts to injury or hypoxia is hypertrophy—the growth of cardiomyocytes without cell division. Being the heart one of the most conserved organs at the molecular level in vertebrates (Poss et al., 2002; Buckingham et al., 2005; Lepilina et al., 2006; Olson, 2006; Srivastava, 2006), and, considering that different vertebrate species have different cardiac regeneration rates -high in teleost fish, moderate in urodele amphibians, and almost negligible in mammals (Scaddind, 1977)-, it is possible to study some differentiation, regeneration and pathology processes using vertebrates models evolved before mammals such as the fish. Despite being a well-recognized model to study cardiogenesis, the zebrafish is an under utilized organism for studying cardiac remodeling, possibly since myocytes hypertrophy has not been reported in adult zebrafish. Thus, the aim of this research is to characterize the miRs involved in cardiac hypertrophy induced with phenilephrine (PE) in *ex-vivo* culture. Moreover, since the role of miRs during the cardiac regeneration process in zebrafish is

largely unknown, in this research, it was evaluated the gene expression timing in adult regenerating hearts *in-vivo* operated by 20% of ventricle. Regenerating the human heart has thus become a major goal in experimental medicine, discovering the molecular mechanisms of cardiac regeneration and hypertrophy in zebrafish can suggest new strategies to control heart pathologies and activate genes able to repair cardiac damages.

MATHERIALS AND METHODS

Ex-vivo hearts culture and induction of hypertrophy with phenilephrine (PE)

Hearts were removed from adult zebrafish, letally anesthetized with Tricaine (MS222, Sigma), see below. After one wash in osmolar L15 media (Leibovitz's L15 media, Sigma) added with 5% FCS, 5% glutammin, 5% penicillin/streptomicin), hearts were transferred in 12 wells dish petri with 1 ml of L15 media and incubated at 28°C for 8 hours. Hearts were than transferred in L15 media without FCS, and in the presence of 100 (n=10), 200 (n=10), 500 (n=40) µM of phenilephrine (PE, Sigma) for 48, or 72 hours to study the hypertrophy induction. At the end of treatment, some hearts were fixed in Bouin or 4% PFA in PBS and then embedded in paraffin or included in OCT for cryostat sections. Others hearts were used for RNA extraction.

Resection of 20% of ventricular apex of zebrafish heart

The animal experiments were performed according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Adult zebrafish (6-18 months old, n=60) were anesthetized for 3 minutes in 0.168 mg/l Tricaine (MS222, Sigma) and placed dorsally in a humid sterile sponge, with ventral side up. The surgery was done following the protocol developed by Poss et al., (2002). Brefly, a small incision (scalpel 0.15mm, Fine Science Tools Inc. US.) was made just under the gills, in correspondence to where the heart is located. The pericardial sac was isolated and punctured to expose the ventricle by gentle abdominal pressure. Approximately 20% of the ventricle apex was removed by iridectomy scissors (Fine Science Tools Inc. US.). With this protocol, 90% of fish was survived. After surgery, fish were returned to water and stimulated to breathe with air bubbles from an air pump. After 1, 2, 3, 7 and 30 days post amputation of ventricular apex (dpa), fish (n=9 for each timing point considered) were lethally anesthetized with Tricaine and the whole hearts were used for histology (n=3 for each timing point) or for RNA extraction for qRT-PCR analysis (n=6 for each timing point).

Histology and immunohistochemistry

i. Sections and histology

Hearts collected from adult zebrafish were fixed in Bouin's fluid for 8 hours at 4°C or fixed in 4% PFA for 24 hours at 4°C. an overnight incubation in PBS containing 10% sucrose was performed to prevent cryo-damage before immersion in liquid nitrogen or the consequent dehydration process (for paraffin embedding). Cryostat sections (7 μm thick) were prepared (Bright-GB), mounted onto poly-L-lysine-coated slides (Sigma) or APES treated slides and used for immunohistochemistry or normal staining. Dehydration (only for paraffin embendding) was performed in degreeded alchool series, transferred into in 100% toluen and finally included in paraffin. Hearts section of 7 μm obtained using a Reichert-OME microtome were placed in slides treated with APES. Dewaxed sections were rehydrated and stained by Mallory's trichrome or Masson' s trichrome staining methods. Sections were observed with an Axioskope microscope (Zeiss) supplied with image-analysis programs.

ii. Fluorescence

Cryosections were let hawed at R.T. for 20 minutes (under sterile flow) and permeabilization was performed with 0.5% TRITON in PBS for 15 minutes. Blocking of specific sites was performed with 2% BSA in PBS for 20 minutes at 37°C. 1:500 phalloidin in 2% BSA in PBS and 1:100 DAPI were added for 1 hour at 37°C in the dark. After one wash in PBS, slides were mounted with glicerol: phosphate tampon 9:1 and kept at 4°C in the dark until observation at the confocal microscope (Elipse 90-Nicon C eclipse, Nikon).

iii. Statistical analysis

Two-five specimens per group were used and, in each specimen, 5-8 consecutive sections of the heart were examined. The stained myocytes of heart (ventricle portion) were calculated on 1 mm^1 area by a computer-assisted image analysis system by an observer unaware of treatments. The marked area occupied by myocytes was calculated as mean ± standard deviation from the mean. Numerical results were analyzed by means of a two-tailed Student's t-test. For qRT-PCR, significant differences (probability values) between the experimental groups and controls/non treated animals were calculated using 1-way, unpaired Student's *t* test. All experiments were repeated 3 times and presented as average standard error of the mean.

Total RNA extraction and qRT-PCR

Total RNA from various samples was extracted from fresh tissues homogenized with 1 ml/50 mg Trizol (Invitrogen)/tissue. After, 0,2 ml of chloroform were added for ml of Trizol used; samples were vigorously vortexed for 15 sec., left on ice and centrifuged at 13.000 rpm for 20 minutes at 4°C. The supernatant, containing the RNA, has been transferred in a new eppendorf tube. Precipitation was performer adding 0,5 ml of isopropanol (2-isopropanol, Sigma) every ml of Trizol used. Samples were incubated over night at -20°C and centrifuged at 13.000 rpm for 20 minutes at 4°C; upper phase was discarded and pellet has resuspended in 1 ml of 75% ethanol, centrifuged and the pellet has been dried at R.T., before resuspending in dietil-pirocarbonate (DEPEC) water and stored at -80°C. Total RNA has been checked by Pico-drop (Perkin Elmer) and in electrophoretic gel, and used to perform RT-PCR and qRT-PCR. Total RNA has been extracted from one adult zebrafish (body weight: 400 mg), from an adult zebrafish without heart (528 mg), from a pool of 13 hearts (23,6 mg). For the experimental design of hypertrophy, total RNA was extracted from hearts (n=6), cultured (48 or 72 hours) in L15 (Sigma) media without serum and from hearts (n=6), cultured with 500 μM phenilephrine (48 or 72 hours). Total RNA has been analyzed with qRT-PCR to evaluate the expression of miR1, miR133a, miR133b and miR208. For the *in-vivo* heart regeneration experiments, total RNA was extracted from 7 hearts of control (not operated), 1 dpa (n=6), 2 dpa (n=8), 3 dpa (n=6) and 7 dpa (n=6). miRNAs levels have been measured with mirVana qRT-PCR miRNA Detection Kit (Ambion, Inc.), conjugated with fluorescent dye SYBR Green I (Molecular Probes, Carlsbad, CA). Amplification and visualization of specific products has been performed with sequence detection system ABI Prism 7700. As internal control, have been used U6 primers for themplate normalizzazion. Fluorescence signals have been normalized respect to an internal reference and the Cycle of threshold (Ct) has been set in inside the exponential phase of PCR. Relative gene expression has been calculated comparing the cycle times for every PCR target. Ct values of every PCR target have been normalized subtracting the Ct value of the U6 that is represented from ΔCt value. The relative expression level has been calculated using the following equation: relative gene expression=2^{-} (ΔCt sample- ΔC control).

RESULTS

The histology analysis of the zebrafish adult heart (**Figure 1 a**) evidenced the presence of four chamber in series disposed: *sinus venosus*, *atrium*, ventricle and *bulbus arteriosus*. The heart is localized in ventral position, just next and caudally the gill chamber and enveloped by serous pericardium. The hearts cultured in *ex-vivo* with L15 w/o fetal serum, fixed after 48 h or 72 h evidenced similar histology as compare with the fixed hearts left in the body (**Figure 1 a,b**). These latter specimens were used as controls for the *ex-vivo*/hypertrophy experiments. The first experiment evaluated the dose-action of the PE in the 48 h of culture. These experiments revealed that the optimal concentration to obtain the enlargement of the muscle fibers was 500 μM (**Figure 1**). **Figure 2 (c-f)** displays adult zebrafish hearts cultured *ex-vivo* with 500 μM phenilephrine (PE) for 48 or 72 h. The treatment with 500 μM PE for 48 h caused a slight increase of the muscles fibers (**Figure 2 c-d**) and the permanence of PE for 72 h had significantly increased the size of the myocytes ($P< 0.01$), as revealed by image analysis done per muscles area (1 mm^2) (**Figure 2 e,f**). Moreover, in the latter experimental group, the wall of ventricle resulted thickest as compare to control group (**Figure 2 f).** To better evaluate the contractile apparatus and thus the hypertrophy, was performed a staining with phallodine rodamate, and the specimens were analyzed by confocal microscopy (**Figure 3**). The phalloidine had marked the actin filament of the muscle fibers, evidencing also in this case, an increase of muscle fibers in experimental hearts, especially in 72 h cultured hearts ($P< 0,05$).

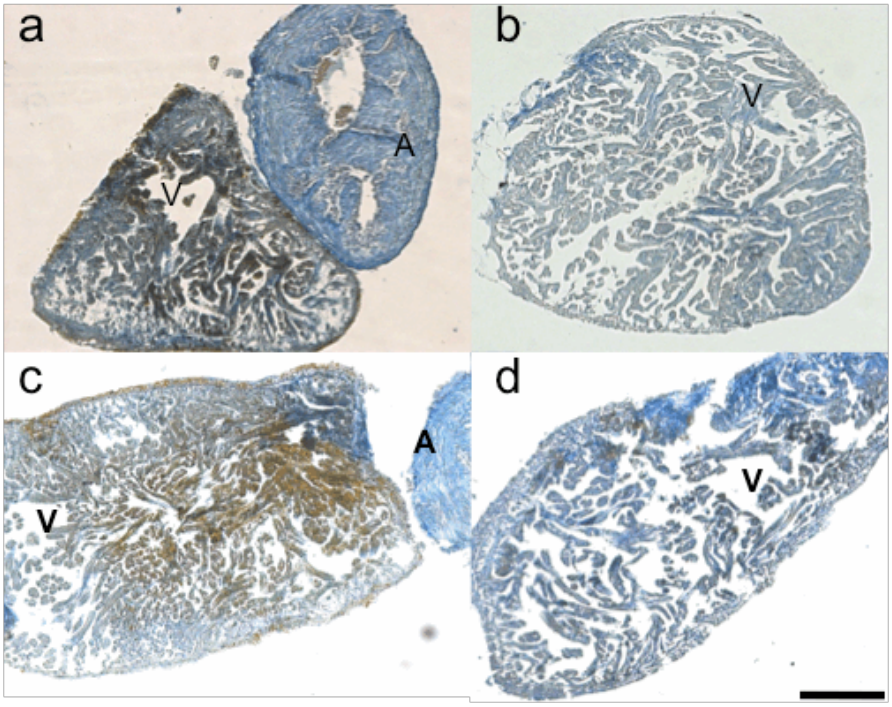

Figure 1. a) Control cultured ex-vivo 48 h; (A=*atrium*; V=ventricle) for 48 hours; bar: 250 µm. **b-d)** experimental hearts (ventricle part) treated with 100 µM PE **(b)**, 200 µM PE **(d)** and for 500 µM PE **(c)** for 48 hours; bar: 200 µm.

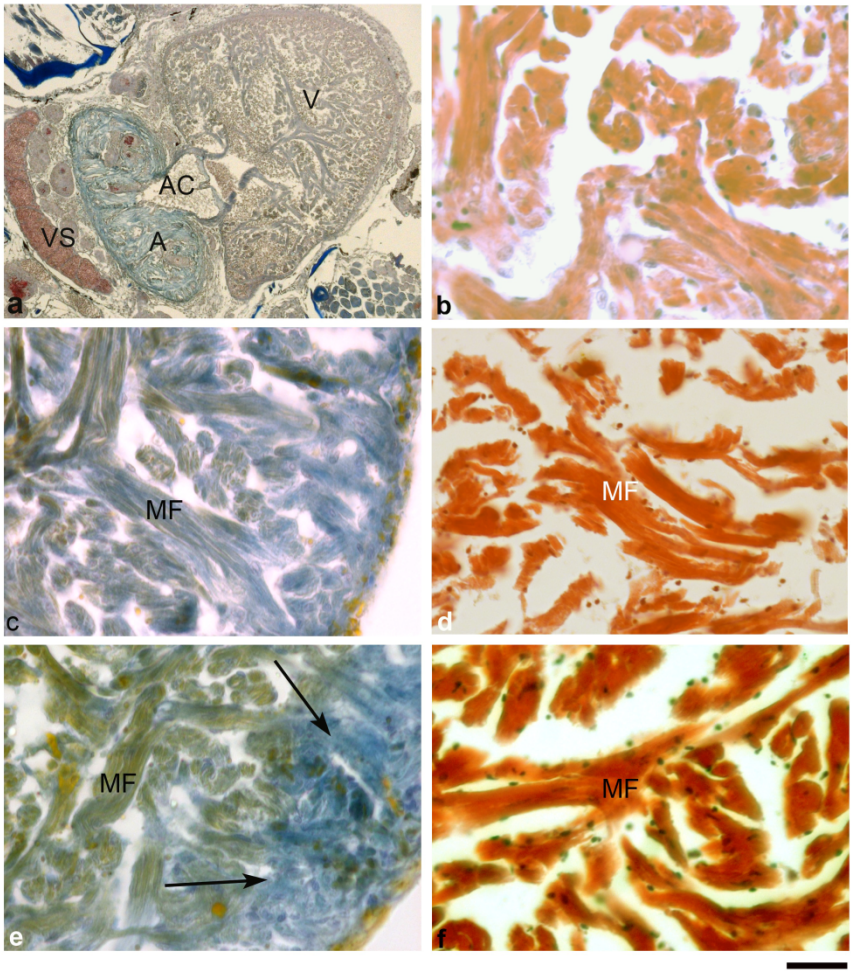

Figure 2. Zebrafish heart cultured with PE. a) Heart in the adult whole zebrafish is placed in ventral position and shows the four chambers in series: *sinus venosus* (SN), *atrium* (A), ventricle (V) and *conus arteriosus* (AC) (Mallory's, trichrome staining); bar: 0,25 mm. **b)** Control zebrafish heart cultured in *ex-vivo* for 48 hours shows similar muscle fibers pattern as compare whit *in-vivo* heart, (Masson's trichrome staining) bar: 40 µm. **c-d)** Experimental hearts cultured with PE. **c)** 48 h cultured heart shows a slight enlargement of muscle fibers (Mallory's trichrome staining), evidenced in **d)** with Masson's trichrome staining (MF= muscles fibers). **e)** Mallory's trichrome staining and the **f)** Masson's trichrome staining evidenced the enhancement of hyperthrophy of the muscle fibers (MF) and of the muscle wall of the heart ventricle (arrows) in 72 h PE cultured organ. (bar: 40 µm).

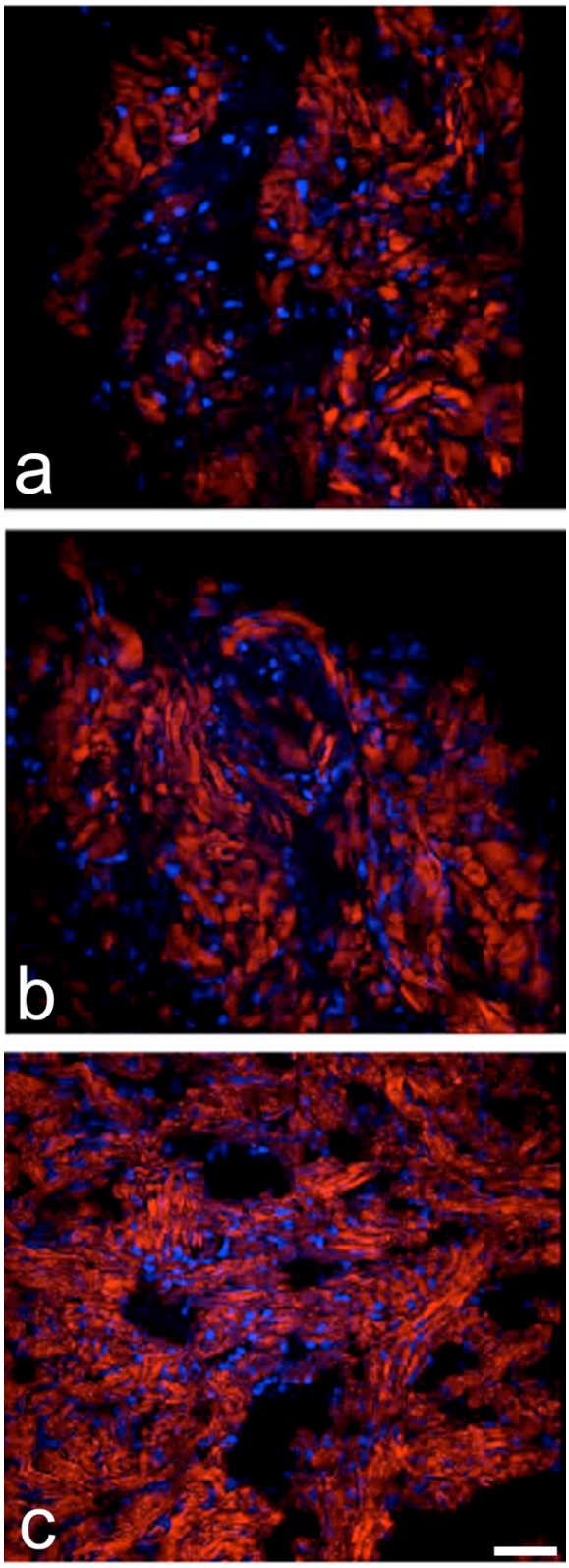

Figure 3. Phallodine rodamate/DAPI staining of non treated **(a)** and PE treated hearts for 48 h **(b)** and 72 h **(c)**. The heart treated with PE for 72 h evidenced an enhancement of the phalloidin staining-actin fibers (red color). Bar 40 μm.

