Nadia Mercader Huber

CURRICULUM VITAE

Name:	Nadia Mercader Huber
Date of birth:	25 October 1974
Place of birth:	Zurich, Switzerland
Nationality:	Swiss, Spanish
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Education

2003	PhD in Biology, Universidad Autónoma de Madrid, Madrid, Spain
1998	Degree in Biology, Swiss Federal Institute of Technology (ETH) - Zürich, Switzerland

Employment History

Since 2020	Full Professor in Anatomy, Developmental Biology and Regeneration Visiting Professor at CNIC, Madrid, Spain
2015–2020	Associate Professor in Anatomy, Developmental Biology and Regeneration Visiting Professor at CNIC, Madrid, Spain
2014–2015	Assistant Professor Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain
2007–2014	Junior Scientist – Department of Cardiovascular Development and Repair – CNIC, Madrid, Spain
2004–2007	Postdoctoral fellow – EMBL Heidelberg, Germany
2003–2004	Postdoctoral fellow – Centro Nacional de Biotecnología CNB-CSIC, Madrid, Spain
1998–2003	PhD Student – CNB-CSIC-Group Miguel Torres, Madrid, Spain

Institutional responsibilities

2020	Vicedean of research Medical faculty, University of Bern (4 year mandate)
Since 2015	Co-director of the Institute of Anatomy, University of Bern, Head of Section Developmental Biology and Regeneration

Current research grants

- H2020-SC1-2019-Single-Stage-RTD REANIMA-874764. New-generation cardiac therapeutic strategies directed to the activation of endogenous regenerative mechanisms. (2020–2024). Co-PI
- ERC Consolidator grant 819717 – TransReg (2019–2023). PI
- SNF Project ForceInRegeneration. 310030L_182575. Deciphering the influence of biomechanics and mechanotransduction during cardiac regeneration in normal and pathological contexts (2019–2021). PI
- HSFP RGP0016/2018 Handling OXPHOS structural heterogeneity and metabolic plasticity (2019–2021). Co-PI
- European Industrial Doctorate Program H2020-MSCA-ITN-2016 4DHeart 722427 (2017–2021). Scientific Coordinator.
- Scherbarth Foundation. The zebrafish as tool for personalized diagnosis of human cancer cell behaviour (2019–2021). Co-PI.

Reviewing activities and participation on evaluation boards

Since 2019	Head of grant committee from Bern Centre of Precision Medicine BCPM (2 year mandate)
Since 2018	Member of the SNSF Commission University of Bern

Evaluation of research projects and fellowships for the Spanish Agency ANEP, the French Agency ANR, The British Heart Foundation, the European Research Council. N. Mercader acts as reviewer for several journals including Circulation, Cell Death Diff, Current Biology, eLife, Development, Developmental Cell, PNAS, PlosOne, Science, Stem Cell Reports, Cell Reports.

Organization of conferences

As main organizer: 10th International Workshop on Cardiomyocyte Biology, Ascona 2021, 10th Swiss Zebrafish Conference 2017, UNIA Workshop 2014 “Cardiovascular Extracellular Matrix in health and disease”, CNIC conference 2013 “Cardiovascular Development, Homeostasis and Repair”.

As Co-Organizer: CNIC conference on Heart regeneration 2020 (postponed); 9th International Workshop on Cardiomyocyte Biology, Ascona 2018; CNIC conference 2016 "Mechanical forces in physiology and disease"; EMBO conference 2016 "The molecular and cellular basis of Regeneration and Tissue Repair", MIC-Symposium Cells in Motion 2016; Weinstein Conference on Cardiovascular Development 2013.

Teaching activities
Coordinator of the subject Embryology and Genetics at Medical Faculty University of Bern and giving Lectures and Tutorials, Lecturer in practical Histology Courses, Professor responsible for the Cutting Edge Microscopy PhD Program at University of Bern (2017–2020).

Awarded prizes and fellowships
Pexeider Prize 2011 for the best oral presentation, ESC Working Group of Anatomy and Embryology Meeting, Liblice.
Ramón y Cajal fellow (2007–2011)
Postdoctoral fellowship Ministerio de Educardion y Ciencia (2006–2007)
EMBO Long Term Postdoctoral fellowship (2004–2006)
Marie Curie PhD fellowship-SNF Internationale Austauschprogramme (1999–2002)

Mentoring
Current members: 5 postdoctoral researchers (Inês Marques, Indre Piragyte-Langa, Myra Chávez, Alexander Ernst, Carolina García-Poyatos), 4 PhD Students (Eleonora Lupi, Benedetta Coppe, Joao Carvalho, Marius Botos).