Cardiac miRNAs expression in zebrafish

A multiple alignment of miRNAs sequences between human, zebrafish and mouse was performed before starting the qRT-PCR experiments in order to detect the miRNAs expression levels in zebrafish hearts as compared with mammals. The multiple alignments showed a high level of homology among the sequences (**Table 1**). The qRT-PCR of the adult no treated hearts evidenced the miR-1, miR-133a, miR-133b expression was also in zebrafish hearts, whereas the miR-208 was unexpressed in whole animal as compare to mouse and human hearts (**Graphic 1**). In zebrafish, miR-1 has a similar expression to miR-133a and miR-133b, it was expressed in the hearth and in other parts of the body (in whole Zf, in Zf without heart and in heart specifically) but with less expression levels as compare with other probes.

```
CLUSTAL W (1.83) multiple sequence alignment

hsa-mir-1-2       UGGAAUGUAAAGAAGUAUGUA- 21
hsa-mir-1-1       UGGAAUGUAAAGAAGUAUGUA- 21
mmu-mir-1-1       UGGAAUGUAAAGAAGUAUGUA- 21
mmu-mir-1-2       UGGAAUGUAAAGAAGUAUGUA- 21
dre-mir-1-1       UGGAAUGUAAAGAAGUAUGUAU 22
dre-mir-1-2       UGGAAUGUAAAGAAGUAUGUAU 22
                  **********************

dre-mir-133b*          -UUUGGUCCCCUUCAACCAGCUA- 22
dre-mir-133c           -UUUGGUCCCUUUCAACCAGCUA- 22
hsa-mir-133b           --UUGGUCCCCUUCAACCAGCUA- 21
mmu-mir-133b           --UUGGUCCCCUUCAACCAGCUA- 21
hsa-mir-133a-1         --UUGGUCCCCUUCAACCAGCUGU 22
mmu-mir-133a-1*        --UUGGUCCCCUUCAACCAGCUGU 22
hsa-mir-133a-2         --UUGGUCCCCUUCAACCAGCUGU 22
mmu-mir-133a-2*        --UUGGUCCCCUUCAACCAGCUGU 22
dre-mir-133a-2         -UUUGGUCCCCUUCAACCAGCUG- 22
dre-mir-133a-1*        -UUUGGUCCCCUUCAACCAGCUG- 22
mmu-mir-133a-1         -GCUGGUAAAAUGGAACCAAAU-- 21
dre-mir-133a-1         AGCUGGUAAAAUGGAACCAAAU-- 22
mmu-mir-133a-2         -GCUGGUAAAAUGGAACCAAAU-- 21
dre-mir-133b           -GCUGGUCAAAUGGAACCAAGUC- 22
                          ****     *  *****  *
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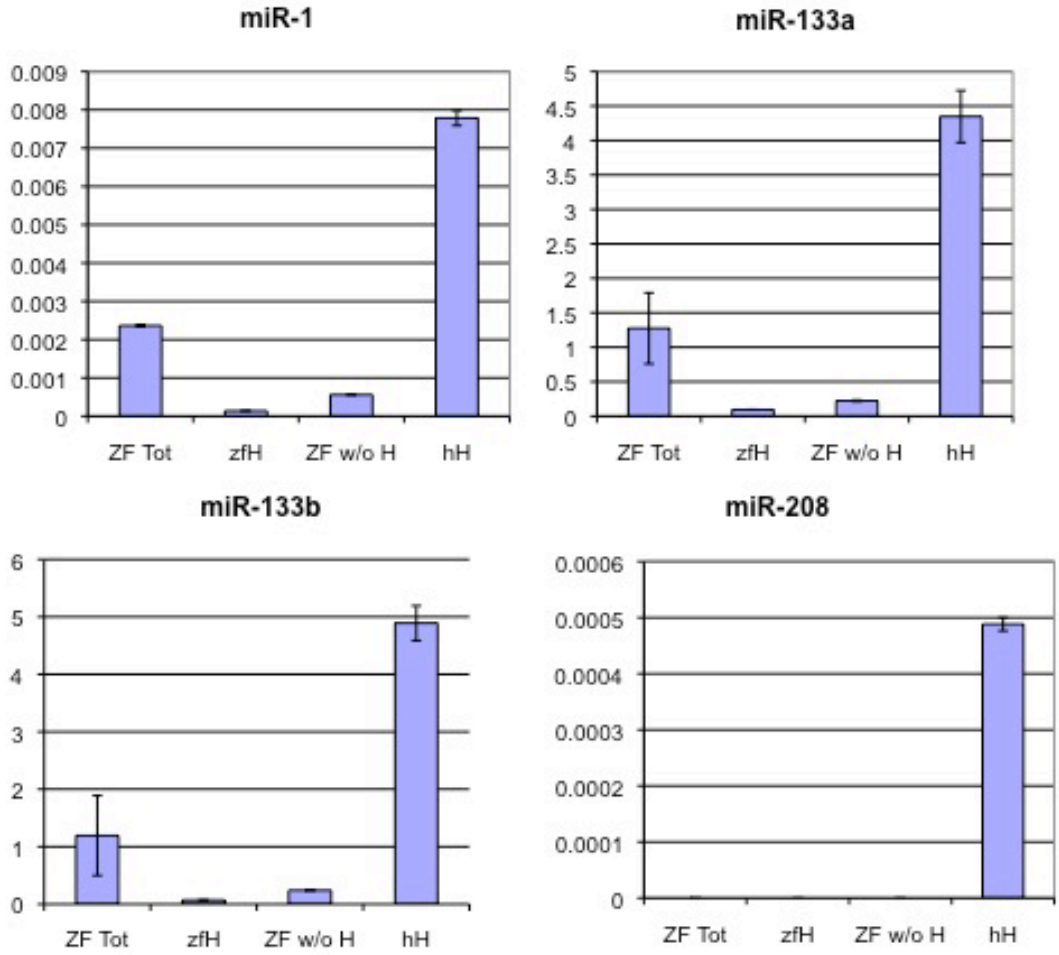
Table 1. Zebrafish (dre), mouse (mmu), human (hsa) miR1-1, miR1-2, miR133a-1, miR133a-2, miR133b and miR133c. No sequences were found for miR-208 in zebrafish. Clustal W multiple alignments.

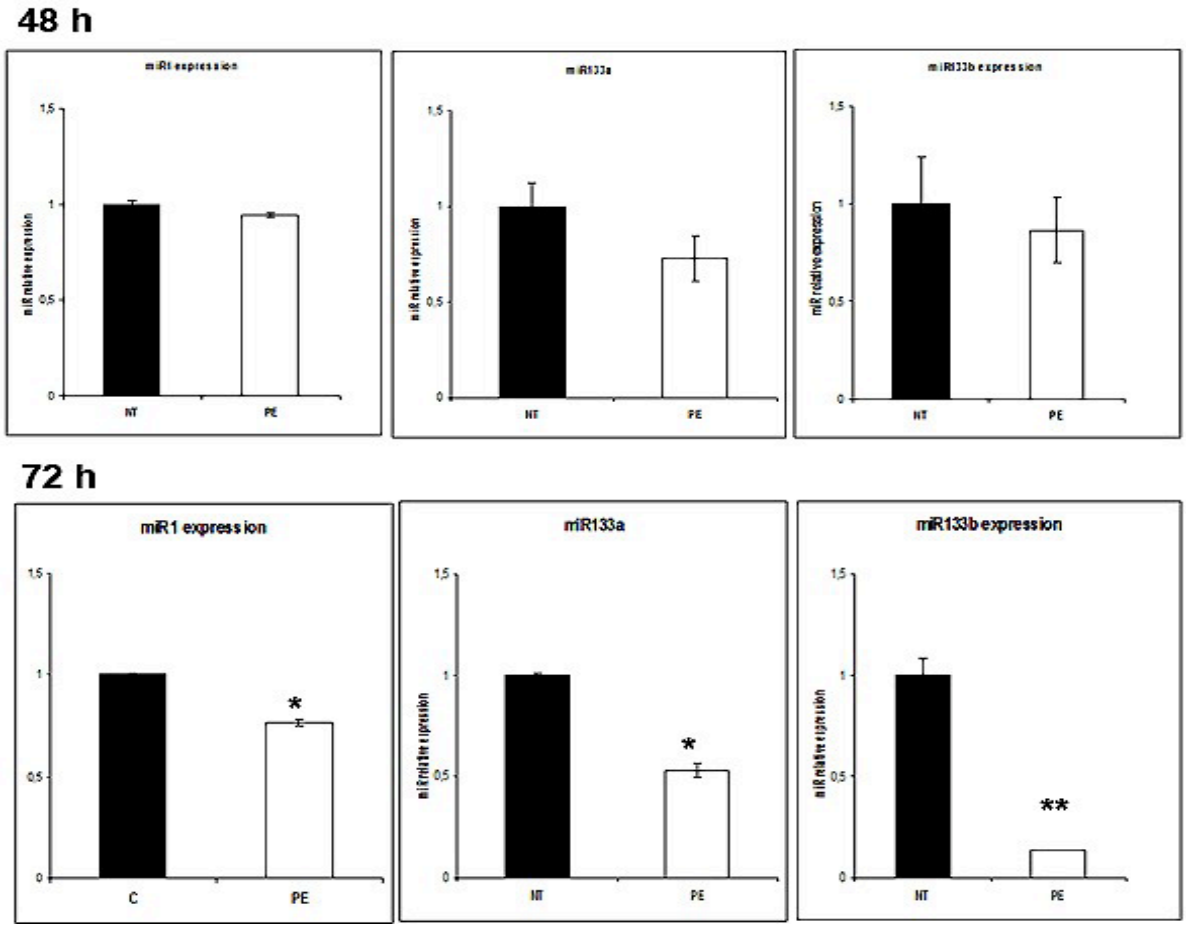


Graphic 1. Relative RNA expression of cardiac miRNAs (miR-1, miR-133a, miR-133b and miR-208) in adult zebrafish (ZF tot), in zebrafish heart (zfH), in zebrafish without the heart (ZF w/o H) and in human heart (hH), used as positive control, in qRT-PCR experiments.

i) PE treatments

Different concentration of PE (100, 200 and 500 μM) was added in *ex-vivo* cultured zebrafish hearts and left for 48 h to assess if the miRs (miR-1, miR-133a and miR-133b) were expressed differently as compared with controls (not treated) hearts. The qRT-PCR results evidenced that only the concentration of 500μM was able to provoked a different expression (not shown). The treatment with 500 μM-phenylephrine for 48h (that is, when the increase in cell size start to be evident, see the previous section) displayed by qRT-PCR analysis a sub-expression trend of miR-1 (0,953 ± 0,001), miR-133a (0,729 ± 0,140) and miR-133b (0,864 ± 0,420) but the difference from controls was not statistically significantly (**Graphic 2**). After 72h the PE treatment provoked a marked decrease of miRs. The major decrease was observed

for miR-133b where the expression was detected about the 75% less (0,235 ± 0,001) as respect to controls (P< 0,001) and 75% and 50% less the miR-1 and miR-133a (P<0,001, miR-133b *versus* miR-133a and *versus* miR-1).



Graphic 2. Relative expression of cardiac miRNAs (miR-1, miR-133a and miR-133b,) detected by qRT-PCR experiments in adult zebrafish heart non treated (C or NT, black columns) or treated with phenil-ephrine 500 μM (PE, white columns) for 48 and 72 h. The analyses done in the 72 h PE treatment revealed a significative decrease as compare with the controls/non treated hearts (versus control, * P<0,05; **P<0,001, two-tail student T test). U6 snRNA was used as normalizer sample.

ii) Surgery and regeneration experiments

The histological and qRT-PCR analysis of the heart in regeneration were done considering different time-points (1, 2, 3, 7 and 30 days) after the surgical removal of ventricular apex. After 1 day, the histological examination of the specimens evidenced a large connective-clot next to the operated area (**Figure 4 a**). After 2-3 days the clot started to be infiltrated by myocytes (**Figure 4 b,c**), but, at 7 days the operated hearts already shown a marked recovery with a reduction of the clot. Finally, at 30 days post-operation, the heart is completely recovered and comparable with control (**Figure 4 e**).

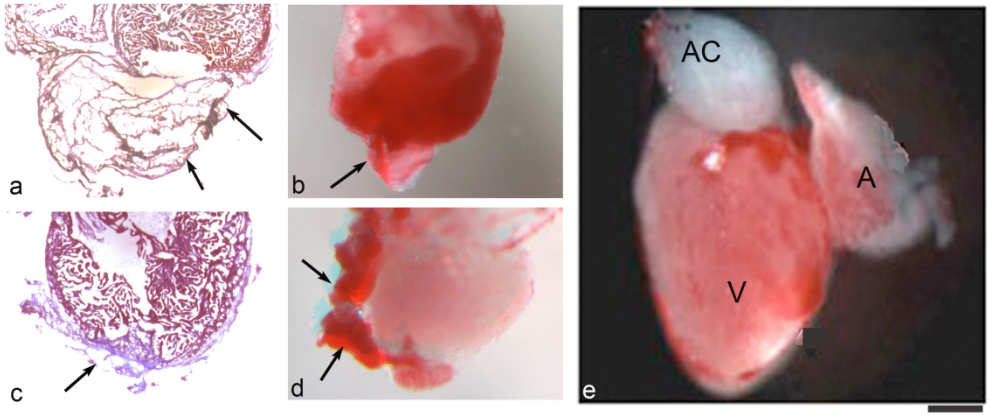
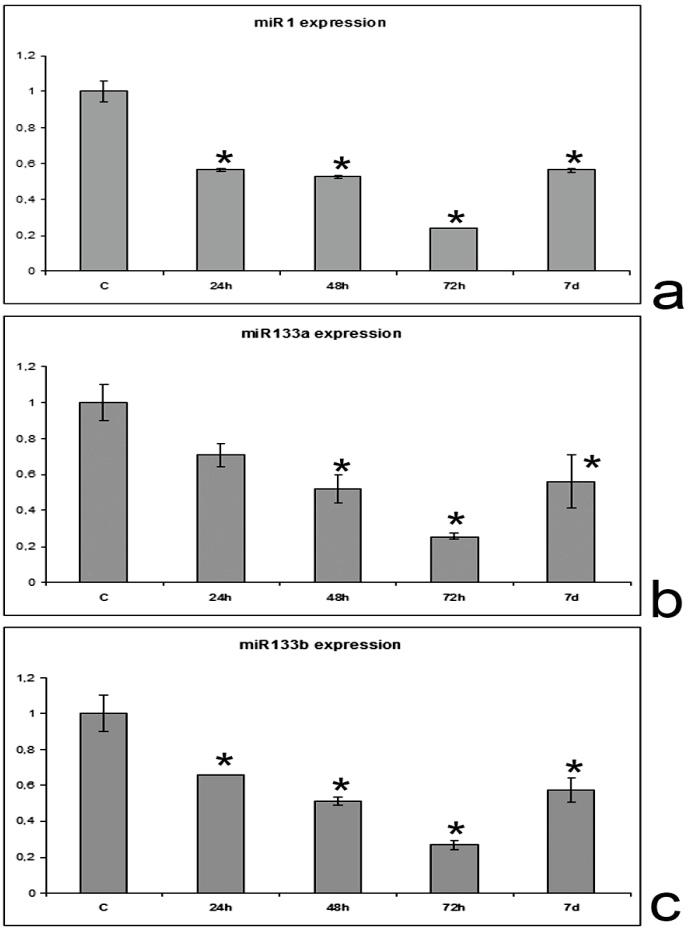


Figure 4. Adult heart of zebrafish in regeneration (Masson' trichrome staining) subsequently the ventricular apex amputation. **(a)** After 24h, the histological section displays a large clot of connective tissue (arrows). **(b)** After 2 days the clot is still large and evidences some infiltrated myocytes (red portion of whole heart, indicated by arrow). **(c)** after 3 days the clot is reduces and new tissue is produced (arrow). **(d)** After 7 days, numerous new-differentiated myocytes are in the operated area (red parts indicated by arrows in the whole-stained heart). **(e)** After 30 days the heart is similar to non operated controls (whole heart). V: ventricle; AC *Conus Arteriosus;* A: atrial chamber. Bar: 100 µm.

In qRT-PCR analysis it was assumed that the non-treated heart (control) has N=1 a expression of miR-1, miR-133a and miR-133b. Generally, the analysis of relative expression of miRs in regenerating hearts evidenced a marked decrease as compare with the controls (**Graphic 3**). Particularly, miR-1 (**Graphic 3 a**) was at least 50% less express in the first 2 days (0,566 ± 0,008 and 0,526 ± 0,004, respectively). Interestingly, at 72h (3 days) the quantitative expression of miR-1 was the 75 % less than in control (0,240 ± 0,003) whereas at

7 days the expression return to be the 50% less than the control (0,564 ± 0,010). At 30 days the miR-1 expression is comparable with the control (not shown). As regard the miR-133a (**Graphic 3 b**) in the first 24 h the expression decreased about the 30% (0,707 ± 0,065), whereas in the day 2, 3, and 7 the trend was similar to that in miR-1 (0,519 ± 0,079; 0,255 ± 0,016; 0,560 ± 0,145, respectively). The miR-133b decreased about the 42% in the first day post-operation (0,657 ± 0,00). At 2 days the expression was the 50% less and at 3 days was around the 75 % less as compare with control (0,513 ± 0,0246 and 0266 ± 0,026) (**Graphic 3 c**). At 7 days the expression of miR-133b was around the 50 % less as respect to control (0,574 ± 0,068) and at 30 days was almost comparable (not shown).



Graphic 3. Relative expression of miRs, by qRT-PCR analysis, in regenerating adult heart after the ventricular apex amputation (at 24h-1 day, 48h-2 days, 72h-3 days and 7 days). **(a)** expression of miR-1. **(b)** expression of miR-133a. **(c)** expression of miR-133b. (*) significantly different from control (P<0,001). One-tail Student T test.

DISCUSSION

Different vertebrate species have different cardiac regeneration process and in last analysis similar but not identical mechanisms of activation of genes responsible to the proliferation and differentiation of cardiomyocytes (Srivastava and Ivey, 2006; Ausoni and Sartore, 2009). Understanding how complex physiological processes are coordinately controlled at the molecular level *in-vivo* is one of the corner stones of research in modern translational medicine. MicroRNAs are involved in negative regulation of post-transcriptional gene expression (Carrington and Ambros, 2003; Lim et al., 2005; van Rooij and Olson, 2007) and are involved in the regulation of mammal heart functions, in development and disease (Lagos-Quintana et al., 2002; Chien, 2007). Thus, they are differently expressed in hypertrophic conditions and during heart regeneration (Chien, 2007). Comparing the expression of some miRNAs between medaka, chicken, mouse and zebrafish, it was ascertain that the timing and location of miRNAs expression was not strictly conserved in vertebrates and the variation of miRNAs expression being more pronounced the greater the differences in physiology (Ason et al., 2006). However, in the present research, the cardiac miRNAs sequence alignments among zebrafish, mouse and human revealed a high homology permitting the use of the same probe in the qRT-PCR analyses. The expression levels of miR-1, miR-133a, miR-133b in adult zebrafish detected in this research, revealed in the whole body, in body without hearts and in the heart of zebrafish were comparable, less the miR-1 that had minor expression levels. Moreover, the higher expression levels of miRs were observed in the whole zebrafish body, confirming that miR-1, miR-133a and miR-133b expression is specific for the muscles and not restricted to the heart (Chien, 2007). MiR-1 is among the most widely conserved miRNAs during evolution: it was found in the genomes of organisms as diverse as *C. elegans*, *Drosophyla*, zebrafish, chicken, mouse, and human (Zhao et al., 2007). An ancient genomic duplication likely resulted in two distinct loci for the miR-1/miR-133 cluster in vertebrates, with identical mature sequences derived from the duplicated loci. Targeted deletion of the muscle-specific miRNAs, miR-1-2, revealed numerous functions in the heart, including regulation of cardiac morphogenesis, electrical conduction, and cell-cycle control (Zhao et al., 2007). miR-1 and miR-133 play an important different roles in modulating muscle proliferation and differentiation (Callis et al., 2007). While miR-1 enhanced myogenesis, over-expression of miR-133 repressed myoblast differentiation and promoted myoblast proliferation (Chien, 2007; Takaya et al., 2009). Since the alignment of miR-208 sequence was failed it was concluded that this miRNA is not expressed in zebrafish,

contrary as reported in mammals heart (van Rooij et al., 2007). MiR-208, encoded by an intron of the αMHC gene and seems to be an essential cardiac-specific regulator of βMHC expression and mediator of stress and T3, is required for cardiomyocytes hypertrophy and fibrosis in the mice heart (van Rooij et al., 2007). However, lacking this miRNA in zebrafish it was concluded that the myocytes physiology and morphology must be controlled in this species by the know miRNAs or, possibly by other still unknown miRNAs. It has been already demonstrated that various forms of injury and stress evoke a hypertrophic growth response in adult cardiac myocytes, which is characterized by an increase in cell size, enhanced protein synthesis, assembly of sarcomeres, and reactivation of fetal genes, often culminating in heart failure and sudden death (van Rooij et al., 2006). Aiming to study the possible role of the miR-1, miR-133a, miR-133b in the morphology of myocardial cells, for the first time it was treated in *ex-vivo* organ-culture. Interestingly, in the present research the qRT-PCR analysis of the heart treated for 72h with 500 μM of PE revealed a significant decrease of miRNAs, whereas 48 h of treatment was not sufficient for provoked a decrease statistically significant. The major decrease was observed for miR-133b where the expression was detected about the 75% less as respect to controls, whereas the other miRNAs, miR-1 and miR-133a had a decrease as compare with controls. In mouse mode was reported that induced-hypertrophy (by aortic constriction) provoked, the expression of different miRNAs regulated temporally, among these miRs, the muscle-specific miR-1 was singularly downregulated as early as day 1 (0,56 ± 0,036), persisting through day 7 (0,29 ± 0,14) (Sayed et al., 2007). In the present study, miR-1 was down regulated already after 2 days, but only after 3 days appeared significant. Moreover, its downregulation seems to be less important as compare with the miR-133b. Sayed et al. (2007) suggested that the downregulation of miR-1 could be necessary for the relief of growth-related target genes from its repressive influence and induction of hypertrophy. Unfortunately, the authors did not test the miR-133 (not of type a nor b). However, other Authors as Carè et al., (2007), demonstrated that in mice and human the down-regulation of miR-133 had a critical role in determining cardiomyocytes hypertrophy after *in-vitro* treatment with PE of neonatal cardiomyocytes. Contrary, the over-expression induced *in-vitro* of miR-1 and miR-133 inhibited the hyperthrophy in cardiac myocytes (Carè et al., 2007; Tatsuguchi et al., 2007) and also in skeletal muscles (McCarthy and Esser, 2007). It will be interesting to determine whether forced overexpression of miR-133 and other miRNAs that are downregulated during cardiac hypertrophy suppress cardiac growth *in-vivo*. In the present research, with the aim to analyze the morphological

hyperthrophy of the PE-treated zebrafish hearts was analyzed the specimens by histology (by specific staining of muscle fibers) and by confocal microscopy (by using the phalloidin-actin-binding as a marker). Due to the auto-fluorescence of the muscle fibers (for the emission from green fluorescent protein as reported by Jackson et al, 2004) the analysis resulted anyway with the enhancement of the fluorescence of the fibers. Thus, the observations done in histology and in immunohistochemistry in PE-treated groups were coincident with hyperthrophy status of myocytes (Care' et al., 2007; Espinoza-Derout et al., 2007). It can be concluded that the miRNAs analyzed are probably involved also in zebrafish in the control of myocytes gene expressions. Further studies are required to better understand if the way of action are the same of this reported in mammals, as well as anti-miR-1 and anti-miR-133 use to verify the hyperthrophy induced by these specific miRNAs (Carè et al., 2007). While the adult mammalian heart shows little or not natural regeneration (cell division in this organ is rare) other non-mammalian species as well as fish possess elevate capacity for cardiac regeneration (Poss et al., 2002). Zebrafish, from larvae to adult, possesses an unclear capacity of restoring the ventricular muscle removed for the 20% by surgical resection, offering new possibilities for experimentally approaching this fascinating biological phenomenon (Lepilina et al., 2006). The discovery of adult cardiac stem cells and the finding that cardiomyocytes have certain capacities to proliferate challenged that concept and raised significant interest in investigating the molecular mechanisms of myocytes differentiation and hypertrophy. In the present research, the experimental approach was similar to that of Poss et al., (2003) and similarly it was observed a recovery of the heart in the same timing point. Moreover, in original approach of study, the Masson's trichrome staining evidenced the clot infiltrating myocytes, already at 48h post ventricular-amputation. For the first time it was investigated the miRNAs expression during the regeneration trial. The data exerted by qRT-PCR revealed a clear down-regulation of miR-1, miR-133a and miR-133b. The lowest expression was reached for all the miRNAs at 3 dpa (72h). Thus, the data exerted by this research seems to indicate that the activation of genes responsible of the regeneration program are linked with the down regulation of the most important miRNAs of the vertebrate heart. Similarly in mammals, the role of these miRNAs in the myocytes proliferation and differentiation seems to be of negative regulation (Callis and Wang, 2008). Over-expression of miR-1 in the developing mouse heart resulted in reduced ventricular myocytes expansion and decreased the number of proliferating myocytes (Zhao et al., 2005). In particular, miR-133 levels were reported to be high in uninjured fins but low during their regeneration (Yin et al., 2008).

Which is the possible role of the Fibroblast Growth Factors (FGFs) during the development/regeneration program and which is the link with the miRs expression in zebrafish is still unexplored field. In mammals, it was suggested that FGFs, in one-hand, are responsible of activation of developmental genes and, in other hand, are involved in the control of miRNAs expression. For instance, it was observed an increase of the miR-133 and consequently a block of fin regeneration when used a specific inhibitor of the FGF receptor (Yin et al., 2008). Conversely, miR-133 antagonism during FGF receptor inhibition accelerated regeneration through increased proliferation within the regeneration blastema (Yin et al., 2008). miR-133 over-expression prevents skeletal muscle differentiation, suggesting that differential processing from the dicistronic transcript may regulate cellular decisions of differentiation or proliferation (Chen and Lodish, 2005; Chen et al., 2006). FGF-dependent depletion of miR-133 promotes appendage regeneration in zebrafish, as like rapid changes in gene expression that achieve dramatic developmental effects (Yin et al., 2008). Among these developmental effects the apoptosis of myocytes can be also related with miRs expression. Both miR-1 and miR-133 produced opposing effects on oxidative stress-induced apoptosis in H9c2 cells, with miR-1 being pro-apoptotic and miR-133 being anti-apoptotic. (Xu et al., 2007a; Yin et al., 2008). The *Mps1* kinase, an established positive regulator of blastemal proliferation, consists of an *in-vivo* target of miR-133 (Yin et al., 2008). A large body of indication evidenced that dyregulation of miRNAs is linked to muscle-related diseases, such as cardiac hypertrophy (Latronico et al., 2008). As such, there has been intense interest in deciphering the underlying molecular mechanisms and in discovering novel therapeutic targets for suppressing adverse cardiac growth (Wang et al., 2009). In this work, it has been demonstrated that zebrafish can also be used as hypertrophic and heart regeneration model. These findings point out that miRNAs are potentially associated with muscle-related diseases in fish as well as mammals. Converting the observations reported in this paper into biological targeted cardiovascular therapy is a central tenet in translational medicine. The titration of miRs and the study of their expression and targets could be a key act to control the cardiac hypertrophy and overall, the cardiac regeneration.

CHAPTER III

Genes expression during zebrafish heart regeneration *in-vivo* and *ex-vivo*

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Manuscript on preparation

ABSTRACT

Zebrafish (*Danio rerio*) have the remarkable ability to regenerate the heart, by a process referred to as epimorphic regeneration, the regrowth of amputated structures from an anatomical complex stump. Networks of transcription factors regulate heart development, maintenance, and regeneration in a dose-dependent manner, but the effects of translational regulation on the titration of these pathways are largely unknown. Here, with In Situ Hybridization (ISH) experiments, it was tested in regenerating hearts the presence and the timing expression of genes involved in FGF regulation pathway. *In-vivo* experiments showed that the response to the injury started already at 3 hours post amputation (hpa), just around the surgered site, by the reactivation of developmental genes such as *dusp6* (*mkp3*), *erm*, *pea3*, *raldh2*, and *sef*. Between 6 and 12 hpa all the amputated hearts evidenced the expression of other genes such as *etv5* and *sprouty4*, showing the reactivation of development genes. With the aim to optimize the media to reproduce the regeneration process, it was also tested the ability of zebrafish heart to survive in *ex-vivo* cultures after the amputation of ventricular apex. Injured hearts cultured with BCI, FGF-2, Thrombin, and with a cocktail of FGFs were able to maintain expression of *raldh2* between 7 and 14 days post amputation (dpa), whereas PDGF and Basement Membrane Extract (BME) supplemented with FGFs cocktail allowed the *raldh2* expression until 30 dpa. Regenerating hearts in *ex-vivo* conditions were able to survive and make contractions, and surprisingly showed different degree of cell replication in dependence to the culture media. The highest level of duplication, detected by BrdU incorporation, was observed at 14 dpa in hearts cultured with addiction of BCI. However operated hearts *in-vivo* showed a complete regeneration after 30 dpa whereas *ex-vivo* cultured the hearts displayed only a partial regeneration. Moreover, in *ex-vivo,* a big diminution of the clot site was observed in hearts cultured with BCI, suggesting that this compound could interact with FGF pathway, stimulating and supporting the heart regeneration process.

INTRODUCTION

Although considerable progress has been made in pharmacological management, the possibly to stimulate the heart regeneration processes in mammals and overall in humans, seems to be a long-standing goal in cardiovascular medicine. The striking genetic and anatomic similarity between zebrafish and humans has also propelled the increasing characterization and analysis of zebrafish models of human diseases, and in all process correlated to a perturbation of normal developmental programs, including hematopoietic and

cardiovascular disorders (Wang et al., 1998; Ernest et al., 2000; Basset and Currie, 2003. For a review see Lieschke and Currie, 2007). The zebrafish heart evidenced a remarkable ability to regenerate large portions of myocardium after amputation of approximately 20% of the ventricular apex (Poss et al., 2002), although the mechanisms underlying this process have not been well characterized. Good candidates to drive the development and the regeneration programs could be the Fibroblast Growth Factors (FGFs). FGFs comprise of a large family of secreted polypeptides thought to signal in a dose-dependent manner through receptor tyrosine kinases (Böttcher and Niehrs, 2005). It was demonstrated their role in regulation of atrial/ventricular proportionality and the establishment of cardiomyocyte number and their differentiation (Tjwa et al., 2003). Furthemore, Roehl and Nüsslein-Volhard (2001) suggested that *pea3* (*Etv4*) and *erm* (*Etv5*), PEA3/Ets transcription factors family, as targets of FGF8 signaling, because both genes are expressed or downregulated by ectopic expression of *fgf8*. Moreover, among the studied developmental markers, the Dual Specificity Phosphatase 6 (*Dusp6*) also known as Map kinase phosphatase 3 (M*kp3*, Zebrafish Information Network), had the important function to dephosphorylate activated signaling factors as p44 and p42 ERKs (Tsang et al., 2004). *"Similar Expression to fgf genes" (Sef-il17rd-*Zebrafish Information Network) has been identified as an antagonist of FGF signaling in zebrafish and subsequently in mouse and human, but not in invertebrates (Fürthauer et al., 2002; Darby et al., 2009). *Sef* encodes a putative type I transmembrane protein that antagonizes the Ras/mitogen-activated protein kinase pathway in all three species (Rong et al., 2007). Zebrafish *Sef* (*zfSef*) antagonizes FGF activity during embryogenesis by acting as a feedback-induced antagonist of the Ras/MAPK-mediated FGF signaling. *Sef* expression is positively regulated by FGF and ectopic expression of *Sef* in zebrafish embryos specifically inhibits FGF signaling (Tsang et al., 2002). Overexpression of *sef* in zebrafish results in a ventralized phenotype (Tsang et al., 2002), similarly to what occurred after *dusp6* and *spry4* overexpression (Fürthauer et al., 2001; Tsang et al., 2004), and contrary to the occurred after *fgf8* overexpression (Fürthauer et al., 1997). Comparative analysis revealed *raldh2*, a rate-limiting enzyme for the synthesis of retinoic acid, as one of the most highly induced genes across the three regeneration platforms. *In situ* localization and functional studies indicate that *raldh2* expression is critical for the formation of wound epithelium and blastema (Mathew et al., 2009). Genes that are expressed during zebrafish heart development could be reactivated during the regeneration process (Lepilina et al., 2006). Studying the regenerative process in zebrafish at genomic and molecular level can help to understand how cardiac regeneration is

naturally optimized and why this characteristics has been lost during the evolution. Furthermore, study the FGFs role in differentiation of myocytes during the regenerative process *in-vivo*, could improve the culture conditions to try to reproduce the heart regeneration process in *ex-vivo*, and can help the formulation of new drugs.

MATHERIALS AND METHODS

Resection of 20% of ventricular apex of zebrafish heart and *ex-vivo* organ culture

The animal experiments were performed according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. The surgery was performed according to the methodology developed by Poss et al., (2002). Briefely, adult zebrafish (6-18 months old) were anesthetized for 3 minutes in 0.168 mg/l Tricaine (MS222, Sigma) and placed dorsally in a humid sterile sponge, with ventral side up. A small incision (scalpel 0.15mm, Fine Science Tools Inc. US.) was made just under the gills, in correspondence to where the heart is located. The pericardial sac was isolated and punctured to expose the ventricle by gentle abdominal pressure. Approximately 20% of the ventricle apex was removed by iridectomy scissors (Fine Science Tools Inc. US.). A kimwipe was used to blot the incision, because the wounds bleed profusely for few seconds until beginning to clot. Body wall incision was not sutured and in few days the injured part was recovered. With this protocol, 90% of fish was survived. After surgery, fish were returned to water and stimulated to breathe with air bubbles from an air pump. Animals usually begin to swim within few minutes. After 24 hours, or after 7 days, fish were lethally anesthetized with Tricaine and the whole hearts were removed and placed in Hank's solution (supplemented of penicillin/streptomycin 2:100) for the *ex-vivo* cultures. Samples were then transferred in 24-well plates with Leibovitz's L15 medium (GIBCO-BRL) supplemented by penicillin/streptomycin (1ml/100ml Gibco, 20.000 US, 100X), L-glutamin 0.3 mg/ml (Invitrogen), Insulin 20 US/ml (Sigma), $CaCl_2$ 0.8mM, Hepes 25 mM (Sigma). Plates were maintained in incubator at 28°C and medium was changed every 4 days. Hearts were maintained in culture *ex-vivo* for 48 and 72 hours post amputation (hpa) and 7, 14 and 30 days post amputation (dpa). For each time, 5 hearts were used for proteomic studies and 3 hearts were fixed over night in 4% PFA for ISH studies. and 3 hearts were treated for 7 days with BrdU (see BrdU labeling) and then used for cryosections.

Whole-mount in situ hybridization (ISH)

The *in situ* labeling was performed as previously described (Poss et al., 2000), with few modifications, using the markers: *dusp6, erm, etv5, pea3, raldh2, sef, sprouty4*. Probes were synthesized using T3, T7 or SP6 RNA polymerase (Promega). Briefly, zebrafish hearts of control, and hearts at different time points after the amputation of around 20% of the ventricle area, *in-vivo* and *ex-vivo*, were fixed in 4% paraformaldehyde overnight at 4°C, rinsed two times in 1X phosphate buffered saline (PBS) buffer and in methanol, and stored at -20°C in methanol until used. After hydratation stepwise through graduate ethanol in 1X PBS and in PBS-0.1% Tween (PBT), samples were treated with 20 μg/ml proteinase K in PBT for 30 min and hybridized at 65°C with 50% formamide. The reaction was developed with BM purple (BMannheim) and stopped with PBS and fixed with 4% PFA at room temperature. Hearts processed for whole-mount *in situ* hybridization were mounted in 100% glycerol and photographed using a Leica MZ16 microscope and Q Imaging Retiga 1300 digital camera. Experiments were performed in triplicate.

Organ culture supplemented with growth factors and chemical compounds

To optimizing the heart organ culture *ex-vivo*, to the Leibovitz's L15 medium were added different compounds as described: (1) a cocktail of FGFs, composed of 5 ng/ml of Recombinant Human Cardiotrophin-1 (Peprotech Inc.), 2 nM Thrombin (Sigma), 40 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGFb, FGF-2) (Invitrogen, Biosource), 10ng/ml Fibroblast Growth Factor-4 (Sigma) and 10 ng/ml Platelet-Derived Growth Factor-BB (Sigma); (2) 10 μM BCI, a chemical compound, dissolved in 0,5% DMSO. Medium with only DMSO was used as control; (3) 200 ng/ml Platelet-Derived Growth Factor-BB (PDGF); (4) 100 ng/ml Recombinant Human Cardiotrophin-1 (CT); (5) 100 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGF-2); (6) 10 mM Thrombin (TR); (7) 150 μl/cm^2 Cultrex Basement Membrane Extract (BME), Trevigen, according to the manufactures directions, and added with L15; (8) BME added with FGFs cocktail. Plates were maintained in incubator at 28°C and medium was changed every 4 days.

Histology

Hearts were fixed in 4% PFA overnight at 4°C, washed two times in 1% PBS and cryopreserved for several hours in a sucrose gradient (10-20-30%). Samples were included in cryogen embedding medium (Instrumedics Inc), frozen at -80°C for 30 minutes before

cryosectioning. Longitudinal sections of 10 μm were stained with Hematoxylin and eosin (H.E.) and with Masson's trichrome procedure (Sigma Diagnostic Procedure), for fibrin, collagen and nuclei.

BrdU labeling

Bromodeoxyuridine (BrdU), a chemical that is only incorporated into cells undergoing *de novo* DNA synthesis, was added to the fresh media at the concentration of 3.0 mg/ml. BrdU was added during the last 7 days of *ex-vivo* culture. The determination of BrdU was made with a primary antibody Mouse Anti-BrdU BD Bioscience (Fischer) diluited 1:100 in PBST (PBS, 0,1% Tween) and with a secondary antibody Cy3 diluited 1:500 in PBST, over night at 4°C in the dark. Fluorescence was checked with red filter in a Leica MZ16FA microscope and pictures made with a Q Imaging retiga EXI digital camera. Quantification of BrdU incorporation was performed counting BrdU positive cells in the cut site in 5 sections of three hearts for each condition and were calculate averages and standard deviations.

RESULTS

Whole mount ISH

***In-vivo* experiments**

From the analysis of the localization of considered genes, it is possible generalize the timing of events observed. 1) After the surgery of the 20% of ventricular apex, all the genes were at least detected from 1 day post amputation (dpa). 2) The localization of the probes were firstly detected in the area near the amputation site and consequently in the whole heart. 3) After some days (time dependent from the gene considered) the expression in the heart turn confined in the area where the amputation was done. Particularly, at 3 hours post amputation (hpa) expression of *dusp6, erm, pea3, raldh2*, and *sef*, were visible in the clot site of hearts maintained *in-vivo* (**Figure 1**). The maximum expression of those genes, peacked in two times: at 6 and 12 hpa. At 6 hpa were detected a slight expression of *etv5* and *sprouty4* reaching an intense expression level at 12 hpa (**Graphic 1**). Their localization seemed to be exclusively in the cut site. The regeneration process *in-vivo* was very efficient (**Figure 2)**; at 48 hpa a large clot was visible in the cut site that was completely replaced with muscular tissue by 30 dpa, and 15-20 days later the non operated and operated hearts were hystologically totally comparable (data not shown).

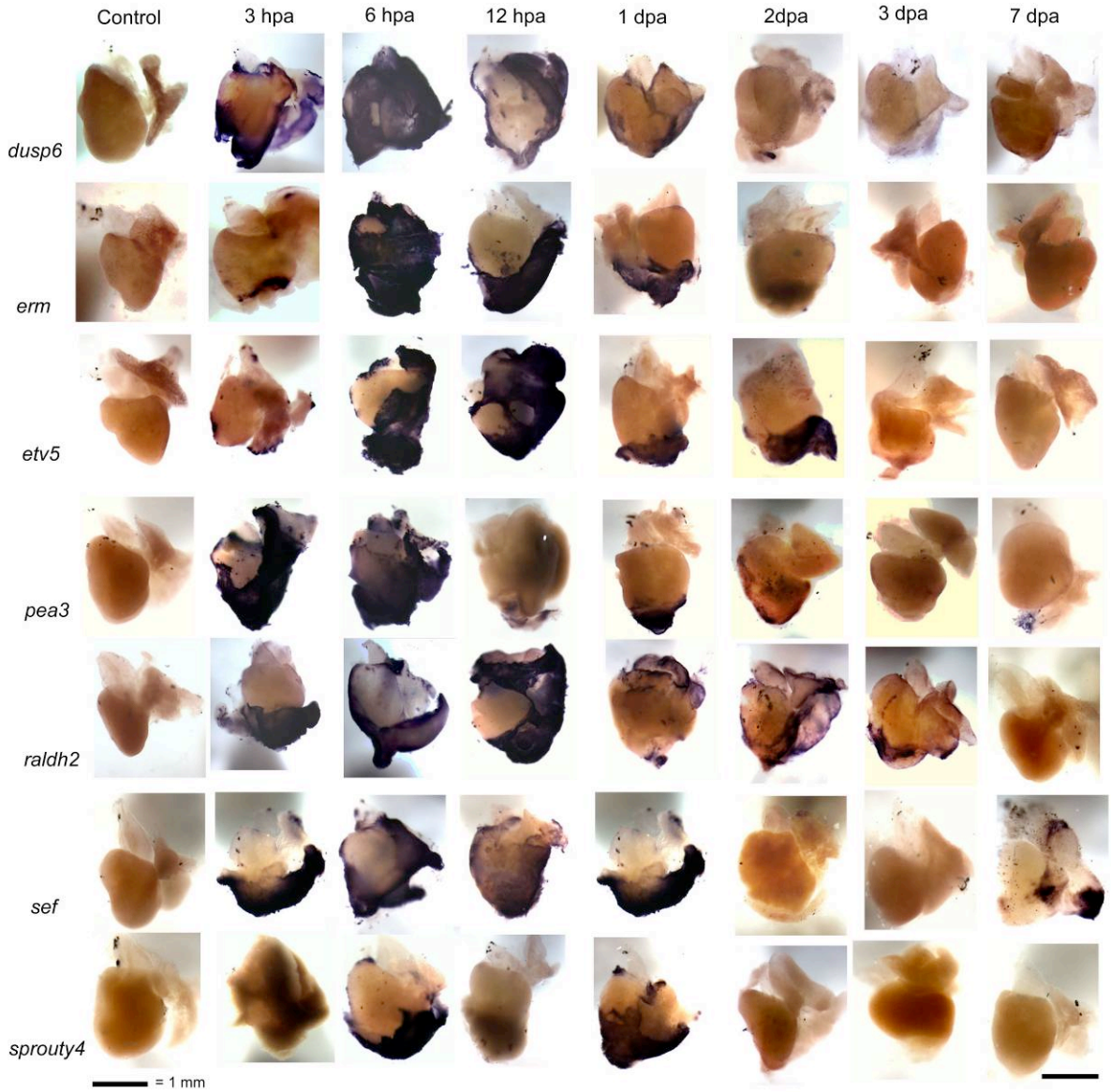
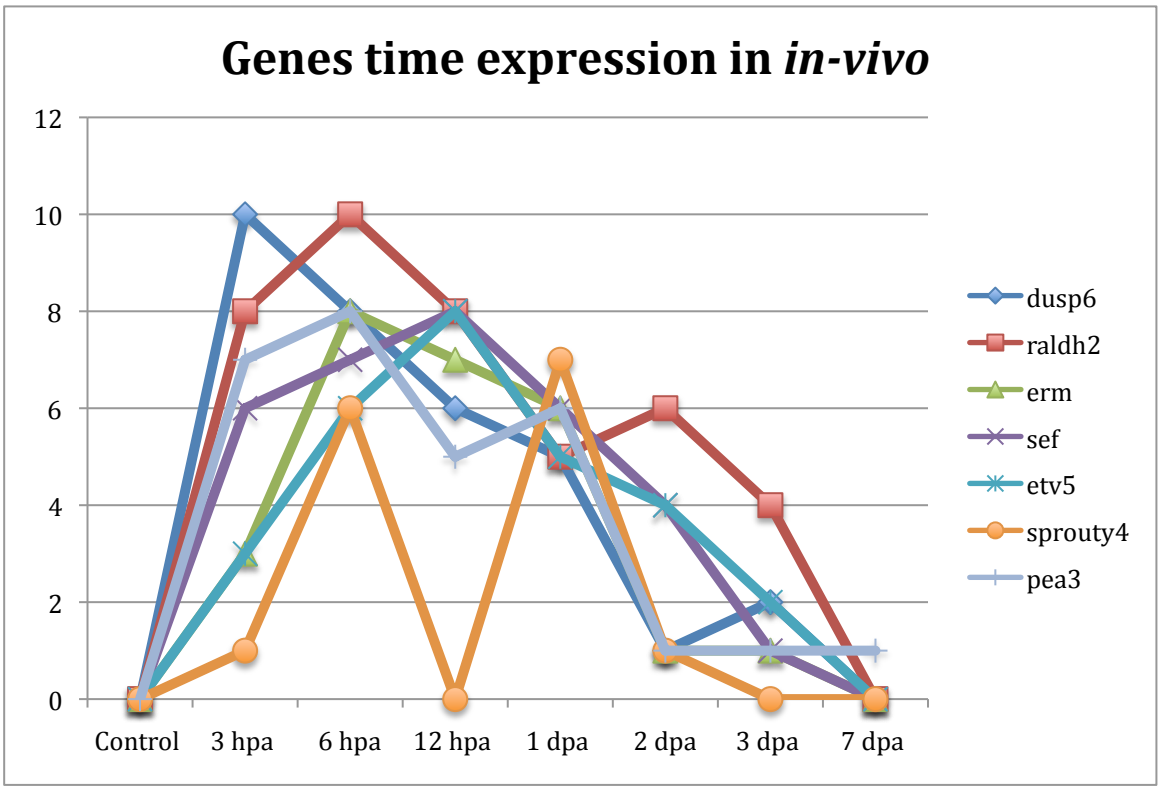


Figure 1. Localization of different genes in zebrafish hearts *in-vivo,* at different time point after the surgery, detected with ISH experiments. (hpa)= hours post amputation; (dpa)= days post amputation.



Graphic 1. Genes time expression in *in-vivo* heart regeneration detected with ISH. Value 0 corrisponds to no expression, value 10 corresponds to maximum expression levels. (hpa)= hours post amputation; (dpa)= days post amputation.

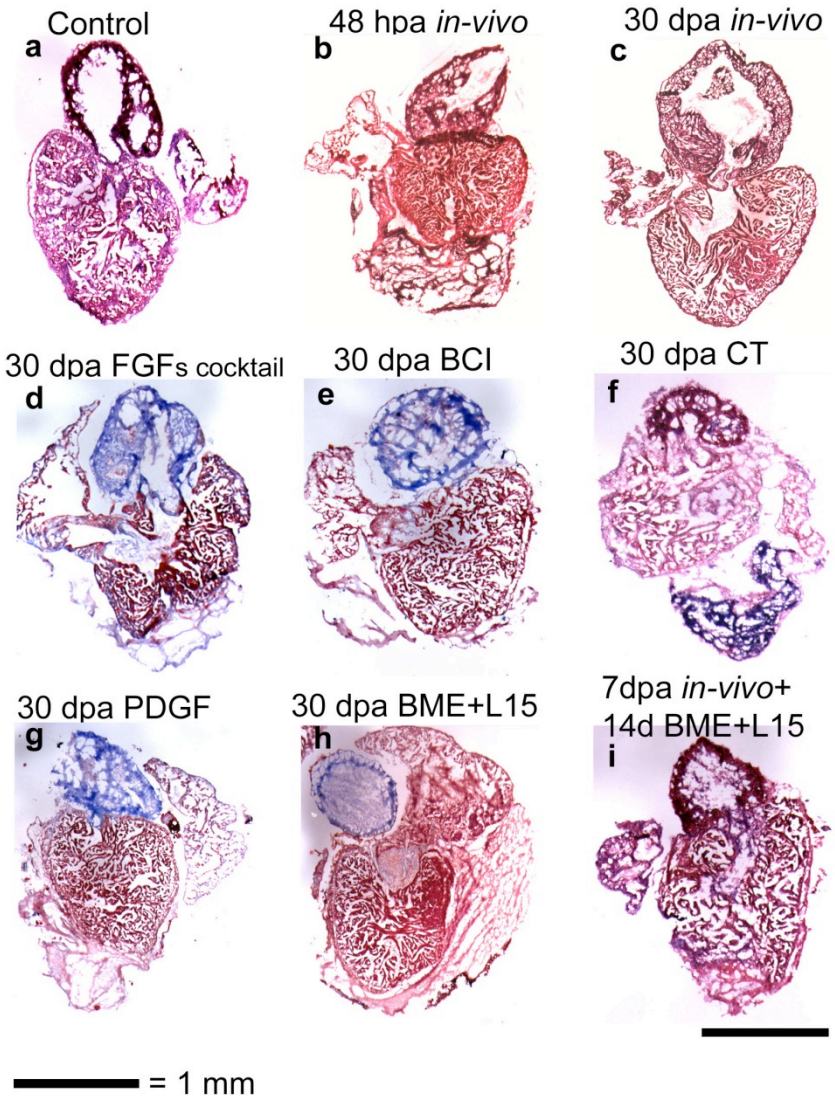


Figure 2. Masson staining of zebrafish heart (**a**) control not amputated, (**b**) 48 hpa *in-vivo,* and (**c**) 30 dpa *in-vivo*. (**d-h**) Hearts at 30 dpa *ex-vivo*, put in plate after 1 dpa *in-vivo*, in presence of (**d**) Leibovitz's L15 medium (L15) supplemented with FGFs cocktail (5 ng/ml Recombinant Human Cardiotrophin-1, 2 nM Thrombin, 40 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGFb, FGF-2), 10ng/ml Fibroblast Growth Factor-4 and 10 ng/ml Platelet-Derived Growth Factor-BB), (**e**) 10 µM BCI, (**f**) 5 ng/ml Cardiotrophin (CT), (**g**) 10 ng/ml Platelet-Derived Growth Factor-BB (PDGF) (**h**) BME+L15=150 µl/cm^2 Cultrex Basement Membrane Extract supplemented with Leibovitz's L15; (**i**) Amputated heart left regenerate 7 days in *in-vivo* conditions and 14 days in *ex-vivo* culture with BME+ L15. (hpa)= hours post amputation; (dpa)= days post amputation.

***Ex-vivo* experiments**

In *ex-vivo* cultures, the expression of *raldh2* in hearts was monitored after the treatment with different culture media (**Figure 3)**. The higher levels of expression was detected at 7 dpa for all the media, except for the hearts cultured with Thrombin and with FGFs cocktail, that reached the maximum expression level later, at 14 dpa. At 30 dpa *raldh2* was not expressed in the hearts *in-vivo* (data not shown), but it was expressed in hearts *ex-vivo* cultured with: i) BME+FGFs cocktail, ii) FGF-2, and iii) PDGF. However, at 30 days after the surgery, in the heart ex-vivo was still visible a big in the area where the amputation was made, and no complete regeneration was observed as in *in-vivo* experiments.

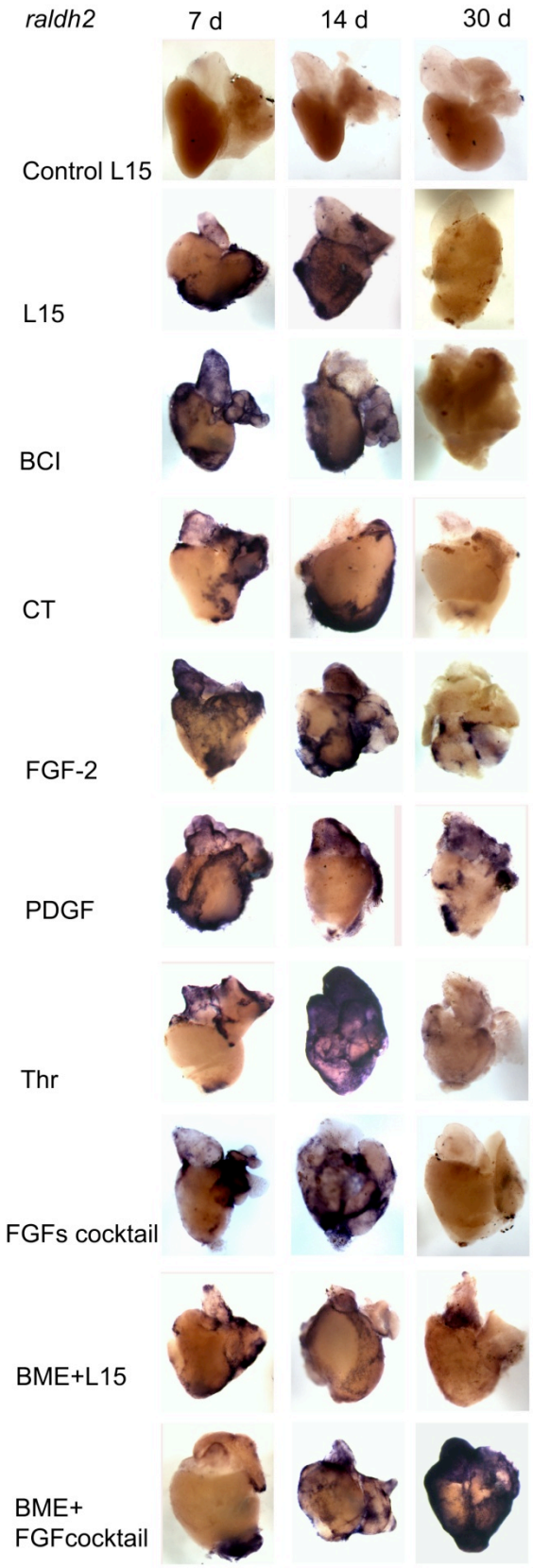

raldh2
7 d
14 d
30 d
Control L15
L15
BCI
CT
FGF-2
PDGF
Thr
FGFs cocktail
BME+L15
BME+
FGFcocktail

Figure 3. Localization of *raldh2* in zebrafish hearts *ex-vivo*, cultured with different media, at different time point after the surgery. All the hearts were maintained *in-vivo* until 1 dpa and then cultured *ex-vivo*. (d)= days in *ex-vivo* conditions. L15= Leibovitz's L15 medium; BCI=10 μM BCI, dissolved in 0,5% DMSO; CT= 100 ng/ml Recombinant Human Cardiotrophin-1; FGF2= 100 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGF-2); PDGF= 200 ng/ml Platelet-Derived Growth Factor-BB; Thr= 10 mM Thrombin; FGFs cocktail= 5 ng/ml Recombinant Human Cardiotrophin-1, 2 nM Thrombin, 40 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGFb, FGF-2), 10ng/ml Fibroblast Growth Factor-4 and 10 ng/ml Platelet-Derived Growth Factor-BB; BME=150 μl/cm^2 Cultrex Basement Membrane Extract; BME+ FGFs cocktail= BME added with FGFs cocktail.

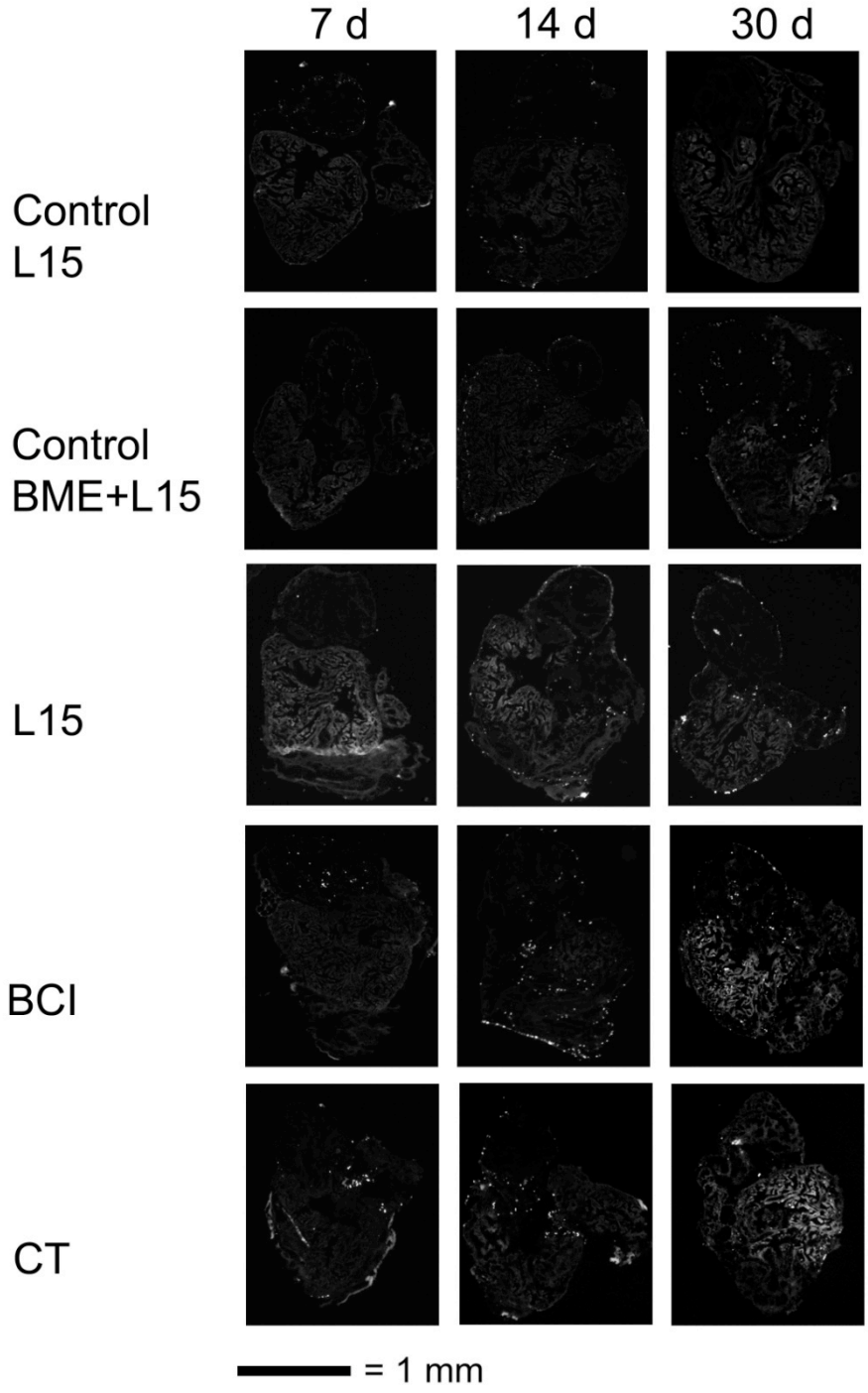
Organ culture *ex-vivo* in L15 medium and in FGF supplemented media

Zebrafish hearts (operated or not) in *ex-vivo* conditions cultured with in L15 were able to contract for several months. In culture, several cells, with apparently myocytes and fibroblast typology, were released from the hearts from 5-6 hpa. The latter type, around the fifth day of culture, helped the organ to attach at the bottom of the plate. However, the regeneration process in operated hearts were apparently delayed for several days and even not comparable with *in-vivo* condition. For example, a visible diminution of the clot size and/or a regeneration of the ventricle apex were not observed after 30 days of culture, nor in 15 days later (not shown). The same culture supplemented with different growth factors as 1) cardiotrophin, and 2) PDGF, 3) the chemical BCI, 4) BME, and 5) a cocktail of FGFs did not show the complete absorption of the clot area and the final regeneration of the amputated ventricle area. However, in presence of BME and L15, a remarkable decrease of clot size was seen in operated hearts leaved *in-vivo* for 7 days and then cultured *ex-vivo* for a total of 30 days.

BrdU labeling

Figures 4 shows the cell duplication occurred in the hearts in the presence of different media, during the last 7 days of *ex-vivo* culture. The hearts of control not submitted to surgery, cultured with L15, evidenced a few activity of cell duplication, especially at 14 days, in all the heart surface. An increase of BrdU incorporation from 14 days untill 30 days were observed adding BME. In both culture conditions, the higher activity of cell replication was visible at 14 days of culture (**Graphic 2**). Similarly, the hearts submitted to the surgery and

cultured with L15, Thrombin, Cardiotropin, BCI and FGFs cocktail, showed the maximum level of cell duplication at 14 dpa. At this time point, the higher number of $BrdU^+$ cells was detected in the hearts treated with BCI. In culture media supplemented by PDGF, FGFs cocktail and BME+L15 $BrdU^+$ cells were observed already at 7 dpa. Very low cell duplication activity was visible in hearts cultured with FGF2 and with Thrombin. At 30 dpa, few cells positive to the BrdU were observed in the hearts cultured with BCI, FGFs-cocktail, BME+L15 and BME+FGFs cocktail not only near the cut site, but in the total surface of the heart.


7 d
14 d
30 d
Control
L15
Control
BME+L15
L15
BCI
CT
= 1 mm

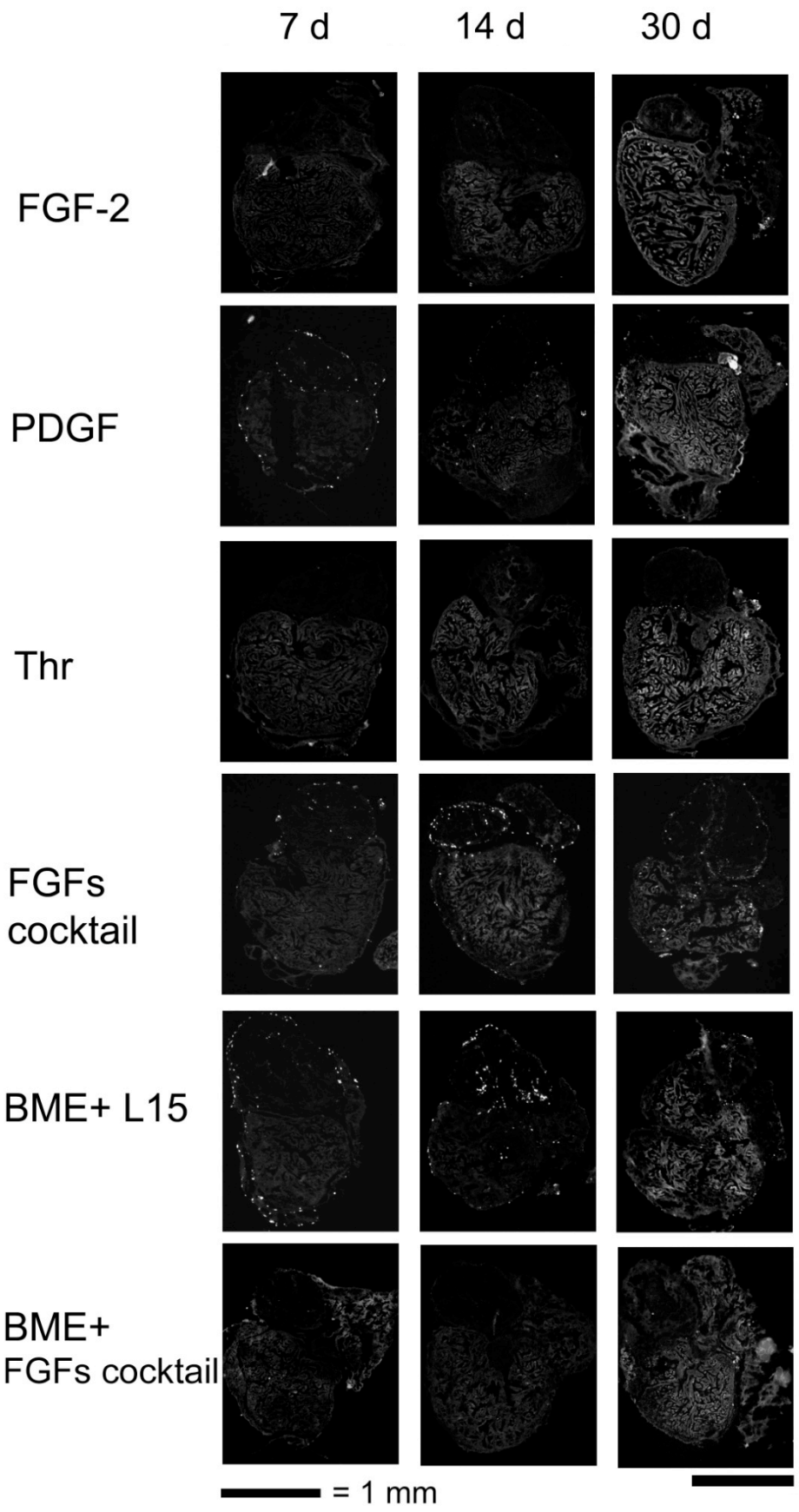
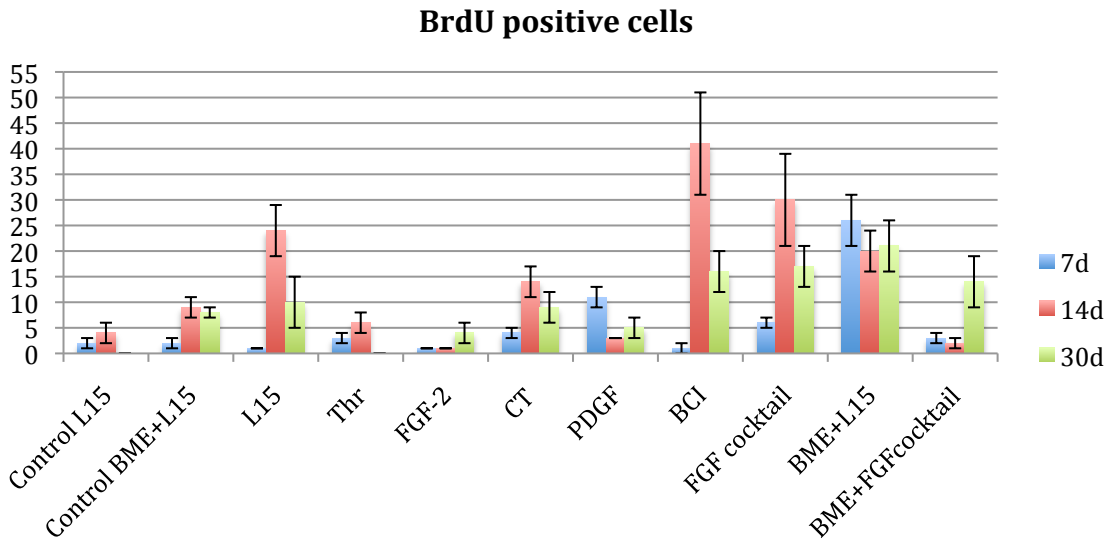

7 d
14 d
30 d
FGF-2
PDGF
Thr
FGFs cocktail
BME+ L15
BME+ FGFs cocktail