Past PhD and Postdoctoral fellows: Marina Peralta (PhD in 2013, received Marie Curie fellowship to join IGBMC), Juan Manuel González-Rosa (PhD in 2014, received EMBO fellowship, now PI at Harvard Medical School), Héctor Sánchez-Iranzo (PhD in 2015, received EMBO fellowship, starting own research group in Januray 2021 at KIT, Germany), Laura Andrés-Delgado (Postdoctoral fellowship from Spanish Ministry of Science and Competitiveness from 2013 to 2015, now lecturer at Universidad Autónoma de Madrid), Marcos Sande (PhD in 2019), Andrés-Sanz Morejón (MSc in 2015, obtaining Spanish national Arquímedes Prize for Best Master Thesis, PhD in 2020, obtained SNSF early postdoc mobility grant to work at EMBL Heidelberg).

SELECTED PUBLICATIONS

Sande-Melón M, Marques IJ, Galardi-Castilla M, Langa X, Pérez-López M, Botos MA, Sánchez-Iranzo H, Guzmán-Martínez G, Ferreira Francisco DM, Pavlinic D, Benes V, Bruggmann R, **Mercader N.** Adult sox10(+) Cardiomyocytes Contribute to Myocardial Regeneration in the Zebrafish. ***Cell Rep.*** 2019 Oct 22;29(4):1041-1054.e5. doi: 10.1016/j.celrep.2019.09.041. PubMed PMID: 31644901; PubMed Central PMCID: PMC6856760.

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Andrés-Delgado L, Ernst A, Galardi-Castilla M, Bazaga D, Peralta M, Münch J, González-Rosa JM, Marques I, Tessadori F, de la Pompa JL, Vermot J, **Mercader N.** Actin dynamics and the Bmp pathway drive apical extrusion of proepicardial cells. ***Development.*** 2019 Jul 4;146(13). pii: dev174961. doi: 10.1242/dev.174961.

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Kovacic JC, **Mercader N,** Torres M, Boehm M, Fuster V. Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: from cardiovascular development to disease. ***Circulation.*** 2012 Apr 10;125(14):1795-808.
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Peralta M, Steed E, Harlepp S, González-Rosa JM, Monduc F, Ariza-Cosano A, Cortés A, Rayón T, Gómez-Skarmeta JL, Zapata A, Vermot J, **Mercader N.** Heartbeat-driven pericardiac fluid forces contribute to epicardium morphogenesis.
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González-Rosa JM, Martín V, Peralta M, Torres M, **Mercader N.** Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. ***Development.*** 2011 May;138(9):1663-74. doi: 10.1242/dev.060897. Epub 2011 Mar 23.

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HEART DEVELOPMENT AND REGENERATION IN THE ZEBRAFISH

Nadia Mercader Huber

Abstract

In humans, myocardial infarction results in ventricular remodeling, progressing ultimately to cardiac failure, one of the leading causes of death worldwide. In contrast to the adult mammalian heart, the zebrafish model organism has a remarkable regenerative capacity, offering the possibility to research the bases of natural regeneration. In our group, we have investigated the cellular and molecular mechanism of heart regeneration in the zebrafish. We further also take advantage of this model organism to study embryonic development of the heart. Understanding the developmental processes of heart formation might help to unravel the causes of congenital heart disease. Here, I will summarize some of our contributions to this research field.

Introduction

The heart is among the first organs to acquire its function. Long before its development is completed, it starts beating, and puts in motion the blood flow, controlling in this manner the overall progression of the organism's development. Blood flow is important not only because it promotes oxygenation of the embryonic tissues. Blood flow forces themselves act as biomechanical signals sensed by endothelial cells of the vasculature and the endocardium, the inner lining of the heart. In this manner, the heartbeat induces, for example, the development of the cardiac valves. Congenital heart defects are among the most common type of birth defects and can have both environmental and genetic underlying causes. They can have early phenotypic consequences, but they can also manifest only later in the adult. A good understanding of cardiac development constitutes therefore an essential asset to promote and preserve health.

Apart from inherited predispositions, environmental stressors and nutritional habits can impact cardiovascular health during adulthood. Heart failure is among the leading causes of death worldwide. Coronary artery occlusion, for example as a consequence of atherosclerosis, can lead to myocardial infarction (MI) and millions of cardiac muscle cells can die as a result of interrupted blood flow. Fortunately, rapid intervention protocols are currently significantly reducing the mortality after MI. Nonetheless, reperfusion injury after ischemia still poses an important stress to cardiomyocytes, leading to their elimination. As a reaction to the lost myocardium, cardiac fibroblast start proliferating and producing extracellular matrix (ECM). This fibrotic response avoids cardiac wall rupture and is therefore lifesaving. However, the surplus of fibroblasts has a long-term detrimental effect. The cardiac muscle loss affects cardiac contractility and fibrotic tissue disrupts electrical propagation. As a response, remaining cardiomyocytes that now need to work more undergo hypertrophy, and secondary interstitial fibrosis occurs throughout the heart. These events, altogether defined as ventricular remodeling, lead to arrhythmias and cardiac dysfunctions ultimately leading to heart failure.

The zebrafish is an excellent model to study cardiovascular development (Figure 1). Zebrafish *(Danio rerio)* are small freshwater ray-finned fish that reach around 3 cm in length. They display characteristic horizontal blueish and white stripes along the trunk and fins. Their natural habitat is the Ganges basin in Northern India and Bangladesh. It became a popular animal model in research around 1980 thanks to the work of George Streisinger, a researcher who originally held zebrafish as a pet and then converted it into an experimental model. An important boost for the model occurred when geneticists such as Nobel Prize Winner Christiane Nüsslein-Vollhardt used the facts that large clutches of animals are easily available, that embryo development is rapid and that zebrafish embryos are transparent allowing to visualize development of all organ primordia, to perform mutagenesis screens in the zebrafish. This led to the identification of a multitude of new genes leading to developmental alterations and, in this way, allowed the study of gene function during the formation of body parts and organs. Importantly, more recently, the sequencing of the zebrafish genome confirmed that over 70% of genes are conserved between humans and zebrafish. As such, the zebrafish repre-

sents an excellent model to interrogate function of genes involved in human disease progression. Furthermore, imaging technologies and genome editing nowadays even allow for the study of gene function and organ development in vivo at the single cell level.

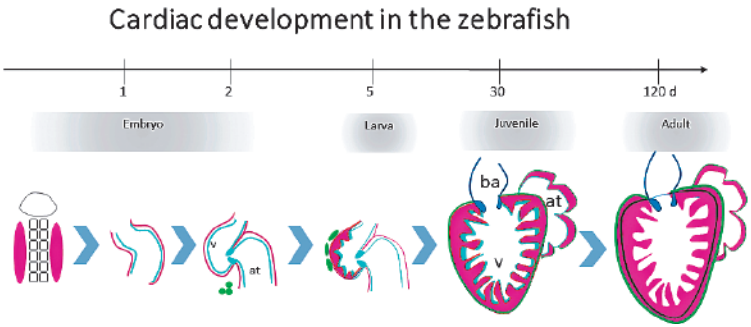

Figure 1. Cardiac development in the zebrafish. *Cardiac precursors derive from the lateral plate mesoderm that migrate rostrally and fuse to form a primordial heart tube during the first 24 hours after fertilization (24 hpf). The heart tube is formed by the cardiac muscle (myocardium, pink) and an inner endocardial layer (blue). Heartbeat starts at 25 hpf. Subsequently, the heart tube loops, forming the atrial and ventricular chamber, connected by the atrioventricular valve. At that time, a third layer is added to the heart, the epicardium. Epicardial precursors derive from the proepicardium (green cells) that detach from the pericardial wall and reattach to the myocardial surface. Around 5 days postfertilization (5 dpf), the heart becomes trabeculated. A bulboventricular valve starts forming. In juveniles a third outer myocardial layer is added by trabecular cardiomyocytes that breach the initial primordial layer and form the cortical layer.at, atrium; ba, bulbus arteriosus; v, ventrile.*

While initially the attention to the zebrafish was centered on its role as a model in developmental biology, the work from Ken Poss and others incorporated the zebrafish as a central vertebrate animal model to understand organ regeneration (Figure 2). While in humans, the loss of cardiomyocytes upon MI or other cardiovascular events leads to the irreversible loss of cardiomyocytes, the zebrafish has the extraordinary capacity to renew the lost myocardium in response to injury. Since this initial observation, research has advanced to understand the underlying mechanisms.