= 1 mm

Figure 4. BrdU reactions in zebrafish hearts cryosections.; (d)= days in *ex-vivo* conditions. Hearts were treated with BrdU during the last 7 days of culture. L15= Leibovitz's L15 medium; BME+L15= 150 µl/cm^2 Cultrex Basement Membrane Extract supplemented with L15; BCI= 10 µM BCI, dissolved in 0,5% DMSO; CT= 100 ng/ml Recombinant Human Cardiotrophin-1; FGF2= 100 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGF-2); PDGF= 200 ng/ml Platelet-Derived Growth Factor-BB; Thr= 10 mM Thrombin; FGFs cocktail= 5 ng/ml Recombinant Human Cardiotrophin-1, 2 nM Thrombin, 40 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGFb, FGF-2), 10ng/ml Fibroblast Growth Factor-4 and 10 ng/ml Platelet-Derived Growth Factor-BB; BME+ FGFs cocktail= BME added with FGFs cocktail.



Graphic 2. Numbers of BrdU$^+$ cells in cryosections of zebrafish hearts cultured in *ex-vivo,* with relative standar deviations. All the hearts were maintained *in-vivo* until 1 dpa, then cultured *ex-vivo,* and treated with BrdU during the last 7 days of culture (d)= days in *ex-vivo* conditions. L15= Leibovitz's L15 medium; BME+L15= 150 µl/cm^2 Cultrex Basement Membrane Extract supplemented with Leibovitz's L15 medium; Thr= 10 mM Thrombin; FGF2= 100 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGF-2); CT= 100 ng/ml Recombinant Human Cardiotrophin; PDGF= 200 ng/ml Platelet-Derived Growth Factor-BB; BCI=10 µM BCI, dissolved in 0,5% DMSO; FGFs cocktail= 5 ng/ml Recombinant Human Cardiotrophin-1, 2 nM Thrombin, 40 ng/ml Recombinant Human Fibroblast Growth Factor-basic (FGFb, FGF-2), 10ng/ml Fibroblast Growth Factor-4 and 10 ng/ml Platelet-Derived Growth Factor-BB; BME+ FGFs cocktail= 150 µl/cm^2 Cultrex Basement Membrane Extract added with FGFs cocktail.

DISCUSSION

Teleost fish have a remarkable ability to regenerate organs and tissues, capacity that is limited in mammals. For this reason, zebrafish results a useful biological model to understand the cardiac regeneration process and these knowledge could be useful for translational medicine; in fact after a surgical amputation of around 20% of the ventricle apex, there is a complete restoration of muscular tissue, in a process that involves FGFs and takes between 30 and 60 days (Poss et al., 2002; Lepilina et al., 2006). During the heart regeneration the formation of a blastema and the induction of developmental markers were also dependent by FGF factors (Lepilina et al., 2006). The clot that seals the apex matures within several days (1-4 day post-amputation -dpa), into fibrin, a complex milieu containing serum factors and degenerated erythrocytes. Functional cardiomyocytes and new contractile muscle infiltrated the injured area and sealed off the wound. Interestingly, the regenerated myocardium displayed a transiently hypertrophied compact zone, from 21-30 dpa, most likely reflecting a compensatory reaction to the hemodynamic overload subsequent to myocardial loss (Lepilina et al., 2006). Scarring and collagen deposition, characteristic of damaged mammalian hearts, did not occur. Remarkably, 60 days after surgery, the zebrafish heart appeared roughly normal both histologically and based on examination of heartbeat. In contrast, mouse hearts subjected to similar damage induced by freezing do not regenerate, but instead form scar tissue (Wills et al., 2008). In the infarcted mammalian ventricle, fibrin deposition attracts fibroblasts and inflammatory cells, and is a precursor to scarring.

Using a comparative microarray analysis in zebrafish Mathew et al., (2009) demonstrated that various tissues, including the heart, respond to amputation/injury with strikingly similar genomic response. However, regeneration of the zebrafish heart appears to differ from that described in other tissues, including the newt limb and zebrafish fin, where differentiate cells (muscle, cartilage, and skin) adjacent to the wound site, first dedifferentiate to form a blastema or mass of pluripotent cells, which then give rise to a fully formed and patterned limb or fin (Biga and Goetz, 2006). The formation of the blastema that regenerates these tissues requires successive process of dedifferentiation, transdifferentiation, and pattern formation. The zebrafish myocardium is a relatively simple structure, composed of one major cell type, so the initial step of dedifferentiation observed during limb regeneration may not be required for cardiac regeneration. Using double transgenic animals with both nuclear-localized DsRed2 fluorescent reporter for cmlc2 (cmlc2:nRFP, where RFP is red fluorescent protein) and enhanced green fluorescent protein (EGFP), that can reveal temporal and spatial

characteristics of promoter activation and inactivation, Lepilina and colleagues (2006) demonstrated that new myocardium arises from undifferentiated progenitors cells and maintained by a dynamic epicardial injury response.

In-vivo experiments

In the present research it was shown that the *in-vivo* regeneration process involved many genes that were correlated with the FGFs pathways. The group of FGF activity modulators includes transmembrane proteins that interact with the FGFR, as *Sef*, and cytoplasmatic proteins such as *dusp6* and *Sprouty*. In the first 3 hours after the surgery the activation was restricted around the clot area. Between 6 and 12 hpa there is a gene activation of all heart surface suggesting that the surgery causes a response of all the organ and enforced the idea that FGFs are implied in the regeneration process. The genes implied in the activation of the regeneration and correlated to FGFs were *dusp6, erm, pea3, raldh2*, and *sef*. These genes were normally involved in the zebrafish embryogenesis and silent in the adult (Major and Poss, 2007). It is known that *dusp6* expression is controlled by FGF ligands and receptors, however it has been controversial as to which signaling pathway downstream of the receptor is required for *dusp6* gene transcription. Functional studies in zebrafish revealed that FGF signaling is necessary and sufficient for *dusp6* expression (Tsang et al., 2004). *Dusp6* and *sef* are two genes that exhibit almost identical expression to *fgf8* and *fgf3* during development the Ets transcription factors *erm* and *pea3*, and the FGF inhibitors *sef* and *spry.* (Li et al., 2007a; Tsang et al., 2004). Also, it was determined that *dusp6* is a negative feedback regulator of FGF signaling in the zebrafish embryos as it is in mammalian cells, and that *dusp6* expression is initiated by maternal β-catenin activity prior to any detectable FGF/RAS/MAPK signaling. It is known that *dusp6* expression is controlled by FGF ligands and receptors. *Pea3* subfamily members are FGF-dependent. In zebrafish, the only *Pea3* identified members, *pea3* and *erm* are regulated by *fgf8* and *fgf3* (Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). Overexpression of *sef* in zebrafish results in a ventralized phenotype (Tsang et al., 2002), similarly to what occurred after *mkp3* and *spry4* overexpression (Fürthauer et al., 2001; Tsang et al., 2004), and contrary to the occurred after *fgf8* overexpression (Fürthauer et al., 1997). In this study was evidenced that at 1 dpa, the expression of the genes studied, was localized not only in the cut site as was observed during the first hours after the surgery: this observation, suggests that the genetic response to the injury is very rapid and probably the clot site stimulates all the epicardium to react. Thus, the area near the cut site starts the activation of

the whole heart and then is the last part to switch off. Moreover, the injury site is also responsible of silencing the re-activated genes when the regeneration process is concluded. In fact this part was here demonstrated to be the last to actuate the switch off of regeneration process. At 2 dpa is visible only the expression of *etv5* and *sprouty4* (*Spry4*), evidencing that these genes were activated later respect to the other genes and that they remain on for longer time. Similar to *Sef*, *Sprouty4* encodes an FGF signaling antagonist (Fürthauer et al., 2002). Also, *Sprouty* proteins are activated both transcriptionally and postranslationally through the Ras-ERK1/2 pathway upon FGFR activation. Raya and colleagues (2003) demonstrated that adult zebrafish have a remarkable capacity to regenerate the heart in a process that involves up-regulation of *msxB* and *msxC* genes. Heart regeneration in zebrafish is accompanied by up-regulation of components of the Notch pathway, followed by members of the *Msx* family. These genes are not expressed during zebrafish heart development, indicating that regeneration involves the execution of a specific genetic program, rather than redeployment of a developmental program. Also, the same Authors demonstrated that components of the Notch pathway are also up-regulated during zebrafish fin regeneration, suggesting that this pathway may play a general role in the activation of the regenerative process.

***Ex-vivo* experiments**

In *ex-vivo* culture it has seen that zebrafish hearts cultured with L15 are able to contract for several months, even if the regeneration process apparently delayed and was not as efficient as *in-vivo*. Several reasons can cause the failure of regeneration in *ex-vivo*. Firstly, the lack of stem cells capable to reactivate the silent development/regeneration genes. In fact, the operated hearts culture *ex-vivo*, evidenced just a few hours after, numerous cells leaving the organ. These cells resembled similar to those observed in mammals models (Messina et al., 2004). It was demonstrated in mouse and in human that these cells had adult stem cells identity and were capable, at occurrence, to differentiate in cardiomyocytes (Barile et al., 2007). Sometimes, after 5 days around the hearts were also observed fibroblast that allowed the organ attachment to the plate. These elements were also observed in mammals models, confirming the similarity of regeneration process in vertebrates (Luo et al., 2009). Secondly, the gene re-activation seemed to be more complicated and not exclusively driven by FGFs. In fact with the aim to understand the role of FGFs in the regeneration process and to improve this process in *ex-vivo*, the medium was also supplemented with different growth factors and with a cocktail of FGFs. However, similarly with the first experiment with L15 medium, the

treatment of *ex-vivo* operated hearts using FGFs cocktail revealed not efficient as well as i*n-vivo* process: at 30 dpa, the clot was still present in all the hearts cultured with different media. These findings suggest that the presence of FGFs were not sufficient *in se* to activate the efficient regeneration process. Since FGFs display pleiotropic actions and are appealing candidates for signals, it is difficult to exactly determine their time of action and the combination of these factors with others (Watanabe et al., 2001). The question if the process is simply delayed in culture or if is blocked by the absence of necessary key factors could take an answer by the analysis of gene expression in operated hearts cultured. In *ex-vivo* cultures the presence of *raldh2* was detected at 7 dpa, thus, several days later the *in-vivo* experiments. In mammals, *raldh2* expression is regulated by Wnt signal and FGFs/ERK signaling (Halilagic et al., 2007). It is possible hypothesize that Wnt signal is delayed or blocked in *ex-vivo* conditions, whereas is active in *in-vivo* experiments. Interestingly, a significant diminution of the clot size, and a quite complete regeneration, occurred in the hearts removed from the fish at 7 dpa- instead of at 1 dpa- and then cultured with L15 supplemented with BME. These results suggests that the first days after the surgery were critical for the regeneration process and that until 7 days several growth factors were involved and not exclusively FGFs. Thirdly, the factors expressed during the first days after the amputation in *in-vivo* regeneration were necessary not only for the differentiation but also for improve the myocytes proliferation. In fact, by incorporation of BrdU, it was demonstrated in this study that the operated hearts were able to duplicate the myocytes in *ex-vivo*. However, the amount of cells was not sufficient to allow the regeneration. Only the culture medium composed by L15 supplemented with PDGF, FGFs cocktail and BME were able to efficiently stimulate the cell proliferation, confirmed by the large incorporation of BrdU in cells that starting from 7 dpa. It has been demonstrated by Lien et al., (2006), that PDGF was up-regulated in regenerating zebrafish hearts and that a chemical inhibitor of PDGF receptor decreased DNA synthesis in adult zebrafish cardiomyocytes both *in-vitro* and *in-vivo* during regeneration. The BME could improve the cell replication because it is a gelatinous membrane that can embody the heart and restrict the release of the cells important for the regeneration in the culture media. Interesting, the $BrdU^+$ cells were localized not only near the cut site, but in the total surface of the heart, enforcing the idea that the activation from the injured site is capable to stimulate the whole organ. An increase of $BrdU^+$ cells was seen between 14 and 30 dpa, adding the BME in the culture media, showing that this particular media is improving the culture conditions. In this study, it was studied the activation of some genes related to FGFs

induction during the *in-vivo* and *ex-vivo* heart regeneration process. Moreover, it was developed an *ex-vivo* system to reproduce the cardiac regeneration, to understand the role of different growth factors and chemicals involved in this process. *Raldh2* that is expressed during the *in-vivo* heart regeneration is also expressed in *ex-vivo* process too, even if in retard. These results will help to understand the distribution and cell type-specific expression of FGF during the heart regeneration process and can give important insight into the action of FGF signaling on adult zebrafish, and in last analysis vertebrate heart. In fact various growth factors, and small molecules could be used to induce mammalian cardiomyocyte proliferation in the hope that these molecules can be utilized for cardiac regeneration.

CHAPTER IV

Proteomic of the adult zebrafish heart

Maria Azzurra Missinato[1,2], *James Carroll*[2], *Nicla Romano*[1], and *Michael Tsang*[2]

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Manuscript on preparation

ABSTRACT

The use of zebrafish (*Danio rerio*) as a model to study human hematopoietic and cardiovascular disease could contributes valuable leads toward the current understanding human pathophysiology in these organ systems. Aimed to know the protein pattern expressed in adult zebrafish heart, a proteomic approach has been employed in this study. One hundred and fifteen proteins were identified using MALDI/TOF-MS analysis. Identified proteins belong to different families, including metabolic enzymes (glyceraldehydes-3-phosphate dehydrogenase, mitochondrial malate dehydrogenase, ATP synthase), proteins of signal transduction pathways (transcription factor Lmx1b, Mcm10 protein), growth factors (vascular endothelial growth factor, insulin-like growth factor), cytoskeletal components, globin family proteins (myoglobin, ba1 globin), and structural proteins (four and a half LIM domains, actin, desmin). For the first time, an overall study of proteins that are naturally expressed in the adult zebrafish heart has been done. This study can give a basic contribute in understanding the biological knowledge of the vertebrate heart.

INTRODUCTION

During last decade zebrafish has become a widely used and powerful vertebrate model system for studies of the molecular mechanisms of ontogeny (Quian et al., 2005), pathology (Eisen, 1996), and pharmacology (Drummond et al., 1998). The adult zebrafish heart is composed of two main chambers, the *atrium* and the ventricle, and of two additional chambers, constituted by *sinus venous* and *bulbus arteriosus*, all chambers derived during the ontogeny by a transformed arterial vase (Thisse and Zon, 2002). In mammals, the heart is composed by four main chambers (2 atrial chambers and 2 ventricles), and the additional *bulbus arteriosus*, one for each ventricle. Generally, vertebrate heart possesses autoregulatory mechanisms that enable it to adapt its force of contraction to continually changing demands (Bending et al., 2006). The molecular components of the cardiac mechanical stretch sensor are mostly unknown but of immense medical importance, since dysfunction of this sensing machinery is suspected to be responsible for a significant proportion of human heart failure (Kim et al., 2002). Although the zebrafish genome is only half the size of the human genome, the genetic structure is remarkably similar. Also, the utility of the genome sequence increases with the quality of annotation of protein-encoding genes and the creation of transgenic zebrafish has been used to monitor protein expression patterns during development (Udvadia

and Linney, 2003). It has been proposed that proteomics should complement the genome-wide expression profiling (Love et al., 2004). In fact, with proteomics, that allows the separation of thousand of proteins in a single analysis, it is possible confirm and revise existing genome annotations, and also discover completely new genes (Findlay et al., 2009). Since there are just few proteomic studies in zebrafish, and not one focused on the heart, here, for the first time, it is reported a proteomic analysis of the adult zebrafish heart. The peptide fragments obtained from the digestion of proteins separated by two-dimensional gel electrophoresis (2DE), were analyzed using mass spectrometry (MS); the peak lists generated from the MS data were analyzed using software and used to search existing *Danio rerio* protein databases to identify the protein. Understanding the type of proteins that are naturally expressed in the adult zebrafish heart is not only important as basic biological knowledge, but also, because it constitute a reference point to compare proteins expression in various cardiac diseases.

MATHERIALS AND METHODS

Proteomics of the zebrafish heart

i) Samples preparation

2DE gel system was used to detect the protein expression of adult zebrafish hearts (n=8) followed by MALDI-TOF/MS protein identification. Protein identification was carried out using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and the Mascot and ProFound search engines. The zebrafish hearts were removed and homogenized (Teflon pestle) in 20 µl of labeling buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate), pH 8.5 adjusted with 1N HCl, clarified by ultracentrifugation (100,000 x g, 23^{o}C, 1.0 hr) and stored at −80 °C.