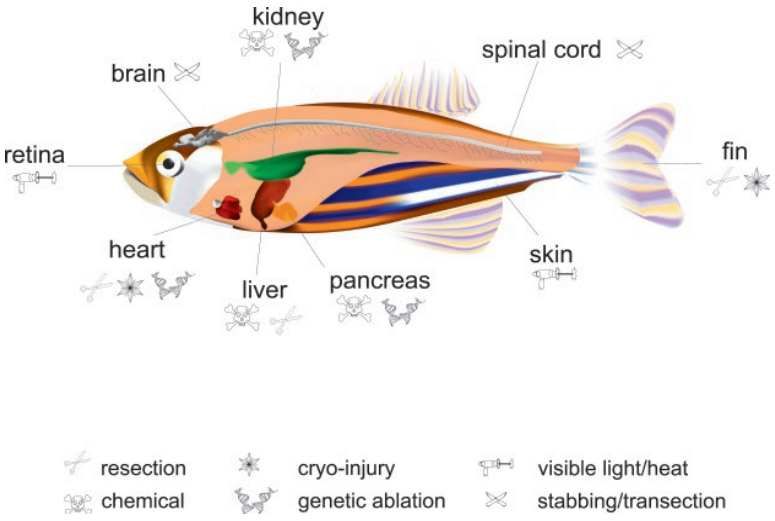

Figure 2. Organ regeneration in the zebrafish. *Highlight of some of the organs and tissues used for regeneration studies in zebrafish. Commonly used injury models are marked for each organ. Adapted from (Marques et al., 2019).*

Results

Zebrafish as a model to study epicardium formation

During heart development, the epicardium is the last layer to be added. The epicardium plays important roles in cardiac development and homeostasis, as a source of progenitor cells for the coronary vasculature and cardiac fibroblasts as well as a source for signalling molecules influencing myocardial growth (Quijada et al., 2020). Several hypotheses were put forward to understand the development of this outer mesothelial layer. Initially, it was thought that the outer myocardial cells transdifferentiate into epicardial cells. Examinations in several species revealed the presence of a cluster of cells close to the inflow tract of the heart that expressed epicardial marker genes. This structure was defined as the proepicardium. Two further hypotheses were discussed: (1) proepicardial cells are transferred to the myocardium to form the epicardium through a cellular and ECM based bridge or, (2) proepicardial cell clusters detach from

the proepicardium, are released into the pericardial cavity and subsequently attach to the heart. To prove any of the hypotheses, in vivo imaging is necessary. Therefore, we decided to make use of the zebrafish model to interrogate the mechanisms of epicardium formation (Figure 3). We first generated a transgenic reporter line in which green fluorescent protein (GFP) is expressed specifically in the proepicardium and epicardial cells. Next, we used this line to image proepicardium and epicardium formation in vivo. Anaesthetized zebrafish were imaged using high-speed confocal imaging between 48 and 60 hpf, the time point of proepicardium formation and the time when first epicardial cells start to be present. We found that proepicardial cells indeed detach from the dorsal pericardium and are released into the pericardial cavity. They are advected for several minutes in the pericardial cavity until finally adhering to the myocardial surface. This process continues until most of the myocardium is covered. Importantly, blocking the heart beat impaired advection of cells from the proepicardium to the myocardium (Peralta et al., 2013). In this manner, we found that the heartbeat promotes morphogenesis not only by putting in motion the blood flow, but also by promoting pericardial flow forces outside the heart. In a follow up work, we wanted to understand in more detail how proepicardial cells emerge from the pericardial mesothelium. We found that cells delaminate from the mesothelium through apical extrusion, a process that had been extensively studied in the context of epithelial homeostasis but not much in the context of embryonic development. Here we found that, rather than being eliminated, as happens during epithelial homeostasis, extruded proepicardial cells survive and form the epicardium upon attachment to the myocardium (Andres-Delgado et al., 2019). Again, *in vivo* imaging was crucial to make this discovery.