ii) 2-D Electrophoresis

Sample was subjected to isoelectric focusing (IEF) using an Ettan IPGphor (GE Healthcare/Amersham Biosciences) and 13 cm linear, pH 3–10 Immobiline DryStrips (GE Healthcare/ Amersham Biosciences). The IPGphor system was programmed to perform IEF with the following settings: (i) 12 hour rehydration, (ii) 500 V, 1.0 h, “Step-n-hold”; (iii) 1000 V, 1.0 h, “Step-n-hold”; (iv) 1500 V, 1 h, “Step-n-hold”; (v) 4000 V, 1 h, “Step-n-hold”; and (vi) 8000 V, 60,000 Vh, “Step-nhold”. The IPG strip was subjected to two 15-min

equilibration steps with 1% DTT in SDS buffer (3 M urea, 50mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS and trace bromophenol blue). The 2DE was carried out on a 8-16% SDS-polyacrylamide gradient gel with a constant current of 30 mA until the tracking dye was 1 cm above the bottom of the gel. Silver staining of the 2D gel was performed using Silver Stain Plus Kit (Bio-Rad) according to the manufacturer's instructions. Gel was also stained with Comassie Blue (Bio-Rad) Gel images were created by acquiring digitized images (400-600 dpi) using a Canon LiDE 30 flatbed scanner. Once scanned, gels were stored in 1.0% acetic acid (Fisher) at 4^{o}C until spots of interest were excised for mass spectrometry.

iii) In-gel trypsin digestion of proteins

Protein spots of interest were manually picked (1.0 mm to 3.0 mm in diameter) and rinsed in distilled water. Silver ions were removed by adding fresh destaining solution (15 mM potassium ferricyanide and 50 mM thiosufate in distilled water) (Invitrogen, Carlsbad, CA) to each spot and incubating for 20 min. Samples were then rinsed with distilled water, destained for blue comassie with 40% methanol 10% acetic acid, and equilibrated with 200 mM ammonium bicarbonate (Fisher). Samples were rinsed in 50% acetonitrile (Fisher) in 20 mM ammonium bicarbonate and dehydrated in 100% acetonitrile. Samples were digested *in-situ* with 200-300 ng trypsin (Sigma) (14 hours, 37^{o}C) and peptides were extracted twice with 50% acetonitrile, 2.5% TFA (Fisher) in distilled water and dried using a CentriVap Speed Vacuum. Extracted, dried peptides were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) (ABI) and 0.5 µl was spotted onto the target for MALDI-TOF analysis. MALDI-TOF was performed using the 4800 Proteomics Analyzer (ABI, Foster City, CA). Mass spectra were individually calibrated using internal trypsin peaks (842.51 and 1045.56 *m/z*) with Data Explorer software available from ABI. Proteins were identified using ProteinProspector (University of California, San Francisco; http://prospector.ucsf.edu/) set to a mass accuracy of +/- 50 ppm to compare unknown mass fingerprints to those of known proteins in the NCBI non-redundant database using a species-specific filter for *Danio rerio.*

RESULTS

Lysate from the adult zebrafish hearts proteins separated using a 2DE is shown in **Figure 1**. The protein separation in the first dimension was done according the Isoelectic Point (PI), the pH at which a particular protein carries no net electrical charge. The second dimension allows the separation of proteins according to molecular weights (MW). MALDI-

TOF/MS/MS data analysis using ProteinProspector analyzed 115 proteins, a number of which had a unique NCBI annotation. It was possible to identify several metabolic enzymes (glyceraldehydes-3-phosphate dehydrogenase, mitochondrial malate dehydrogenase, ATP synthase), proteins of signal transduction pathways (transcription factor Lmx1b, Mcm10 protein), growth factors (vascular endothelial growth factor, insulin-like growth factor), cytoskeletal components, globin family proteins (myoglobin, ba1 globin), and structural proteins (four and a half LIM domains, actin, desmin). Particularly, glyceraldehydes-3-phosphate dehydrogense, revealing the same molecular weight (MW 35784 Da) in numerous different spots (**Spots # 37, 38, 42, 44**), were identificates as probable isoforms. Tha ATP synthase with MW of 59744 Da was identified in three spots (**# 67, 68, 78**) in the 2D gel (**Figure 1**). As regard the enzyme isocitrate dehydrogenase, two isoforms were founded: the isoform 3 (NAD+) alpha, with MW of 39984 Da (**Spots # 56, 57**), and the isoform 2 (NAD+) mitochondrial, with MW corresponding to 50397 Da (**Spots # 62, 63, 64, 65**). One isoform of the enzyme mitochondrial aconitase (MW 84860), was founded in two consecutive spots (**Spots # 104, 105**). Regarding the fructose bisphosphatase aldolase, were identified the isoform a (**Spots # 41, 43)**, with MW of 39741 Da and the isoform c (**Spot # 50**), corresponding to 39260 Da.

In the 2D gel of the zebrafish heart it was possible also identified proteins with structural function. More in particular, two isoform of actin were founded; the alpha 2, characteristic of smooth muscle of aorta (**Spots # 59, 60, 75**), with MW of 41995 Da, and the alpha 1b, characteristic of skeletal and cardiac muscle (**Spots # 61, 73, 77)**, with MW of 41887 Da. Also, several forms of myosin were founded: the light polypeptide 7, with regulatory function, (**Spot # 13** - MW 19021), the myosin binding protein C, cardiac specific (**Spot # 114** - MW 142312), and the ventriculat myosin heavy chain-like (**Spots # 113, 115** - MW 223315).

In this study were also identified several proteins of globin family, as myoglobin (**Spots # 3, 4** – MW 15583), cytoglobin 2 (**Spot # 11** – MW 20585), and microglobulin (**Spot # 15** - MW 23835). As regard the Ba globin, two isoforms with similar MW were founded: the ba1 globin (**Spots # 5, 6**), corresponding to 16389 Da, and the Ba1 globin (**Spot # 7**), corresponding to 16403 Da.

A complete list of all the proteins identified in this study, with the corrispondent values of MW (Da), the Isoelectic Point (PI), score, and accession number, is reported in **Table 1**.

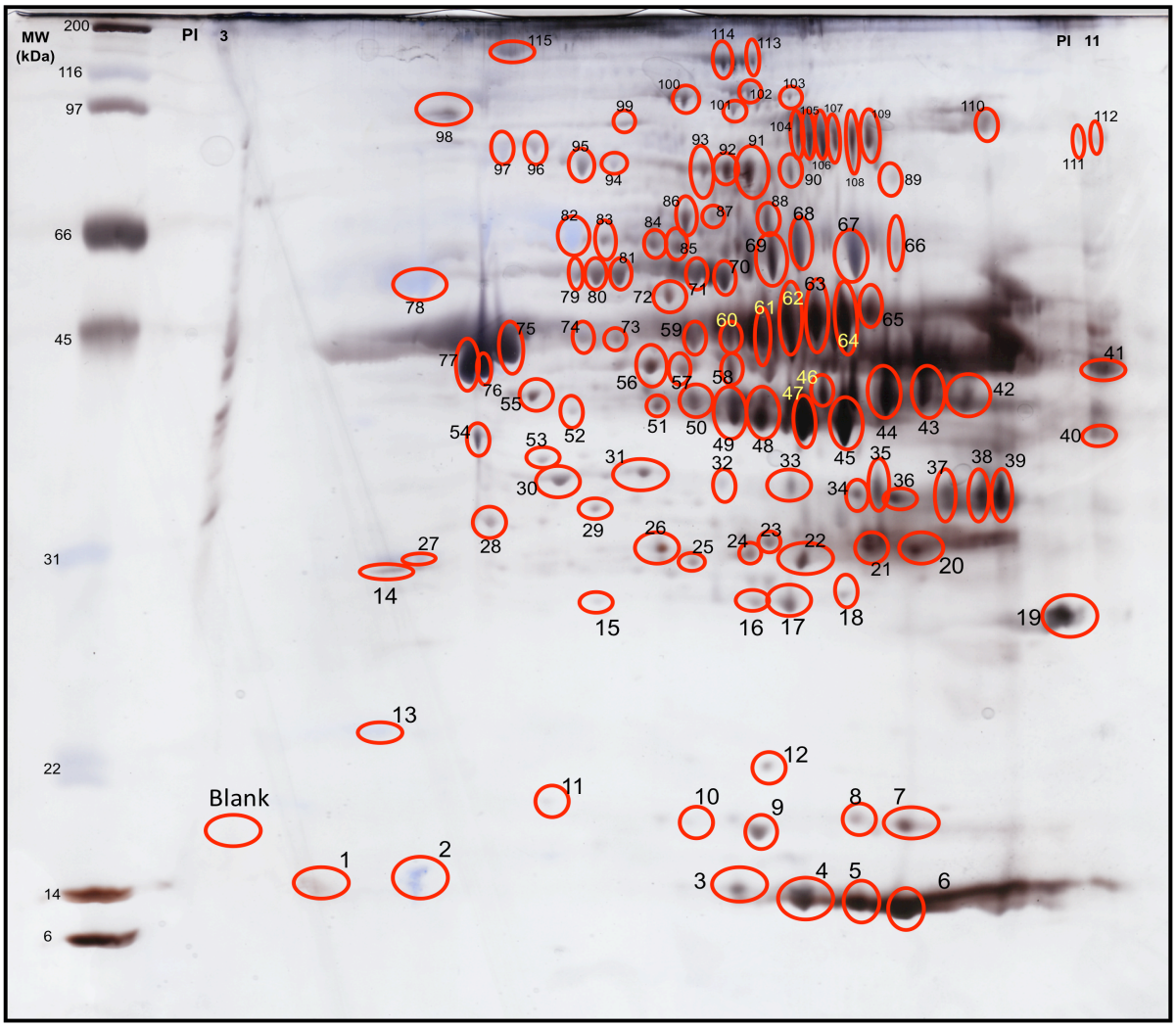


Figure 1. Two-dimensional electrophoresis gels (2DE) of proteins expressed in 8 zebrafish hearts, and spots of 115 proteins used for identification in MALDI/TOF-MS.

Spot #	Protein name	MW (Da)	pI	% Cov	Accession #
1	Rbp2b protein	15659	5.8	38.5	66267453 M
2	transcription factor Lmx1b	13543	4.6	50.4	61287134
3	myoglobin	15583	7	46.3	41053652 M
4	myoglobin	15583	7	46.3	41053652 M
5	myoglobin	15525	7.9	63.3	37695556

6	ba1 globin	16389	7.7	87.8	18858329 M
7	Ba1 globin	16403	7.7	45.9	92096378
8	PREDICTED: similar to Myosin-Id	20875	9.5	31.1	189526020
9	PREDICTED: similar to Myosin-Id	20875	9.5	24	189526020
10	nucleoside diphosphate kinase B	17124	6.8	51.6	41053595 M
11	cytoglobin 2	20585	5	23.5	145279651 M
12	PREDICTED: similar to phosphoinositide-3-kinase, regulatory subunit 6	26669	6.9	27.2	189520820
13	myosin, light polypeptide 7, regulatory	19021	4.7	23.5	29725603 M
14	apolipoprotein A-1	30257	5.1	60.7	18858281 M
15	alpha-1-microglobulin/bikunin precursor, like precursor	23835	5.2	50	41055365 M
16	kinase 1 binding protein 1	28063	7.7	25.3	66472792 M
17	Prdx3 protein	26866	8.9	31.2	60688276 M
18	PREDICTED: hypothetical protein LOC100006474	26668	8.2	28.9	189534558
19	PREDICTED: similar to Wnt4b protein	25168	9	36.4	125843849
20	insulin-like growth factor binding protein 2b	29436	8	26.4	187608371 M
21	regulator of microtubule dynamics 1	34131	8.2	24.1	157311701 M
22	triosephosphate isomerase B	26828	6.4	37.5	82245450 M
23	regulator of microtubule dynamics 1	34131	8.2	33.6	157311701 M
24	Rho-class glutathione S-transferase	26409	6.8	27.9	113682261 M
25	insulin-like growth factor binding protein 1a	28200	7.8	21.4	40254671 M

26	Four and a half LIM domains	32019	8.9	28.9	166797040
27	calbindin 2, like	31125	5	31.7	41152295 M
28	PREDICTED: hypotetical protein	31398	5.3	21.8	189515152
29	Mcm10 protein	37690	5.5	25.9	62202893
30	electrontransfer-flavoprotein, alpha polypeptide	35091	6.9	42.6	38707985 M
31	3-hydroxyisobutyrate dehydrogenase b	34618	8.3	28.3	41055658 M
32	coiled-coil domain containing 89	42157	5.6	32.8	167621496 M
33	coiled-coil domain containing 89	42157	5.6	34.2	167621496 M
34	four and half LIM domains protein 2 isoform a	32480	8.1	20.8	74013664
35	novel protein similar to vertebrate WNT1 inducible signaling pathway protein 1 (WISP1)	34937	8.7	25.6	148726365
36	PREDICTED: similar to zinc finger protein 502	41796	9.2	29.2	189534640
37	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	45.5	169403947 M
38	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	61	169403947 M
39	wingless-type MMTV integration site family, member 8a	43379	8.9	24.5	220678939
40	PREDICTED: similar to WNT1- inducible-signaling pathway protein 3 precursor (WISP-3)	41746	9.2	30.1	189532808
41	fructose-bisphosphate aldolase A	39741	8.5	40.4	41282154 M
42	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	55.3	169403947 M
43	fructose-bisphosphate aldolase A	39741	8.5	40.4	41282154 M
44	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	61	169403947 M
45	mitochondrial malate dehydrogenase	35420	8.4	56.7	47085883 M
46	mitochondrial malate dehydrogenase	35420	8.4	38.6	47085883 M

47	mitochondrial malate dehydrogenase	35420	8.4	47.2	47085883 M
48	mitochondrial malate dehydrogenase	35420	8.4	49.3	47085883 M
49	mitochondrial malate dehydrogenase	35420	8.4	35.3	47085883 M
50	aldolase c, fructose-bisphosphate	39260	6.2	27.8	35902900 M
51	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10	40337	6.7	20.8	48734808
52	PREDICTED: similar to Protein zyg-11 homolog	42480	5.8	30.9	189538821
53	PREDICTED: similar to Protein zyg-11 homolog	42480	5.8	13.8	189538821
54	pyruvate dehydrogenase (lipoamide) beta	39309	5.8	52.6	47085923 M
55	PREDICTED: similar to mKIAA0844 protein	38920	8.7	37.1	125842480
56	isocitrate dehydrogenase 3 (NAD+) alpha	39984	7	35.9	46358344 M
57	isocitrate dehydrogenase 3 (NAD+) alpha	39984	7	48.5	46358344 M
58	vascular endothelial growth factor c	45288	8.1	32.1	45387875 M
59	Actin, alpha 2, smooth muscle, aorta	41995	5.2	19.1	49901223
60	actin, alpha 2, smooth muscle, aorta	41995	5.2	35.3	49901223 M
61	actin, alpha 1b, skeletal muscle	41887	5.3	45.6	70778800 M
62	isocitrate dehydrogenase 3 (NAD+) mitochondrial	50397	8.4	33.2	41054651 M
63	isocitrate dehydrogenase 3 (NAD+) mitochondrial	50397	8.4	46.4	41054651 M
64	isocitrate dehydrogenase 3 (NAD+) mitochondrial	50397	8.4	55.7	41054651 M
65	isocitrate dehdrogenase 2 (NADP+), mitochondrial	50397	8.4	43.4	41054651 M

66	wingless-type MMTV integration site family, member 10a	50116	8.7	30.8	229577306 M
67	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	59744	9.1	59.9	116325975 M
68	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	59744	9.1	64.6	116325975 M
69	dihydrolipoamide dehydrogenase	53561	7.6	41.6	41393167 M
70	enolase 3, (beta, muscle)	47472	6.2	46.9	47551317 M
71	PREDICTED: similar to zinc finger protein 721	72333	9.3	44.6	189517662
72	PREDICTED: similar to von Willebrand factor precursor (vWF)	60614	5.1	35.4	189542644
73	actin, alpha, cardiac muscle	41887	5.3	35	70778800 M
74	Prmt1 protein	43567	5.7	28.3	27882547
75	Actin, alpha 2, smooth muscle, aorta	41995	5.2	38.2	49901223
76	PREDICTED: similart to zinc finger protein 568	44108	9.5	23	189518306
77	actin, alpha, cardiac muscle	41887	5.3	23.9	70778800 M
78	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	55001	5.2	28.6	66773080 M
79	myotubularin related protein 7a	75258	6.3	24.4	94536956 M
80	PREDICTED: similar to zinc finger protein 40	53243	9.1	32.2	189534638
81	P2rx4a protein	45045	6.5	29.4	27503429 M
82	PREDICTED: similar to zinc finger protein 160	72858	9.3	25.7	189528537
83	Desm protein	55616	5.6	25	62204269 M
84	novel protein similar to vertebrate dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2)	64141	9.5	29.3	55962523

85	LOC553337 protein	68993	6.8	35.7	61403613
86	PREDICTED: similar to zinc finger protein 160	65587	8.5	29.2	189534592
87	PREDICTED: similar to zinc finger protein 160	72858	9.3	53.3	189528537
88	PREDICTED: similar to zinc finger protein 721	72333	9.3	24.3	189517662
89	PREDICTED: similar to zinc finger protein 721	72333	9.3	28.3	189517662
90	RecName: Full=Myotubularin-related protein 8; AltName: Full=Myotubularin-related protein 6	72504	6.3	18.7	82187381 M
91	LGN	72007	6.9	25	51944894
92	PREDICTED: similar to zinc finger protein 721	72333	9.3	33.3	189517662
93	TPA_exp: transferrin	73532	6.8	36.1	47264590
94	PREDICTED: similar to zinc finger protein 208	79103	9.1	30.5	189528501
95	PREDICTED: similar to mCG142610	91424	9.3	32.5	189528482
96	PREDICTED: similar to zinc finger protein 160	72858	9.3	19.2	189528537
97	myotubularin related protein 8	72324	6.3	22	41055311 M
98	PREDICTED: similar to zinc finger protein 721	72333	9.3	29.9	189517662
99	PREDICTED: ras homolog gene family, member T1 isoform 2	75344	6.4	23.2	189520622 M
100	PREDICTED: similar to mCG142610	122393	9.1	36.4	189534703
	PREDICTED: similar to mCG142610	91424	9.3	34.7	189528482
101	PREDICTED: similar to dynamin 2	86596	6.4	25.8	189519626
102	PREDICTED: similar to pol polyprotein	128147	8.7	29	189515367
103	PREDICTED: similar to zinc finger 142	169377	8.7	25.1	189523652
104	aconitase 2, mitochondrial	84860	7.6	59.1	38707983 M
105	aconitase 2, mitochondrial	84860	7.6	58.4	38707983 M

106	similar to zinc finger protein 721	70257	9.3	25.9	189517658
107	PREDICTED: similar to mCG142610	71778	9.5	23.4	189518369
108	PREDICTED: similar to protein tyrosine phosphatase, receptor type, T	96885	7.1	22.7	189520775
109	PREDICTED: similar to mCG142610	134532	9.3	46.6	189534972
110	PREDICTED: similar to Ca2+-dependent secretion activator	1038986	5.8	15.3	189525131
111	PREDICTED: PTK2B protein tyrosine kinase 2 beta	115235	9.6	14.5	189526015
112	PREDICTED: PTK2B protein tyrosine kinase 2 beta	115235	9.6	21.2	189526015
113	ventricular myosin heavy chain-like	223315	5.5	29.5	163644263
114	myosin binding protein C, cardiac	142312	6.3	18	172072588
115	ventricular myosin heavy chain-like	223315	5.5	30.2	163644263

Table 1. Proteins identified in 8 adult zebrafish hearts, using MS, with relative MW (Da), PI, score and accession number. In yellow, the proteins identified with score <20.

Table 2 reports the proteins identified with MS, classified according to their biological function. They include metabolic enzymes, structural proteins, motor proteins, signaling proteins, receptors, transcription factors, globin family proteins and novel/predicted proteins.

Function	Spot #	Protein name	MW (Da)	pI	% Cov	Accession #
Metabolic enzyme	10	nucleoside diphosphate kinase B	17124	6.8	51.6	41053595 M
	14	apolipoprotein A-1	30257	5.1	60.7	18858281 M
	16	kinase 1 binding protein 1	28063	7.7	25.3	66472792 M
	22	triosephosphate isomerase B	26828	6.4	37.5	82245450 M
	24	Rho-class glutathione S-transferase	26409	6.8	27.9	113682261 M

	30	electrontransfer-flavoprotein, alpha polypeptide	35091	6.9	42.6	38707985 M
	31	3-hydroxyisobutyrate dehydrogenase b	34618	8.3	28.3	41055658 M
	37	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	45.5	169403947 M
	38	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	61	169403947 M
	41	fructose-bisphosphate aldolase A	39741	8.5	40.4	41282154 M
	42	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	55.3	169403947 M
	43	fructose-bisphosphate aldolase A	39741	8.5	40.4	41282154 M
	44	glyceraldehyde-3-phosphate dehydrogenase	35784	8.2	61	169403947 M
	45	mitochondrial malate dehydrogenase	35420	8.4	56.7	47085883 M
	46	mitochondrial malate dehydrogenase	35420	8.4	38.6	47085883 M
	47	mitochondrial malate dehydrogenase	35420	8.4	47.2	47085883 M
	48	mitochondrial malate dehydrogenase	35420	8.4	49.3	47085883 M
	49	mitochondrial malate dehydrogenase	35420	8.4	35.3	47085883 M
	50	aldolase c, fructose-bisphosphate	39260	6.2	27.8	35902900 M
	51	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10	40337	6.7	20.8	48734808
	54	pyruvate dehydrogenase (lipoamide) beta	39309	5.8	52.6	47085923 M
	56	isocitrate dehydrogenase 3 (NAD+) alpha	39984	7	35.9	46358344 M
	57	isocitrate dehydrogenase 3 (NAD+) alpha	39984	7	48.5	46358344 M
	62	isocitrate dehydrogenase 2 (NAD+) mitochondrial	50397	8.4	33.2	41054651 M
	63	isocitrate dehydrogenase 2 (NAD+) mitochondrial	50397	8.4	46.4	41054651 M
	64	isocitrate dehydrogenase 2 (NAD+) mitochondrial	50397	8.4	55.7	41054651 M

	65	isocitrate dehydrogenase 2 (NADP+), mitochondrial	50397	8.4	43.4	41054651 M
	67	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	59744	9.1	59.9	116325975 M
	68	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	59744	9.1	64.6	116325975 M
	69	dihydrolipoamide dehydrogenase	53561	7.6	41.6	41393167 M
	70	enolase 3, (beta, muscle)	47472	6.2	46.9	47551317 M
	74	Prmt1 protein	43567	5.7	28.3	27882547
	78	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	55001	5.2	28.6	66773080 M
	79	myotubularin related protein 7a	75258	6.3	24.4	94536956 M
	90	RecName: Full=Myotubularin-related protein 8; AltName: Full=Myotubularin-related protein 6	72504	6.3	18.7	82187381 M
	93	TPA_exp: transferrin	73532	6.8	36.1	47264590
	97	myotubularin related protein 8	72324	6.3	22	41055311 M
	104	aconitase 2, mitochondrial	84860	7.6	59.1	38707983 M
	105	aconitase 2, mitochondrial	84860	7.6	58.4	38707983 M
Structural function	26	Four and a half LIM domains	32019	8.9	28.9	166797040
	32	coiled-coil domain containing 89	42157	5.6	32.8	167621496 M
	33	coiled-coil domain containing 89	42157	5.6	34.2	167621496 M
	34	four and half LIM domains protein 2 isoform a	32480	8.1	20.8	74013664
	59	Actin, alpha 2, smooth muscle, aorta	41995	5.2	19.1	49901223 M
	60	Actin, alpha 2, smooth muscle, aorta	41995	5.2	35.3	49901223 M
	61	actin, alpha 1b, skeletal muscle	41887	5.3	45.6	70778800 M
	73	actin, alpha, cardiac muscle	41887	5.3	35	70778800 M

	75	Actin, alpha 2, smooth muscle, aorta	41995	5.2	38.2	49901223
	77	actin, alpha, cardiac muscle	41887	5.3	23.9	70778800 M
	83	Desm protein	55616	5.6	25	62204269 M
Motor proteins	13	myosin, light polypeptide 7, regulatory	19021	4.7	23.5	29725603 M
	114	myosin binding protein C, cardiac	142312	6.3	18	172072588
	113	ventricular myosin heavy chain-like	223315	5.5	29.5	163644263
	115	ventricular myosin heavy chain-like	223315	5.5	30.2	163644263
Signaling	1	Rbp2b protein	15659	5.8	38.5	66267453 M
	17	Prdx3 protein	26866	8.9	31.2	60688276 M
	20	insulin-like growth factor binding protein 2b	29436	8	26.4	187608371 M
	21	regulator of microtubule dynamics 1	34131	8.2	24.1	157311701 M
	23	regulator of microtubule dynamics 1	34131	8.2	33.6	157311701 M
	25	insulin-like growth factor binding protein 1a	28200	7.8	21.4	40254671 M
	27	calbindin 2, like	31125	5	31.7	41152295 M
	39	wingless-type MMTV integration site family, member 8a	43379	8.9	24.5	220678939
	58	vascular endothelial growth factor c	45288	8.1	32.1	45387875 M
	66	wingless-type MMTV integration site family, member 10a	50116	8.7	30.8	229577306 M
	91	LGN	72007	6.9	25	51944894