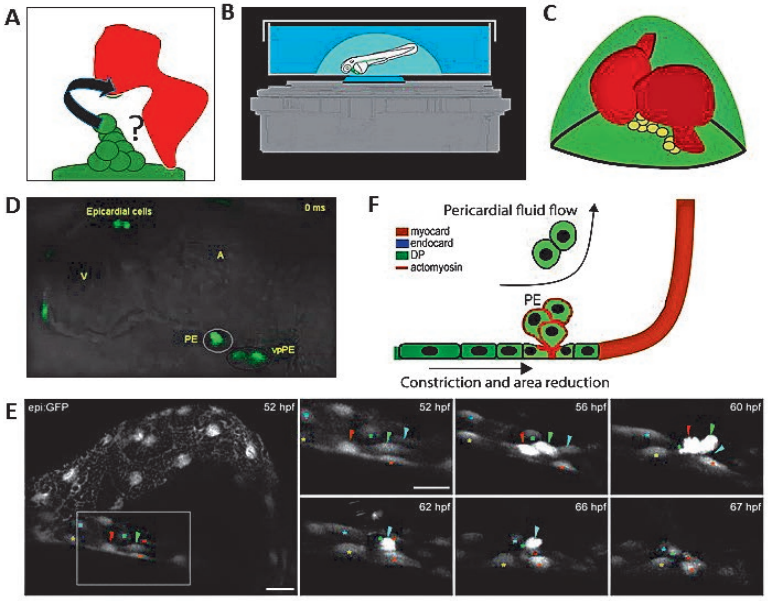

Figure 3. Cellular mechanism of epicardium formation. A, *Question to be answered: how are proepicardial cells transferred to the heart? Heart tube is shown in red, proepicardial cells in green.* ***B,*** *set up for in vivo imaging of epicardium formation using the zebrafish embryo.* ***C,*** *Schematic representation of the heart tube within the pericardial cavity. Proepicardial cells are shown in green. Shown is a frontal view.* ***D,*** *Frame of a video of the epi:GFP line used to image epicardium formation. epi:GFP positive cells are shown in green.* ***E,*** *epi:GFP in vivo time lapse. Individual cells are marked with dots of different colors. PE cells that extrude are marked with an arrowhead.* ***F,*** *Scheme of proepicardium delamination. PE cells are extruded apically into the pericardial cavity in a process that required actin and myosin II. Pericardial flow allows transfer of the cells to the myocardial surface. A, atrium; PE, proepicardium; vpPE, venous pole proepicardium, V, ventricle.*

How does a zebrafish regenerate the heart?

The zebrafish heart is built up similarly to the human heart, with some obvious difference (Figure 4). It is formed by a single atrium, a single ventricle and a prominent outflow tract named bulbus arteriosus. The chambers are separated by an atrioventricular as well as a bulboventricular valve. As in humans, zebrafish hearts are formed by myocardium –

the cardiac muscle – an inner lining of endocardium and an outer epicardium. The myocardium is highly trabecular and as in mammals, contains cardiac fibroblasts. The heart is irrigated by a poorly developed coronary

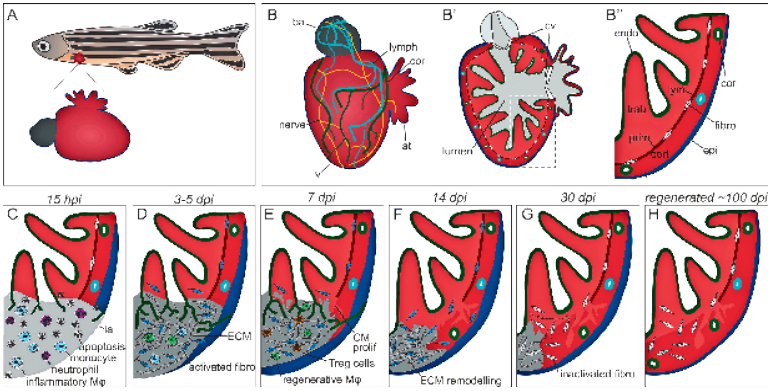

Figure 4. Representation of cardiac regeneration in the adult zebrafish. ***A,*** *Adult zebrafish heart anatomical position.* ***B,*** *Overview of the uninjured zebrafish heart, comprising the atrium, ventricle and bulbus arteriosus. The heart is covered and wired by the epicardium, lymphatic system, coronary arteries, and nerves.* ***B',*** *Section of the zebrafish heart. Cardiac valves separate the chambers.* ***B",*** *Zoomed region of B'. Three myocardial layers can be identified: trabecular, primordial, and cortical myocardium. The endocardium coats the lumen. The cortical layer is covered by the epicardium. Fibroblasts lie between the cortical and trabecular myocardium.* ***C–H,*** *Timeline of cardiac regeneration events upon cryoinjury.* ***C,*** *Fast freezing of the ventricular apex leads to the formation of the injury area. Necrotic and apoptotic cells trigger an inflammatory response characterized by the infiltration and activation of neutrophils, monocytes, and macrophages, among others. Endothelial and epicardial cells are activated and infiltrate the injury area.* ***D,*** *The acute inflammation regresses and activated fibroblasts elicit a fibrotic response by depositing extracellular matrix (ECM).* ***E,*** *Peak of cardiomyocyte proliferation followed by migration along epicardial and endocardial cells.* T_{reg} *cells home to the injured tissue.* ***F,*** *The ECM remodels, and cardiomyocyte proliferation continues.* ***G,*** *Fibroblasts undergo inactivation and the fibrotic scar regresses.* ***H,*** *Complete regression of the fibrotic scar and replenishment by functional myocardium. The cortical myocardial layer remains thickened and the primordial layer does not regenerate. Abbreviations: at, atrium; ba, bulbus arteriosus; CM prolif, cardiomyocyte proliferation; cor, coronary arteries; cv, cardiac valves; ECM, extracellular matrix; epi, epicardium; endo, cardiac endothelium; dpi, days post injury; fibro, fibroblast; ia, injury area; hpi, hours post injury; lymph, the lymphatic system; MΦ, macrophage; prim, primordial layer; trab, trabecular layer; v, ventricle. Picture from (Sanz-Morejon and Mercader, 2020).*