Receptors	81	P2rx4a protein	45045	6.5	29.4	27503429 M
Transcription factors	2	transcription factor Lmx1b	13543	4.6	50.4	61287134
	29	Mcm10 protein	37690	5.5	25.9	62202893

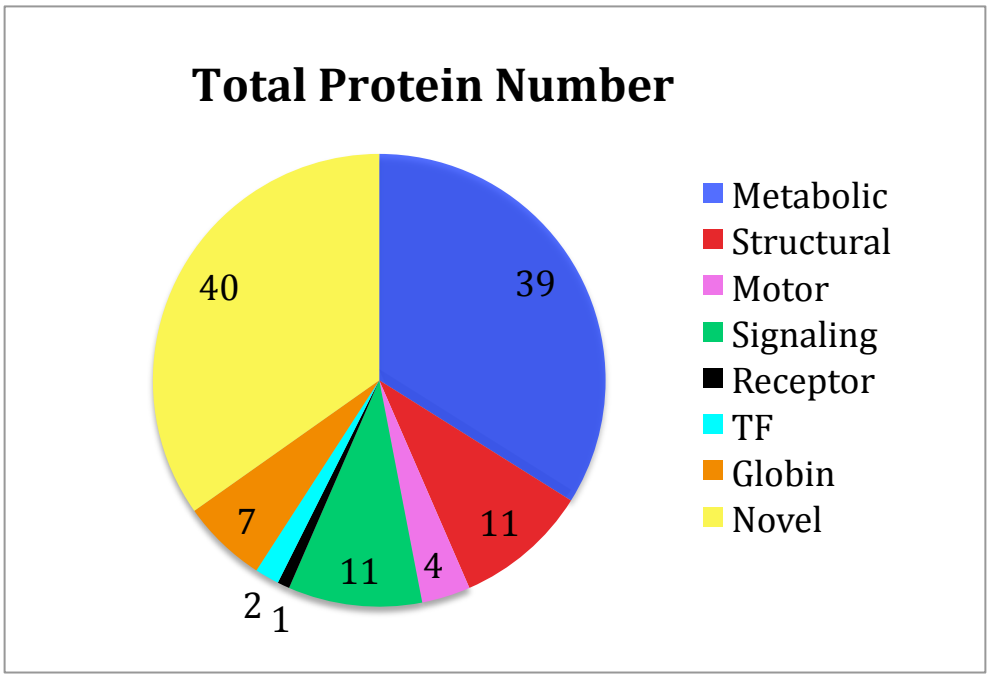
Globin family	3	myoglobin	15583	7	46.3	41053652 M
	4	myoglobin	15583	7	46.3	41053652 M
	5	ba1 globin	16389	7.7	87.8	18858329 M
	6	ba1 globin	16389	7.7	66.9	18858329 M
	7	Ba1 globin	16403	7.7	45.9	92096378
	11	cytoglobin 2	20585	5	23.5	145279651 M
	15	alpha-1-microglobulin/bikunin precursor, like precursor	23835	5.2	50	41055365 M
Novel/ predicted	8	PREDICTED: similar to Myosin-Id	20875	9.5	31.1	189526020
	9	PREDICTED: similar to Myosin-Id	20875	9.5	24	189526020
	12	PREDICTED: similar to phosphoinositide-3-kinase, regulatory subunit 6	26669	6.9	27.2	189520820
	18	PREDICTED: hypothetical protein LOC100006474	26668	8.2	28.9	189534558
	19	PREDICTED: similar to Wnt4b protein	25168	9	36.4	125843849
	28	PREDICTED: hypothetical protein	31398	5.3	21.8	189515152
	35	novel protein similar to vertebrate WNT1 inducible signaling pathway protein 1 (WISP1)	34937	8.7	25.6	148726365
	36	PREDICTED: similar to zinc finger protein 502	41796	9.2	29.2	189534640
	40	PREDICTED: similar to WNT1-inducible-signaling pathway protein 3 precursor (WISP-3)	41746	9.2	30.1	189532808
	52	PREDICTED: similar to Protein zyg-11 homolog	42480	5.8	30.9	189538821
	53	PREDICTED: similar to Protein zyg-11 homolog	42480	5.8	13.8	189538821
	55	PREDICTED: similar to mKIAA0844 protein	38920	8.7	37.1	125842480

	71	PREDICTED: similar to zinc finger protein 721	72333	9.3	44.6	189517662
	72	PREDICTED: similar to von Willebrand factor precursor (vWF)	60614	5.1	35.4	189542644
	76	PREDICTED: similar to zinc finger protein 568	44108	9.5	23	189518306
	80	PREDICTED: similar to zinc finger protein 40	53243	9.1	32.2	189534638
	82	PREDICTED: similar to zinc finger protein 160	72858	9.3	25.7	189528537
	84	novel protein similar to vertebrate dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2)	64141	9.5	29.3	55962523
	85	LOC553337 protein	68993	6.8	35.7	61403613
	86	PREDICTED: similar to zinc finger protein 160	65587	8.5	29.2	189534592
	87	PREDICTED: similar to zinc finger protein 160	72858	9.3	53.3	189528537
	88	PREDICTED: similar to zinc finger protein 721	72333	9.3	24.3	189517662
	89	PREDICTED: similar to zinc finger protein 721	72333	9.3	28.3	189517662
	92	PREDICTED: similar to zinc finger protein 721	72333	9.3	33.3	189517662
	94	PREDICTED: similar to zinc finger protein 208	79103	9.1	30.5	189528501
	95	PREDICTED: similar to mCG142610	91424	9.3	32.5	189528482
	96	PREDICTED: similar to zinc finger protein 160	72858	9.3	19.2	189528537
	98	PREDICTED: similar to zinc finger protein 721	72333	9.3	29.9	189517662
	99	PREDICTED: ras homolog gene family, member T1 isoform 2	75344	6.4	23.2	189520622 M
	100	PREDICTED: similar to mCG142610	122393	9.1	36.4	189534703

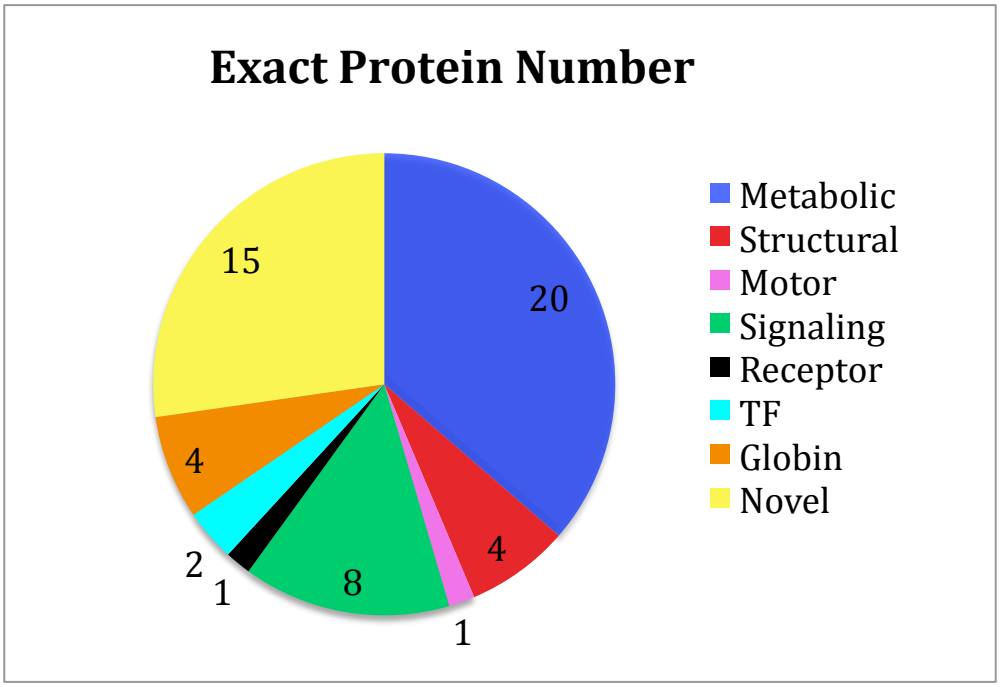
	101	PREDICTED: similar to dynamin 2	86596	6.4	25.8	189519626
	102	PREDICTED: similar to pol polyprotein	128147	8.7	29	189515367
	103	PREDICTED: similar to zinc finger 142	169377	8.7	25.1	189523652
	106	similar to zinc finger protein 721	70257	9.3	25.9	189517658
	107	PREDICTED: similar to mCG142610	71778	9.5	23.4	189518369
	108	PREDICTED: similar to protein tyrosine phosphatase, receptor type, T	96885	7.1	22.7	189520775
	109	PREDICTED: similar to mCG142610	134532	9.3	46.6	189534972
	110	PREDICTED: similar to Ca2+-dependent secretion activator	1038986	5.8	15.3	189525131
	111	PREDICTED: PTK2B protein tyrosine kinase 2 beta	115235	9.6	14.5	189526015
	112	PREDICTED: PTK2B protein tyrosine kinase 2 beta	115235	9.6	21.2	189526015

Table 2. Proteins identified in 8 adult zebrafish hearts, using MS, with relative MW (Da), PI, score and accession number, classified according to their biological functions. In yellow, the proteins identified with score <20.

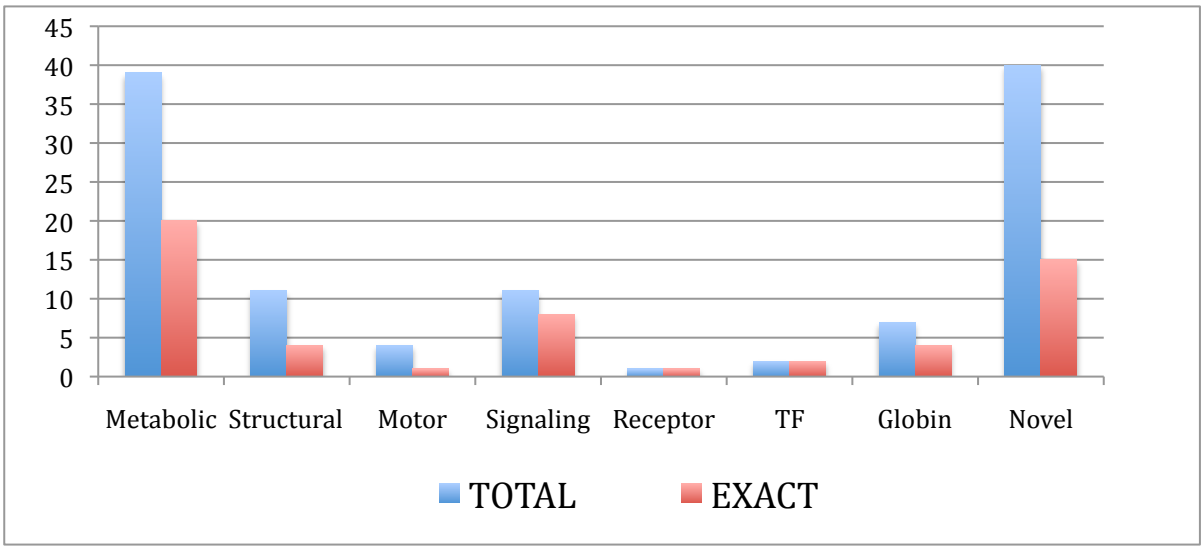
The number of the total proteins present in each biological function is reported in **Graphic 1**. Numerous proteins identified resulted to have a metabolic function. Few proteins had a structural and signaling function. Two proteins were identified as transcription factors and one as receptor. Since sometimes the same protein was found in different spots because of post-transcriptional modifications (e.g. phosphorilation, oxidation, and acetylation), that may change the separation characteristics of the proteins, it was also counted the exact number of different proteins identified in each biological function and these values are reported in **Graphic 2**. It was possible to identify 55 different proteins, 20 of which resulted to have a metabolic function. The total and exact numbers of proteins identified in each biological function class is reported in **Graphic 3**. Numerous proteins with metabolic, structural, and motility function resulted subjected to post-transcriptional modifications.



Graphic 1. Number of total proteins identified, present in each biological function (Total=115).



Graphic 2. Exact number of different proteins identified in each biological function (Total=55).



Graphic 3. Total and exact numbers of proteins identified in each biological function class. The amount of total proteins identified is 115 and the amount of exact proteins is 55.

DISCUSSION

Using zebrafish to study genes and proteins is supported by the sequencing of his 1.7-Gb genome (Sanger Institute's *Danio rerio* sequencing project), which has facilitated the generation of microarrays for large scale expression profiling (Ton et al., 2002; Lo et al., 2003; Mathavan et al., 2005; Quian et al., 2005). Moeover, this fish can be used as vertebrate model for pathogenesis study (Neely et al., 2002; Prouty et al., 2003) such as hematopoietic and cardiovascular diseases (North and Zon, 2003) or for discovering new therapeutic target (Peterson, 2004). Thus, aiming to contribute to the knowledge of the proteomic expression of the heart, it was reported for the first time a proteomic subset of the adult zebrafish heart using a 2DE. Proteome-based technologies are powerful tools for the analysis of protein expression in cells and organs. Such technology can be applied to the identification of potential diseases biomarkers. Among the proteome technologies, two-dimensional electrophoresis followed by mass spectrometric analysis is the most established method, resulting in the identification of thousands of expressed proteins.

Here, we reported for the first time a proteomic subset of the adult zebrafish heart using a 2DE. This study revealed more than one hundred proteins of different function, metabolic enzymes (glyceraldehydes-3-phosphate dehydrogenase, mitochondrial malate dehydrogenase, ATP synthase), proteins of signal transduction pathways (transcription factor

Lmx1b, Mcm10 protein), growth factors (vascular endothelial growth factor, insulin-like growth factor), cytoskeletal components, globin family proteins (myoglobin, ba1 globin), and structural proteins (four and a half LIM domains, actin, desmin). The highest numbers of proteins resulted to have a metabolic function or resulted novel/predicted. In both classes the exact number of proteins resulted almost the half respect to the total proteins identified, suggesting a high level of post-transcriptional modifications, as was described in other classes of vertebrates (Gooley and Packer, 1997; Mann and Jensen, 2003; Kane and Van Eyk, 2009). It was also characterized proteins that are included in the globin family. This finding could be correlated with the naturally presence of blood in the hearts, as already found in proteomic study of mouse heart by Chakravarti et al., (2008). Interestingly, the identification of proteins with functions of signaling, receptor and transcription factors, because if altered, could be correlated with pathology (Simkhovich et al., 2003). It must be take note that, not all the spots processed in this research, gave score >20 due to the low concentration or overlapping of multiple proteins in the same spot. Here it was described some features of the most important proteins identified and, for simplicity, they are separated according to their reported function.

- **Proteins involved in signal transduction pathway**

One calcium-binding protein, calbindin 2, like (**Spot # 27**) was identified in this study. This protein is a cytosolic intracellular Ca2+ regulator that restricts the Ca2+-mediated signals in the cytoplasm (Heizmann and Braun, 1995). In particular, there are several possible functions for the role of calbindin, in the cerebellum such as the protection of intracellular organelles or the regulator of intracellular signaling (Hong et al., 2009). The functional role of calbindin is the partial stabilization of intracellular calcium levels, which can confer protection against different proapoptotic stimuli.

Another protein identified in the heart was the retinol-binding protein (Rbp b2) (**Spot# 1**). In human and rodent genomes there are single copies of genes encoding the cellular retinol-binding protein type I and II (CRBPI and CRBPII) (De Baere et al., 1998). In the adult zebrafish have been identified duplicate genes for both CRBPI and CRBPII (Liu et al., 2004), with crucial roles in organogenesis, vision, reproduction, growth and immunity (Costaridis et al., 1996; Cameron et al., 2002). Rbp belong to the large family of low-molecular-mass (~15 kDa) intracellular lipid-binding proteins (iLBP) that bind fatty acids, retinoids, and steroid (reviewed by Ong et al., 1994 and by Bernlohr et al., 1997).

- **Proteins involved in cell and energy metabolism**

In the present research were identified glycraldehyde-3-phosphate dehydrogenase (**Spots # 37, 38, 42, 44**), and isocitrate dehydrogenase (**Spots # 56, 57, 62, 63, 64, 65**). A recent proteomic work concerning ventricular remodeling in rats affected by heart failure, shows that energy metabolism proteins as glycraldehyde-3-phosphate dehydrogenase, alphaB-crystallin, peroxiredoxin 2, and isocitrate dehydrogenase are linked to echographic parameters according to heart failure severity (Cieniewski-Bernard et al., 2008). Interesting, glycraldehyde-3-phosphate dehydrogenase was also detected in the rat heart (Shi et al., 2008), and, comparing with this study, the spots in the 2DE resulted in the same position. Other matches in the positions of the spots in the gels were founded for triosephosphate isomerase, another protein involved in the energy metabolism, and for actin and myosin, proteins with structural and motor function, respectively (See below).

ATP synthase, here identified in **Spots # 67, 68**, and **78**, represent a critical enzyme in the energetic pathways of cells because it is the enzyme complex that produces the majority of cellular ATP (reviewed by Velours et al., 2009). It has been shown to be involved in several cardiac phenotypes including heart failure and preconditioning, a cellular protective mechanism. Also, in mammals hearts, ATP synthase is subjected to several post-translational modification, as reviewed by Kane and Van Eyk, (2009).

Another protein involved in energetic metabolism identified in this study was the mitochondrial aconitase, 2 (**Spots # 104, 105**). Enzymatic isoforms of aconitase, which are integral to the TCA cycle, and the mitochondrial respiratory complexes (Complexes I, II, and III), seems to be involved in electron transport, incorporate iron-sulfur clusters and are responsible for mitochondrial respiration and energy production (Rouault and Tong, 2008).

Fructose 1,6-bisphosphatase (FBPase) [EC 3.1.3.11] was also identified in the heart proteome (**Spots # 41, 43, 50**). This enzyme catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate (Benkovic and deMaine, 1982). Tejwani, (1983) investigated the colocalisation of aldolase and FBPase in cardiomyocytes. The results indicate *in-vivo* interaction of these two enzymes. Also, in the cardiomyocytes cytoplasm, these enzymes were found to co-localize at the Z-line and on intercalated discs.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [EC 1.2.1.12], here identified in **Spots # 37, 38, 42,** and **44,** is an enzyme that catalyze the sixth step of glycolysis, but it is also implicated in non-metabolic processes, as transcription activation (Sirover, 1997).

Changes in the expression of a variety of proteins involved in energy metabolism have been detected in heart failure by performing proteome analysis (Arrell et al., 2001).

In this study, **Spot # 101** was identified as similar to dynamin 2. Dynamin (Dyn) is a 96kDa enzyme and a member of a novel group of GTPases which was initially identified as a microtubule-binding protein with a role in vectorial movement. Dyn proteins exhibit a high degree of evolutionary conservation (Diatloff-Zito et al., 1995). A unique 3.6-kb transcript is found in all human tissues examined and it is more abundant in skeletal muscle and heart (Diatloff-Zito et al., 1995).

- **Proteins with structural function**

Cardiac and skeletal muscles have a similar sophisticated organization of contractile proteins into sarcomeres and are collectively referred to as striated muscles. Defects in a large set of sarcomeric proteins such as cardiac b myosin heavy chain, atropomyosin, and cardiac a-actin have been demonstrated to induce cardiac hypertrophy (Berry et al., 2007). Here have been identified several isoform of actin (**Spots # 59, 60, 61, 73, 75, 77**), and of myosin (**Spots # 13, 113, 114, 115**). Cardiac myocytes are normally surrounded by a fine network of collagen fibers. Sarcomeres are the functional unit of contraction in cardiac muscles, which consist of thick and thin filament proteins. Several proteins that are post-translationally modified in the cardiac sarcomere have now been identified in mammals, including cardiac myosin binding protein-C (cMyBP-C), cardiac troponin I (cTnI), cardiac troponin T (cTnT), α-tropomyosin (α-TM) and the myosin light chain (MLC) (Jacques et al., 2008; Scruggs et al., 2009). Moreover, dephosphorylation of cMyBP-C, which accelerates its degradation, has been shown to associate with the development of heart failure in mouse models and in humans and its phosphorylation play in regulating cardiac function and heart failure (Hamdani et al., 2008). Elucidating the function of cMyBP-C is clinically important because mutations in this protein have been linked to cardiomyopathy in more than sixty million people worldwide (Lloyd-Jones et al., 2009). Smooth muscle a-actin (42 kDa), b-actin (41 kDa), c-actin (41 kDa), myosin heavy chain (223 kDa) and the actin-binding protein filamin A (281 kDa) were identified as the major serotonylated proteins serotonylated because they are among the most abundant in a smooth muscle-rich tissue (Watts et al., 2009).

Another structural protein identified in this study resulted Desm protein (**Spot # 83**), a 52kD protein that is a subunit of intermediate filaments in skeletal muscle tissue, smooth muscle tissue, and cardiac muscle (Bär et al., 2004). Desmin provides structural and

functional integrity by coordinating mechanical stress transmission, organelle positioning, organization and assembly of sarcomeres, signal transduction, and apoptosis. Mutations in the gene encoding desmin (*DES*) are associated with a variable clinical phenotype referred to as desminrelated myopathy (OMIM #601419), a clinically heterogenous group of disorders encompassing myopathies, cardiomyopathies, conduction disease, and combinations of these disorders (Li and Dalakas, 2001; van Tintelen et al., 2009).

- **Proteins for lipid transport**

In this study it was identified apolipoprotein A-1 (**Spot # 14**). Apolipoproteins are proteins that bind to fats and can have different functions as enzyme cofactors, lipid transport, and ligands (Mahley et al., 1988). The major apolipoproteins include aopE, apoB, apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, and apoC-III (Tall and Small, 1978). Apolipoprotein E (apoE) has a prominent role in the transport and metabolism of plasma cholesterol and triglycerides resulting from its ability to interact with lipoprotein receptors (Mahley, 1988; Weisgraber, 1994). Besides its role in cardiovascular diseases, accumulating evidence has suggested that apoE polymorphism is an important determinant of risk for the development of neurodegenerative diseases, such as Alzheimer's disease. It has been demonstrated that *apoE* gene can be found in a non-mammalian vertebrates and is highly expressed during zebrafish embryonic development (Babin et al., 1997). Whole-mount in situ hybridization was performed to analyze the pattern of *apoE* expression during development of the zebrafish paired and unpaired fins (Monnot et al., 1999).

- **Proteins of the globin family**

Several proteins of globin family were identified in this study, as myoglobin (**Spots # 3, 4**), Ba globin (**Spots # 5, 6, 7**), cytoglobin 2 (**Spot # 11**), and microglobulin (**Spot # 15**). Globins are a family of heme-containing proteins that reversibly bind oxygen and they have been described in bacteria, fungi, protists, plants, and animals (reviewed by Hardison, 1996). Four mammalian globins have been identified so far (hemoglobin [Hb], myoglobin, neuroglobin [Ngb], and cytoglobin [Cygb]) (Burmester et al., 2002). The crystal structures of these globins have been characterized, and all are heme-containing respiratory proteins (Pertutz, 1962 for Hb; Kendrew, 1963 for Mb; Pesce et al., 2003 for Ngb; de Sanctis et al., 2004, for Cygb). Hb is localized in erythrocytes and has a major role to facilitate the transport of oxygen in the blood. Myoglobin is localized in the cytoplasm of skeletal and cardiac

muscle, acts in intracellular oxygen storage, and enhances oxygen diffusion to the mitochondria for use in oxidative phosphorylation. In addition, Hb and myoglobin can act as scavengers of bioactive nitric oxide. Ngb and Cygb are two recently described members of the globin family; Ngb has a yet unidentified function in nerve cells. Cygb has been identified as a 20.9-kDa protein in virtually all human, mouse, and zebrafish tissues. It is expressed in apparently all types of human tissue and therefore has been called cytoglobin (CYGB). Cygb (but not Ngb) was also present in canine kidney, liver, lung, and heart tissue. Mouse and human CYGBs comprise 190 amino acids; the zebrafish CYGB, 174 amino acids. Among them, Cygb is the most recently discovered O2-binding globin in rat hepatic stellate cells as stellate cell activation associated protein. Recently, a fifth globin protein, globin X, was reported in fishes (Roesner et al., 2005). Phylogenetic analysis suggests that an ancient globin differentiated into a cellular globin that gave rise to Mb and Cygb, which share a common ancestor before the split of jawless and jawed vertebrates about 450 million years ago (Xi et al., 2007).