vasculature, as well as a lymphatic system and is innervated by sympathetic and parasympathetic nerve fibres.

As a first response to injury, the epicardium and endocardium start to re-express developmental genes and proliferate. The epicardium undergoes epithelial-to-mesenchymal transition and inflammatory cells home to the heart. The damaged muscle becomes replaced by fibrotic tissue. Simultaneously, CMs initiate proliferation and regenerate the injured myocardium, while the transiently deposited extracellular matrix (ECM) gets eliminated (Sanz-Morejon and Mercader, 2020).

The initial model to study heart regeneration in the zebrafish was based on resection of the ventricular apex. In this model, ¼ of the ventricle is amputated with dissection scissors in the anaesthetized animal (Poss et al., 2002). After the initial formation of a fibrin clot the heart heals within 30 days, including the regeneration of epicardial layer, the myocardium and endocardium. We wondered whether zebrafish would also be able to regenerate upon a different lesion, involving tissue damage rather than tissue loss. We reasoned that this could be to some extent more similar to a pathological situation. Therefore, we established ventricular cryoinjury as a protocol to damage the zebrafish heart (González-Rosa and Mercader, 2012). Injuring the cardiac ventricle by freezing was developed simultaneously in our group as well as the groups of Anna Jazwinska and Gilbert Weidinger (Chablais et al., 2011; González-Rosa et al., 2014; Schnabel et al., 2011). Using this model, we found that, similar to cardiac resection, zebrafish could also regenerate the heart upon tissue damage. However, we noted that regeneration took longer, around double time being complete at 130 days postinjury (dpi). An important second difference to resection was that a massive fibrotic response preceded myocardial regeneration (Figure 5). This simple observation was very important, because it revealed that fibrosis is not blocking regeneration, a common belief among scientists as well as clinicians at the time. Moreover, it showed that fibrosis is not irreversible in the zebrafish. That means that the capacity to regenerate in the zebrafish is not relying on not producing fibrosis but, at least among others, to get rid of the fibrotic tissue.

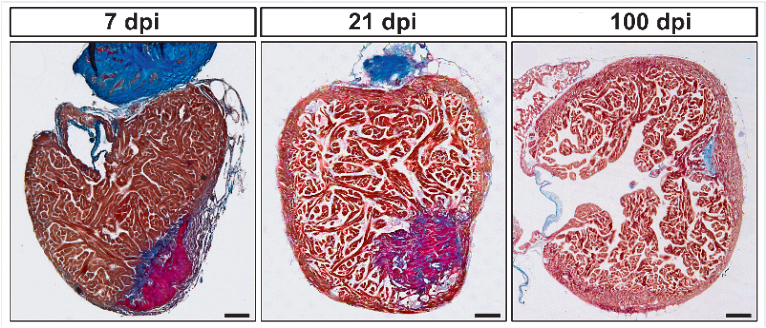

Figure 5. Cardiac regeneration and fibrosis regression upon cryoinjury of the cardiac ventricle in the zebrafish. *AFOG histological stainings on heart sections at 7, 21 and 100 days post cryoinjury (dpi). Myocardium is stained brown, collagen, blue and granular tissue and fibrin red. Note that at 7 dpi, the apex of the ventricle exhibits granular tissue and collagen staining. At 21 dpi, a new myocardial outer layer has been formed engulfing the injured area. At 100 dpi only small remnants of collagen deposits are visible. Pictures adapted from (Gonzales-Rosa et al., 2011).*

Fibrosis as a process compatible with regeneration

We next were interested in understanding where the fibrotic tissue was coming from as, at that time, there had not been any reports on the presence of fibroblasts in the zebrafish heart. So, which cells are generating ECM upon injury in the zebrafish? Are there yet to be identified fibroblasts in the zebrafish heart or are other cell types generating ECM in this species? I was extremely lucky to get funding from the European Research Council through an ERC Starting Grant which enabled us to tackle these questions (337703 "zebraHeart"). First, we set up to analyse the expression of some genes known to mark fibroblasts in humans and other animal models such as the mouse. One gene caught our attention, namely *periostinb (postnb).* It was strongly upregulated in response to injury. We regenerated a transgenic reporter line, to study the dynamics of *postnb* expression as well as the transcriptional profile of *postnb*-positive cells. We found that indeed, *postnb*-positive cells had a transcriptional profile reminiscent of fibroblasts. Interestingly, in the zebrafish, the cells were not only expressing genes related to ECM production and remodelling but also genes related to vasculogenesis and neurogenesis, also includ-

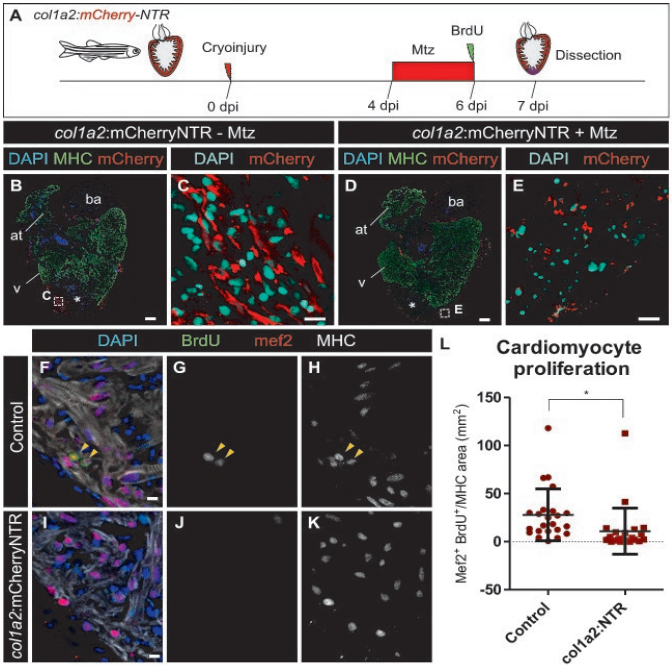

Figure 6. Genetic ablation of collagen 1a2 expressing cells impairs cardiomyocyte proliferation in the cryoinjured heart. A, *Schematic illustration of experimental set up. A transgenic line using a* col1a2 *regulatory sequences from a BAC to drive the expression of nitroreductase (NTR) fused to mCherry was used for this experiment. Adult animals were cryoinjured and treated with Metronidazol (Mtz) from 4 to 6 days postinjury (dpi). Mtz administration leads to cell death of NTR expressing cells. BrdU injection was performed one day prior to fixation to assess cardiomyocyte proliferation.* ***B–E,*** *Immunofluorescence on heart sections of* col1a2:mCherry-NTR *treated with Mtz (B,C) or untreated controls (D,E). C and E are zoomed views of panels B and D, respectively. mCherry is shown in red, myosin heavy chain (MHC) in green and nuclei (DAPI) in blue for B and D, and in cyan for C and E. Note that in Mtz-treated fish,* col1a2:mCherry-NTR *labels cells with fragmented nuclei and the homogeneous expression as shown in the WT heart is lost.* ***F–K,*** *Immunofluorescence using anti-mef2 (red) and anti-MHC (white) to mark cardiomyocytes and anti-BrdU (green) in* col1a2:loxP-tagBFP-loxP-mCherry-NTR *(control) and* col1a2:mCherry-NTR *treated with Mtz and BrdU as described in A. Nuclei are counterstained with DAPI (blue).* ***L,*** *Quantification of BrdU+ cardiomyocytes in* col1a2:mCherry-NTR *and control hearts. Shown are individual measurements as well as median±intercuartile range; *** P=0.0004 by Mann-Whitney test, n = 23 fish per condition, from 2 different experiments. For each point, 3 whole heart sections of a ventricle were quantified. Scale bars, 10 µm (**C,E,F,I**), 100 µm (**B,D**). Figure from (Sanchez-Iranzo et al., 2018b).*

ing several growth factors. This suggested, that not only were fibroblasts not interfering with regeneration, but that fibroblasts might be actively involved in the regeneration process. Indeed, we performed genetic ablation of collagen 1 alpha 2 producing cells and found that cardiomyocyte proliferation was impaired (Sanchez-Iranzo et al., 2018b) (Figure 6).