In conclusion, this proteomic study allowed the identification of more than one hundred proteins naturally expressed in adult zebrafish heart, having different biological functions. This study can give a basic contribute in understanding the biological knowledge of the vertebrate heart and can constitute a reference point to compare proteins expression in various cardiac diseases.

CHAPTER V

Proteomic of the zebrafish heart during the *in-vivo* regeneration process

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Manuscript on preparation

ABSTRACT

Many complex cellular, developmental, homeostatic and regenerative processes depend on precise spatiotemporal regulation of protein levels, some of which function as "rheostats" to execute programs in a quantitative fashion. The heart of vertebrate is one of the most conserved organs at the molecular level and is the organ most affected by disease in childhood and adult populations. Using the model organism zebrafish (*Danio rerio*), here it was tested the hypothesis that proteins are differently expressed during the heart regeneration. Numerous studies have considered the gene expression during the heart regeneration process, but so far, there is no study regarding the proteomic aspect. Applying two-dimensional differencial gel electrophoresis (DiGE) combined with MALDI-TOF/TOF tandem mass spectrometry, here were identified proteins differentially expressed at 3 days after amputation of around 20% of the ventricle apex, compared with hearts not injured. Among more than 100 proteins spots detected in 2D-gels, 3 were up-regulated in injured hearts at 3 days post amputation (dpa). MS analysis allowed the identification of proteins differentially expressed as compared with control, such as: ATP synthase, hyaluronan mediated motility receptor, and desmuslin. Information concerning the global alteration protein pattern during heart regeneration will be helpful for a better understanding of the therapeutic strategies.

INTRODUCTION

A prerogative of modern biomedicine is to repair damaged organs, stimulating their natural regenerative ability (Scott and Stainier, 2002). Regeneration consists in a complex biological process by which animals can restore the shape, structure and function of body parts lost after injury, or experimental amputation. Unfortunately, not all of our organs are equally competent to regenerate, and it is known that heart and central nervous system are particularly resistant to regeneration after injury and that they form scar tissue (Poss et al., 2002). The amazing capacity of Teleost to regenerate fins, brain, retina or lens, has made these animals the preferred subject for research on vertebrate regeneration (Lien et al., 2006; Raya et al., 2003). In particular zebrafish (*Danio rerio*) represents an experimental tractable vertebrate model for the study of diseases and heart regeneration (Lepilina et al., 2006; rev. Lieschke and Currie, 2007). With the full sequence of the zebrafish genome and mutagenesis screens, a lot of genes have thus been examined, and several proteins were identified (Talbot and Hopkins, 2000; Amsterdam, 2006) However, only a limited number of studies were done

regarding the zebrafish proteome during development, and so far, studies are focused neither on regeneration nor in the heart. Previous researches have shown that adult zebrafish can effectively regenerate injured hearts *in-vivo* (Poss et al., 2002; Lepilina et al., 2006). Although some indications had pointed on the involvement of several genes, molecular mechanisms and expressed proteins that potentially can regulate the regeneration process remains unclear. Resolving the proteomic alterations that control these changes can provide important insights, undetectable by gene expression analysis alone. Aiming to know the protomic profile of adult zebrafish heart regeneration, in the present research Differential Gel Electroforesis (DiGE) experiments, combined with MALDI-TOF/TOF tandem mass spectrometry were applied. DiGE is a specialized two-dimensional electrophoresis technique that allows to visualize those proteins that differ between two samples. More in particular, here were analized proteins differentially expressed in the zebrafish heart at 3 days after amputation of around 20% of the ventricle apex, compared with hearts not injured.

MATHERIALS AND METHODS

Resection of 20% of ventricular apex of zebrafish heart

The animal experiments were performed according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. The surgery was done following the protocol developed by Poss et al. (2002). Brefly, adult zebrafish (6-18 months old) were anesthetized for 3 minutes in 0.168 mg/l Tricaine (MS222,Sigma) and placed dorsally in a humid sterile sponge, with ventral side up. A small incision (scalpel 0.15mm, Fine Science Tools Inc. US.) was made just under the gills, in correspondence to where the heart is located. The pericardial sac was isolated and punctured to expose the ventricle by gentle abdominal pressure. Approximately 20% of the ventricle apex was removed by iridectomy scissors (Fine Science Tools Inc. US). Body wall incision was not sutured and in few days the injured part was recovered. With this protocol, 90% of fish was survived. After surgery, fish were returned to water and stimulated to breathe with air bubbles from an air pump. After 3 days, fish were lethally anesthetized with Tricaine and the whole hearts were removed and washed three times in Hank's solution to remove blood from the organs tissues.

Proteomics of the Zebrafish heart

i) Samples preparation

Difference gel electrophoresis (DiGE) system was used to detect the protein expression of 3 adult zebrafish hearts control and 3 hearts at 3 dpa *in-vivo*, followed by MALDI-TOF/MS protein identification. Protein identification was carried out using matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and the Mascot and ProFound search engines. Zebrafish hearts of control and operated were removed and homogenized (Teflon pestle) in 20 µl of labeling buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate), pH 8.5 adjusted with 1N HCl, clarified by ultracentrifugation (100,000 x g, 23^{o}C, 1.0 hr) and stored at −80 °C.

ii) CyDye labeling

Minimal protein labeling with CyDye DiGE fluor, Cy2 was carried out according to manufacturer's instructions (GE Healthcare/Amersham Biosciences). Briefly, the sample was thawed on ice and centrifuged at 16,000 g for 10 min at 4 °C. After 30 min of incubation on ice in the dark, the reaction was terminated with 10 mM ethanolamine.

iii) 2-D Electrophoresis

Sample was subjected to isoelectric focusing (IEF) using an Ettan IPGphor (GE Healthcare/Amersham Biosciences) and 24 cm linear, pH 3–10 Immobiline DryStrips (GE Healthcare/ Amersham Biosciences). The IPGphor system was programmed to perform IEF with the following settings: (i) 12 hour rehydration, (ii) 500 V, 1.0 h, “Step-n-hold”; (iii) 1000 V, 1.0 h, “Step-n-hold”; (iv) 1500 V, 1 h, “Step-n-hold”; (v) 4000 V, 1 h, “Step-n-hold”; and (vi) 8000 V, 60,000 Vh, “Step-nhold”. The IPG strip was subjected to two 15-min equilibration steps with 1% DTT in SDS buffer (3 M urea, 50 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS and trace bromophenol blue). The 2DE was carried out on a 8-16% SDS-polyacrylamide gradient gel with a constant current of 30 mA until the tracking dye was 1 cm above the bottom of the gel.

iv) Image acquisition

Fluorescent images of each CyDye were acquired using a Typhoon 9400 variable mode imager (GE Healthcare/Amersham Biosciences). After the acquisition of the fluorescent

image, proteins separated by 2D-gel analysis were stained using the Silver Stain Plus Kit (Bio-Rad) according to the manufacturer's instructions. Gel was also stained with Comassie Blue (Bioorad). Gel images were created by acquiring digitized images (400-600 dpi) using a Canon LiDE 30 flatbed scanner. Once scanned, gels were stored in 1.0% acetic acid (Fisher) at 4^{o}C until spots of interest were excised for mass spectrometry.

v) In-gel trypsin digestion of proteins

Protein spots of interest were manually picked (1.0 mm to 3.0 mm in diameter) and rinsed in distilled water. Silver ions were removed by adding fresh destaining solution (15 mM potassium ferricyanide and 50 mM thiosufate in distilled water) (Invitrogen, Carlsbad, CA) to each spot and incubating for 20 min. Samples were then rinsed with distilled water, destained for blue commassie bue with 40% methanol 10% acetic acid, and equilibrated with 200 mM ammonium bicarbonate (Fisher). Samples were rinsed in 50% acetonitrile (Fisher) in 20 mM ammonium bicarbonate and dehydrated in 100% acetonitrile. Samples were digested *in situ* with 200-300 ng trypsin (Sigma) (14 hours, 37^{o}C) and peptides were extracted 50% acetonitrile, 2.5% TFA (Fisher) in distilled water and dried using a CentriVap Speed Vacuum. Extracted, dried peptides were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) (ABI) and 0.5 µl was spotted onto the target for MALDI-TOF analysis. MALDI-TOF was performed using the 4800 Proteomics Analyzer (ABI, Foster City, CA). Mass spectra were individually calibrated using internal trypsin peaks (842.51 and 1045.56, *m/z*) with Data Explorer software available from ABI. Proteins were identified using ProteinProspector (University of California, San Francisco; http://prospector.ucsf.edu/) set to a mass accuracy of +/- 50 ppm to compare unknown mass fingerprints to those of known proteins in the NCBI non-redundant database using a species-specific filter for *Danio rerio.*

RESULTS

Using a newly developed analytical procedure, here, for the first time, it is reported the DiGE of the zebrafish hearts at 3 dpa *in-vivo* compared to control uninjured hearts **(Figure 1)**. The red spots show the up-expression of proteins after the surgery and the green spots indicate those that are down-expressed respect to the hearts of control.

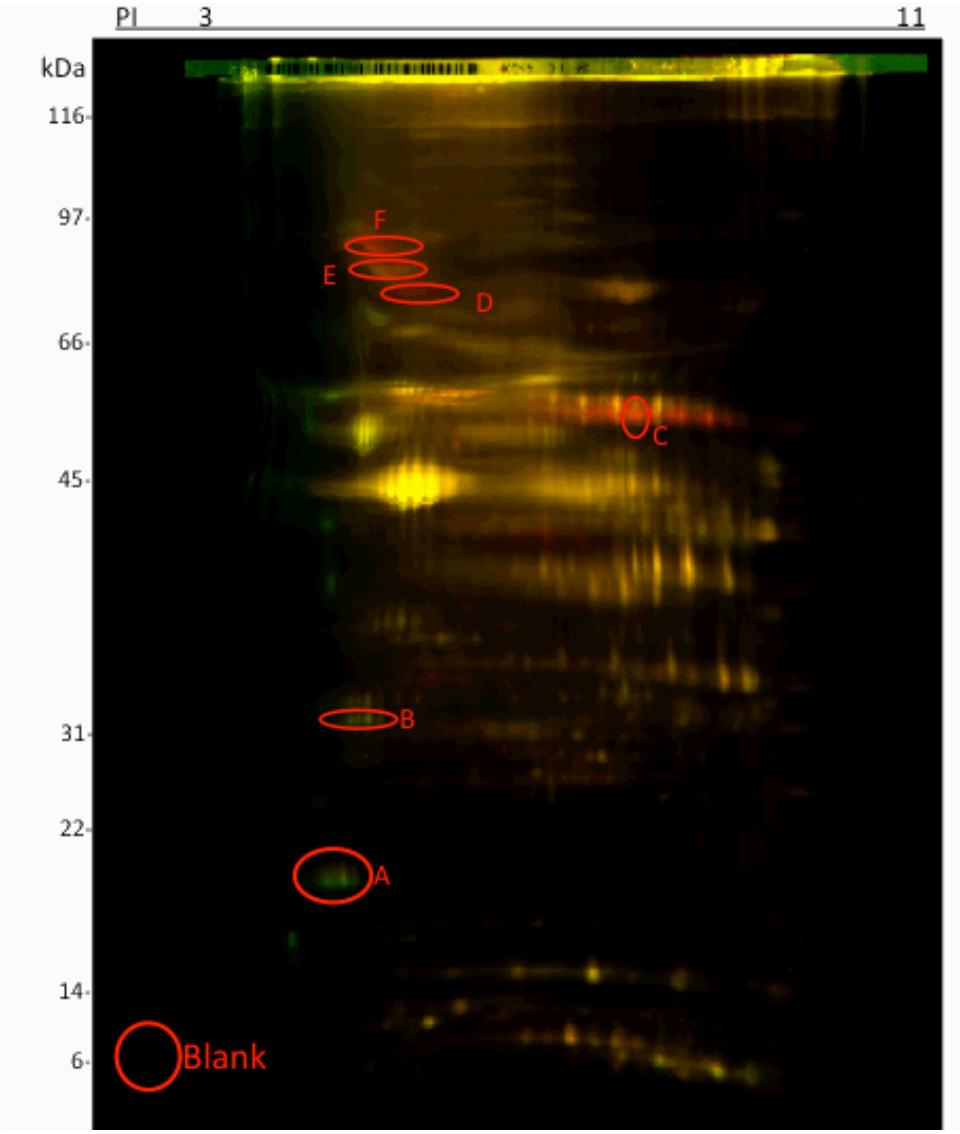

Figure 1. DiGE of proteins differently expressed in zebrafish hearts of control (green), and at 3 dpa (red). In yellow there are the proteins that are equally expressed in the two samples. Circles indicate the spots picked and processed for MS analisyis.

With the aim of avoid preferential binding of proteins with the CyDyes, thus control the specificy of the reaction, the same DiGE separation was performed, inverting the sample labeling. **Figure 2** shows the DiGE of the zebrafish hearts at 3 dpa *in-vivo* compared to the hearts control, not subjected to surgery, with label switched respect to **Figure 1**. In **Figure 2,** the proteins up-expressed after the surgery are labeled with green, and those down-expressed respect to the hearts of control are red.

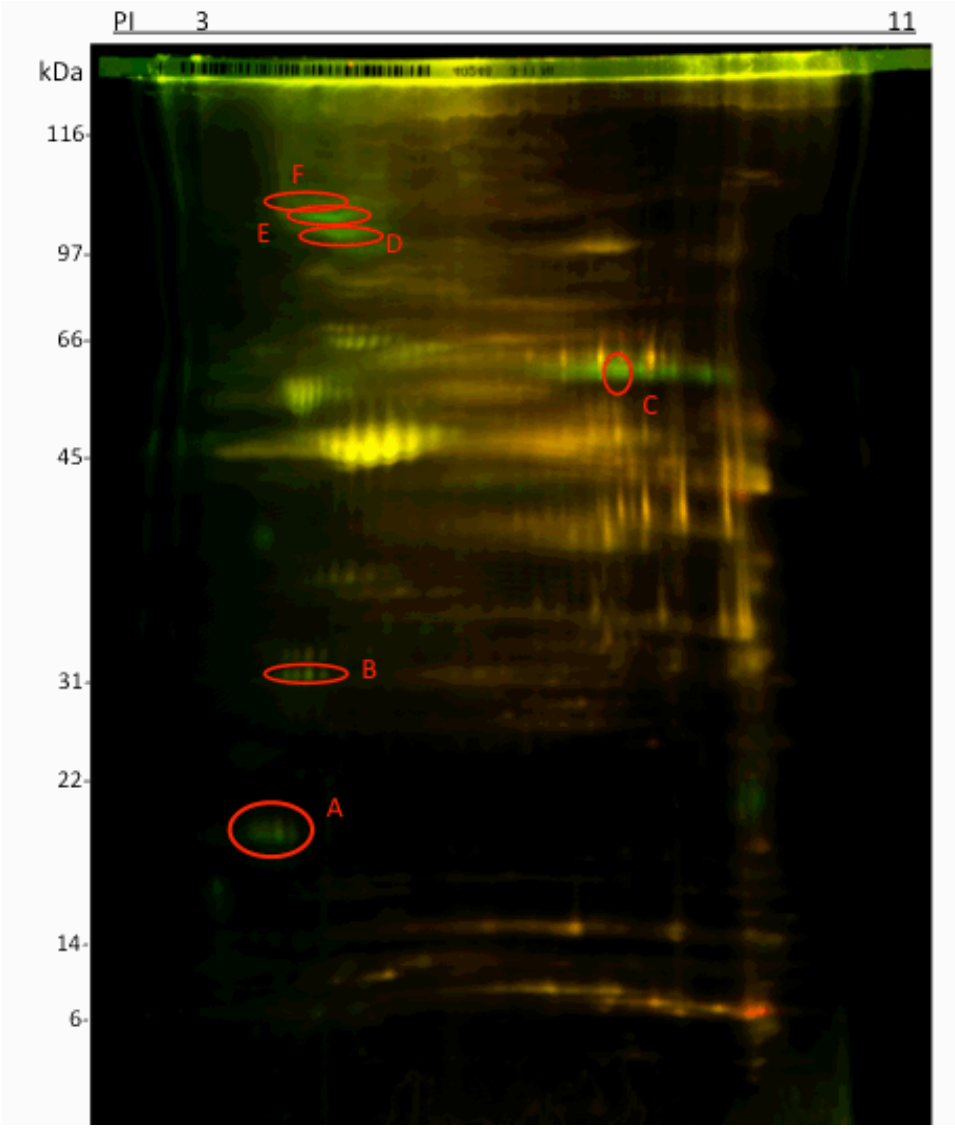

Figure 2. DiGE of proteins differently expressed in zebrafish hearts of control (red), and at 3 dpa (green). In yellow there are the proteins that are equally expressed in the two samples. Circles indicate the spots picked and processed for MS analisys.

Differential expressed proteins spots were picked and processed for MS analysis and then analyzed for the peptide identification. The characteristics of picked proteins (name, molecular weight, isoelecting point, percentage of coverage and accession number) are reported in **Table 1.** In this study were identified one isoform of the myosin protein, (light polypeptide 7, **Spot A**), corresponding to 19021 Da, and an hypotetical protein of 32220 Da (**Spot B**). Both proteins resulted not significative changing between the two samples considered. The three up-regulated proteins at 3 dpa resulted to be: 1) ATP synthase (ATPsyn), corresponding to 59744 Da **(Spot C**); 2) hyaluronan mediated motility receptor

(RHAMM), of 104710 Da (**Spot D, E**), and desmuslin (DMN), with a MW of 122138 Da (**Spot F**).

Spot #	Protein name	MW (Da)	pI	% Cov	Accession #
A	myosin, light polypeptide 7, regulatory	19021	4.7	41.3	29725603 M
B	PREDICTED: hypotetical protein	32220	5	27.8	189526031
C	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	59744	9.1	27.8	116325975 M
D	hyaluronan mediated motility receptor	104710	5.5	20.4	41053824 M
E	hyaluronan mediated motility receptor	104710	5.5	23.1	41053824 M
F	desmuslin	122138	4.9	21.2	113678191 M

Table 1. List and characteristics of the proteins identified in differential gel electrophoresis, compairing zebrafish hearts of control and at 3 dpa *in-vivo*. **Spots A** and **B** represented not significative changing between the two samples. Spots **C**, **D**, **E**, and **F** resulted up-regulated at 3 dpa with respect to the control.

DISCUSSION

Among the proteome technologies, differential two-dimensional electrophoresis (DiGE) followed by mass spectrometric analysis is an innovative, sensitive, and reliable way to detect proteins whose expression is altered between control and treated samples (Gozal et al., 2009). The present work has identified a number of differentially expressed proteins in the adult zebrafish heart subjected to amputation of around 20% of the ventricle area. Three proteins showed altered expression between the groups: 1) ATP synthase (ATPase); 2) hyaluronan mediated motility receptor (RHAMM), and desmuslin (DMN). All were up-regulated in the amputated heart at 3 dpa. Here, for the first time, have been isolated more than 100 proteins naturally expressed in the heart of the adult zebrafish and some of them are differentially expressed (up-regulated) during the regeneration process *in-vivo* at 3 dpa. Switch the labeling of the samples, among identified proteins, myosin, (light polypeptide 7, **Spot A**) and a hypotetical protein (of 32220 Da, **Spot B**), remained labeled with green. This data demonstrates that those specific proteins are more susceptible to bind the Cy3 dye (green) respect to the Cy5 (red). For this reason is not possible to consider these changing

significative. However, the MS identification was performer for all the 6 spots, even if only the spots C, D, E, and F, represented significative changes in proteins levels between the two samples. Those spost were identified as ATP synthase (ATPsyn, **Spot C**), hyaluronan mediated motility receptor (RHAMM, **Spot D, E**), and desmuslin (DMN, **Spot F**).

ATP synthase is a general term for an enzyme that can synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phoshate by using some form of energy (Rev. Velours et al., 2009). Thus, it is a critical enzyme in the energetic pathways of cells because it is the enzyme complex that produces the majority of cellular ATP. ATPsyn has been shown to be involved in several cardiac phenotypes including heart failure and preconditioning, a cellular protective mechanism (Kane and Van Eyk, 2009). ATPsyn was previously purified from Bacillus sp. (Cook et al., 2003), *Streptomyces lividans* (Hensel et al., 1991) and from beef heart mitocondria (Deisinger et al, 1993), demonstrating to be an evolutionary conserved enzyme and heart-expressed in vertebrates. Several reports had pointed on the modulation of the mitochondrial ATPsyn complex in various cardiac phenomena (rev. Das, 2003; Grover, 2008), including disease (Marin-Garcia and Goldenthal, 2008, Murray et al,. 2007). Specifically, it has been observed that there is an increase in ATP hydrolysis by the ATP synthase complex in ischemic myocardium (Grover et al., 2004), which is hypothesized to contribute to the overall depletion of the cellular ATP pool during ischemia. Also, using a proteomic approach, it was demonstrated that in the rabbit heart, elevated preload is associated with up-regulation of proteins involved in fatty acid oxidation, glucose metabolism, and mitochondrial ATPsyn (Schott et al., 2008). In this research, the up-regulation of ATPsyn could be explainded by the energy demand that the regenerating system needs after the activation. Further studies need to be addressed to know whether ATPsyn localizes near the clot, formed after ventricle amputation. It has been suggested that altered expression of proteins from metabolic pathways reflects mitochondrial dysfunction as a feature of the transition from compensated myocardial hypertrophy to the heart failure (Razeghi et al., 2001).

Here, the hyaluronan mediated motility receptor showed to have different expression level during the zebrafish heart regeneration. It has been shown that targeted deletion of the hyaluronan synthase-2 (Has2) gene in mice results in an absence of cardiac jelly and endocardial cushions, a loss of vascular integrity, and embryonic death at E9.5 (Klewer et al., 2006). Despite the requirements for Has2 and its synthetic product hyaluronan (HA) in the developing cardiovascular system, little is known about the normal expression pattern of Has2

or the factors regulating Has2 gene transcription during development. In the same study, Klewer et al., (2006) defined the embryonic expression pattern of Has2 in mice and explored the regulation of Has2 gene expression by Bmp signaling. *In situ* hybridization studies demonstrated dynamic Has2 expression patterns during myocardial cell development and cardiac tube formation, formation of the cardiac endocardial cushions, and cushion invasion by valve primordial cells. In zebrafish Has2 is involved in the earliest left-right asymmetric movements during cardiac morphogenesis (Smith et al., 2008). In fact, using high-resolution 2D imaging of cardiac progenitor cells (CPCs), Authors demonstrated that leftward displacement and rotation of the tube requires Has2 expression. Here, the up-regulation of Has2 could be correlated with the inflammatory changes that occur after the surgery and also with the reactivation of developmental cardiac gene.

Desmuslin is an intermediate filament (IF) and, like other IFs, primarily functions to integrate mechanical stress and maintain structural integrity in eukaryotic cells (Mizuno et al., 2001). While it has been observed in a variety of cell types, it is has been best studied in the sarcomere of skeletal myocytes. It was localized at the Z-disk and has been shown to bind to α-dystrobrevin, α-actinin, and desmin to act as a mechanical linker in transmitting force laterally throughout the tissue, especially between the contractile myofibrils and extracellular matrix (Mizuno et al., 2001). Mutations in the desmin gene can cause desmin myopathies characterized by muscle weakness, cardiac impairment, and intracytoplasmic accumulation of desmin deposits (Dalakas et al., 2000). The up-regulation of the desmuslin founded here in the heart at 3 dpa could be correlated with the activation and replacement of contractile apparatus. Desmuslin, in fact, could have a determinant role in maintaining the cell integrity and restore the ventricle cells damaged near the cut site.

Taken together, this study had demonstrated that the surgery causes not only a gene activation but also a different proteins synthesis. The three proteins resulted up-regulated in the hearts at 3 dpa are involved in the energy methabolism, in the signaling and in maintaining the structure integrity of the cells, so it is reasonable to think that they are involved in the cardiac regeneration process too. Unfortunately, not all the spots that we picked and processed for MS identification, gave a hight percentage of coverage because some of them were low concentrated or because in the same spot it is possible that there are present more than one protein. However, for all the spots we obtained scores > 20 and this allows a still good protein identification. 2DE coupled with MS is currently the key approach for profiling thousands of proteins of a given proteome simultaneously and offer the

possibility to analyze complex biological processes directly on the level of quantitative protein expression. However, this method is labour-intensive and not readily interfaced with protein identification by MS. In addition, hydrophobic, extremely basic proteins and those with a very high or low MW are often difficult to analyze using this approach. In the opposite, the DiGE technique results more sensible than the 2DE and offers the possibility to compare the proteins levels expression between two samples. Here, it has been demonstrate the utility of the DiGE technique as a tool for analyzing the *in-vivo* heart regeneration process in the model organism zebrafish. Indeed, proteome studies have identified important protein sets that are involved in the molecular mechanisms of the zebrafish heart regeneration. This might consequently lead to the definition of biomarkers correlated with human heart and can give the possibility to find novel therapeutic targets for the heart failure.

CHAPTER VI: CONCLUSIONS AND DISCUSSIONS

Injury, disease, and aging all result in a loss of tissue and reduced quality of life. Numerous human conditions could be significantly improved if therapies that encourage tissue regeneration were available. Most adult tissues and organs, especially in humans and other mammals, have lost their regenerative potential. As a result, injury to a tissue or organ usually results in permanent damage from scarring to disability. For example, myocardial infarction (MI) results in large-scale loss of cardiac muscle (often a billion or more myocytes) (Laflamme and Murry, 2005). Repair is one of the major mechanisms needed for survival and proper functioning; however, defective repair with scar formation is an inevitable consequence of tissue damage, compromising the functions of the organ to a certain degree (Harding et al., 2007; Li et al., 2007b). The field of regenerative medicine is aimed at developing strategies to restore individual cell types, complex tissues, or structures that are lost or damaged. Currently, one of the main approaches in the field of regenerative medicine is to guide the process of differentiation of stem cells into specific cell types and then into complex structures (Li et al., 1996; Solloway and Harvey, 2003). The concept of stem cell-based therapies originated with the idea of replenishing damaged tissues with adult stem cells to increase tissues potential for repair (Scorsin et al., 1996; Oh et al., 2003; Cai et al., 2004; Wu et al., 2006b). Alternatively, another strategy is to determine how certain organisms have retained the ability to regenerate their tissues, organs, and appendages (Ausoni and Sartore, 2009). Amphibians, mammals and teleosts have different capacities to repair lost cardiac tissue, and it is from these differences we can learn best how to minimize scarring and maximize regeneration after injury. Zebrafish heart share common structures with those of mammals, serving as a simply model for vertebrate animal studies (Wang et al., 1998; Scott and Stainier, 2002; Sehnert and Stainier, 2002). Regeneration of the zebrafish heart appears to differ from that described in other tissues, including the newt limb and zebrafish fin, where differentiate cells (muscle, cartilage, and skin) adjacent to the wound site, first dedifferentiate to form a blastema or mass of pluripotent cells, which then give rise to a fully formed and patterned limb or fin (Scott and Stainier, 2002; Biga and Goetz, 2006). The formation of the blastema that regenerates these tissues requires successive process of dedifferentiation, transdifferentiation, and pattern formation. The zebrafish myocardium is a relatively simple structure, composed of one major cell type, so the initial step of dedifferentiation observed during limb regeneration may not be required for cardiac

regeneration (Stainier et al., 1993; Glickman and Yelon, 2002). By following the zebrafish regenerative capacity, it is possible to elucidate the transcriptional mechanisms during the differentiation, re-differentiation of tissue or simply differentiation of tissue-resident adult stem cells. The analysis of regeneration in zebrafish, by using cellular and molecular techniques, provides a unique instrument for achieving this goal.

In this study it has been assessed some important genetic, molecular, and protein mechanisms involved in cardiac regeneration in zebrafish. Here, it has been demonstrated that microRNAs (miRNAs), genome encoded small RNAs involved in many biological processes (Carrington and Ambros, 2003; van Rooij & Olson, 2007), are differently expressed during heart regeneration and in hypertrophic conditions. Cardiac hypertrophy, the normal response of mammalian hearts to injury or hypoxia, consists of enlargement of cardiomyocytes and hyperplasia of other cell types in the heart, such as fibroblasts (Poss et al., 2002). Here, for the first time, hypertrophy was induced in zebrafish hearts cultured e*x-vivo,* using phenilephrine (PE)*,* and it has been demonstrated that hypertrophy alters the cardiac miRNAs levels. Interestingly, the qRT-PCR analysis of the hearts treated for 72h with 500 μM of PE revealed a significant decrease of miRNAs, whereas 48h of treatment provoked a decrease-trend that resulted not statistically significant. The major decrease was observed for miR-133b where the expression was detected about the 75% less as respect to controls, whereas the other miRNAs, miR-1 and miR-133a had a decrease as compare with controls. In mice and human the down-regulation of miR-133 had a critical role in determining cardiomyocytes hypertrophy after *in-vitro* treatment with PE of neonatal cardiomyocytes (Carè et al., 2007).

It has been demonstrated that zebrafish is a highly useful biological model to understand also the cardiac regeneration process (Poss et al., 2002); in fact after a surgical amputation of around 20% of the ventricle apex, muscular tissue results completely restored, in a process that involves changing in genes, microRNAs and proteins expression. Other works showed that zebrafish regenerates complex tissue, including fin, and during these processes occur large scale changes in gene expression programs to execute the rapid transformation of quiescent, differentiated tissue to proliferating, actively patterned tissue (Lien et al., 2006; Schebesta et al., 2006). Here, for the first time, it was investigated the miRNAs expression during regeneration. It has been shown that miRNAs are involved in basic cell functions and differentiation, including cardiac myocytes (Zhao et al., 2007). Also, it is known that miR-133 and miR-1, which are included in the same bicistronic unit, are

specifically expressed in skeletal muscle and cardiac myocytes (Chen et al., 2006; Carè et al., 2007;). Notably, miR-133 and miR-1 play key roles in skeletal myoblast proliferation and differentiation, respectively. qRT-PCR experiments revealed a clear down-regulation of miR-1, miR-133a and miR-133b, reaching the lowest expression at 3 days post amputation (dpa). Thus, the data exerted by this research seems to indicate that the activation of genes responsible of the regeneration program are linked with the down regulation of the most important miRNAs of the vertebrate heart. Similarly in mammals, the role of these miRNAs in the myocytes proliferation and differentiation seems to be of negative regulation (Callis and Wang, 2008). On the light of the zebrafish data, further studies will be needed to outcoming the mechanisms that provoked this miRNAs down-regulation, as for example, if exists a gene sequence that encodes for a cis(trans)-transcriptional factor with silencing role of the miRNAs transcription.

Using *In Situ* Hybridization (ISH) experiments, it was shown that during the *in-vivo* regeneration process many FGFs target genes, as *dusp6, erm, pea3, raldh2*, and *sef*, were induced starting from 3 hours post amputation (hpa), especially close to the site of resection. Between 6 and 12 hpa there is a gene activation of all heart surface suggesting that the surgery causes a response of all organ and that FGFs are implied in the regeneration process. At 1 dpa all the genes studied, are expressed only at the site of injury, suggesting that the genetic response to the injury is very rapid and that all the epicardium of the whole heart is activated to react. At 2 dpa is visible only the expression of *etv5* and *sprouty4* (*Spry4*), showing that they are activated later respect to the other genes and that they remain on for longer time. Tsang et al., (2004) identified zebrafish *dusp6* from a random *in situ* screen that showed similar expression to *fgf3*, *fgf8*, the Ets transcription factors *erm* and *pea3*, and the FGF inhibitors *sef* and *spry*. Sugarman, (2007) showed that although adult mammalian cardiomyocytes show very little or no proliferation when cultured, FGF-1 treatment concomitant with p38 MPA kinase inhibition can stimulate their proliferation in culture; for this reason it is reasonable to think that FGFs are involved in cardiac regeneration process. Also, Lepilina et al., (2006) demonstrated that after the surgery, FGF factors are involved in the formation of a blastema and in the induction of developmental markers. Comparative gene expression analysis revealed significant common gene expression changes in larval fin, adult caudal fin, and heart regenerating tissues, suggesting common molecular pathways choreographing the regeneration process (Poss et al., 2000; Lien et al., 2006; Schebesta et al., 2006). The physiological progression of fin regeneration in larvae and adults is similar, as

both initiate with the formation of a wound epithelium, blastema formation, and the distal to proximal propagation of cell proliferation (Poss et al., 2000; Akimenko et al, 2003).

Some molecular mechanisms are likely to be shared by embryonic cardiogenesis and adult cardiac regeneration (e.g., myocardial differentiation programs) (Raya et al., 2004). However, heart regeneration invokes multiple distinct and definitive events, including (1) initiation by injury, (2) activation of quiescent tissue, (3) ostensibly local development as opposed to organ-and organism wide development, (4) simultaneously healing and growth, (5) morphogenesis on large scale to form adult tissue, and (6) possible long-term maintenance or de novo creation of progenitor cells.

In this study zebrafish hearts were cultured *ex-vivo* with L15, and they were able to contract for several months. The mean rate of the adult male zebrafish is 151±30 beats/min (Milan et al., 2006). Unlike the human counterpart, in response to ventricular amputation 2 and 4 dpa ventricular tachycardia or fibrillation was not observed (Sun et al, 2009b) Atrial arrhythmia was recordered only after prolonged sedation. Here, in *ex-vivo* cultures cardiac cells were able to duplicate, showing incorporation of BrdU, but the regeneration process was not as efficient as *in-vivo*; in fact the clot was still present at 30 dpa. The failure of regeneration can be caused by the fact that an abundance of cells migrates from the heart. In some instances, after 5 days around the cultured hearts visible fibroblasts were observed and enabled organ attachment to the plate. Even adding singular or combination of growth factors to the culture media, the *ex-vivo* process was not efficient as compared to *in-vivo* regeneration. The regeneration process in *ex-vivo* is retarded with respect to the natural process that occurs *in-vivo* because (RA) dehydrogenase (*raldh2)* starts to be expressed at 7 dpa. Expression of *raldh2* is regulated by Wnt and fibroblast growth factor/ERK signaling. Lepilina et al., (2006) proposed that the retinoic acid *Raldh2* is involved in RA synthesis, which is required for early chamber specification and anterior-posterior cardiac patterning in mouse, chick, and zebrafish, is important for myocardial maturation and is targeted by miR-138 (Stainier and Fishman, 1992). Since FGFs display pleiotropic actions and are appealing candidates for signals it is difficult to exactly determine their time of action. A significant diminution of the clot size was detected if the heart was removed from the fish at 7 dpa, instead of at 1 dpa, and then cultured *ex-vivo* with BME+L15, suggesting that the fist days after the surgery are critical for the regeneration process and that a lot of growth factors are involved. Also, in all cases $BrdU^{+}$ cells were localized not only near the cut site, but also in the total surface of the heart,

showing that there is an activation of all the heart and a response the entire organ to replace the lost tissue.

For the first time using a two dimensional gel electrophoresis (2DE), here, were identified more than one hundred proteins of the adult zebrafish heart that serve different functions. These include energy metabolism, structural, motility, signal transduction pathway, cell surface receptors, transcription factors, globin family and novel proteins. The highest numbers of proteins identified were metabolic factors followed by novel/predicted proteins. In all cases it was noticed a high level of post-transcriptional modifications. More interesting was the identification of proteins with functions of signaling, receptor class, and transcription factors, because if altered, they are often associated with diseases and pathology.

Using Differential gel electrophoresis (DiGE) followed by mass spectrometric analysis, it was possible identify proteins differentially expressed at 3 dpa. Three proteins (ATP synthase, hyaluronan mediated motility receptor, and desmuslin) were up-regulated in the amputated heart compared with the uninjured hearts. Here it was demonstrated that the surgery caused not only alteration in miRNAs expression, but also alteration of proteins. Regeneration involves the deployment of a complex set of *de novo* mechanisms including dedifferentiation of post-mitotic cells, cell proliferation, pattern generation and, in some cases, trans-differentiation of adult specialized cells to rebuild parts of the body plan after amputation or injury. The three proteins resulted up-regulated in the hearts at 3 dpa are involved in the energy metabolism, in the signaling and in maintaining the structure integrity of the cells, so it is reasonable to think that they are involved in the cardiac regeneration process too. Here, it was demonstrate the utility of the DiGE technique as a tool for analyzing the *in-vivo* heart regeneration process in the model organism zebrafish. Indeed, proteome studies have identified important protein sets that are involved in the molecular mechanisms of the zebrafish heart regeneration. This might consequently lead to the definition of biomarkers correlated with human heart and can give the possibility to find novel therapeutic targets for the heart failure.

Future studies could be directed to isolation and characterization of zebrafish stem cells involved in the heart regeneration. Also, it will be interesting to understand if they are already present in the heart or if they come from different organs and tissues and are activated to respond to the injury in the heart. It will be also interesting try to apply the protocol generated in mouse and human by Messina et al., (2004) to generate zebrafish *in-vitro* cardiospheres, tridimensional agglomerations of cardiac stem cells (CSCs), able to generate electric

impulses. In fact, CSCs and Cardiospheres are a small fraction of the total cells residing in the heart that could be used for autologous heart repair. Multipotent cardiovascular stem and progenitor cells have been found in the fetal heart, which give rise to cardiomyocytes, smooth muscle cells and endothelial cells. The comparisons of the different molecules expressed in cardiospheres within the species will help understanding how restore cardiac functions after damage or injury and will constitute a base to generate drugs for myocardial infarction therapy. Future studies are also likely to take advantage of lineage tracing tools that have been utilized in mice for progenitor cell studies, to define progenitor/progeny relationships during heart regeneration. Also, it will be important to examine new cardiac injury models in zebrafish. In fact, the current model produces a mechanical injury. While this injury might indeed stimulate the strongest possible regenerative response, it will also be interesting to better mimic the infarct models utilized in mammals. Additional molecular studies in the future will increase the resolution of regenerative pathways and bring the idea of cardiac regeneration in humans closer to reality.

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PUBBLICATIONS IN INTERNATIONALS JOURNALS

Manuscripts in preparation

1. *Missinato M.A.*, *Ceci M.*, *Contu R., Condorelli G.*, *Messina E., Tsang M., Romano N.* microRNAs expression in zebrafish (*Danio rerio*) hypertrophy-induced and regenerating hearts.

2. *Missinato M.A.*, *Tsang M., Romano N.* Genes expression during zebrafish heart regeneration *in-vivo* and *ex-vivo.*

3. *Missinato M.A.*, *Carroll J.*, *Romano N.*, *Tsang M.* Proteomic of the adult zebrafish heart.

4. *Missinato M.A.*, *Carroll J.*, *Romano* N., *Tsang M.* Proteomic of the zebrafish heart during the *in-vivo* regeneration process.

Abstract

- **GEI Rome, June 4-7, 2008,** Piergentili R., Ficca A.G., Caccia E., Ceccarelli G., Missinato M.A., Mastrolia L., Romano N. "Clonaggio ed espressione del gene catena gamma del recettore dei linfociti T nella spigola, *Dicentrarchus labrax*.

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