So where are fibroblasts coming from? We found that fibroblasts derived mainly from pre-existing fibroblasts, but also from the epicardium. The endocardium also contributed to ECM production, but in this case, we observed that cells were not fully undergoing epithelial-mesenchymal transition and often remained attached to each other as an endothelial layer. Later, macrophages were also shown to contribute to ECM deposition in the zebrafish (Simoes et al., 2020).

Further, we used Cre/lox based lineage tracing to study the fate of *postnb*-positive cells in the heart (Figure 7). Fibrotic tissue regresses concomitant with regeneration of the new myocardium. Therefore, we expected to gradually loose *postnb*-positive cells at later stages of regeneration. However, the overall number of *postnb*-positive fibroblasts that accumulated after the first week of injury decreased only slightly and even at late stages, in which regeneration should be complete, we still found a considerable amount of *postnb*-positive cells. Clearly, ECM deposits are removed by then, so, what are fibroblasts expressing at such a late time point? We compared the transcriptome of fibroblasts in uninjured hearts, hearts at 7 days postinjury (7dpi) and hearts at 60 dpi. After completion of regeneration, fibroblast returned to a transcriptional state similar to fibroblasts in uninjured hearts, suggesting that activated fibroblasts initially actively contribute to ECM production and later, rather than being eliminated, remained in a quiescent state. Noteworthy, there were few differences in the gene expression profile between fibroblasts at 60 dpi compared to fibroblasts from uninjured hearts, suggesting that even after complete regeneration, the hearts do not recover completely. Indeed, in line with this observation we had previously noticed that cardiac wall contraction is not fully recovered, maybe partly due to the accumulation of these extra fibroblasts (González-Rosa et al., 2014).

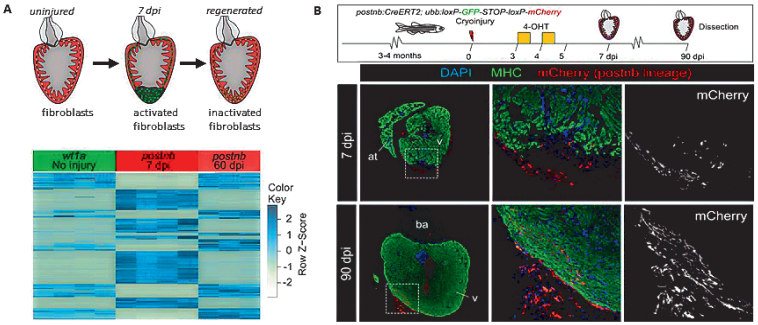

Figure 7. Fate of fibroblasts during heart regeneration. A, wt1a:*GFP allowed labelling of cardiac fibroblasts in the uninjured adult zebrafish heart.* Postnb *expression was used in genetic fate mapping studies of activated fibroblasts in the injured (red, 7dpi) and regenerating (pink, 60 dpi) heart. The transcriptome of* wt1a:*GFP cells was compared to the transcriptome of* postnb-*derived cells at 7 days postinjury (dpi) and 60 dpi. For this, double transgenics* postnCre:ERT2;ubb:Switch *were recombined 3 and 4 dpi and mCherry positive cells FAC-sorted at 7 and 60 dpi. Note that the heat map of* wt1a:*GFP-positive cells is more similar to the gene signature from 60 dpi* postnb-*derived cells than to those from 7 dpi* postnb-*derived cells.* ***B,*** *Lineage tracing of* postnb-*derived cells during heart regeneration. Upper panel shows a schematic representation of the experimental set up and transgenic lines used. Lower panels are immunofluorescence stainings of heart sections and zoomed views of injury area. Note that* postnb-*derived cells (red) are present at 7 as well as 90 dpi, a stage at which regeneration is nearly complete. MHC, myosin heavy chain, marker for myocardium. Image adapted from (Sanchez-Iranzo et al., 2018b).*

Inflammation as a further prerequisite of regrowth

Similar to fibrosis, inflammation has been classically associated with deleterious effects during regeneration, but inflammation is clearly required for wound healing. Indeed, a properly controlled inflammatory response, determines whether a damaged tissue undergoes fibrotic healing or proceeds to regeneration (Godwin et al., 2017b). In a first phase, damage-associated molecular patterns (DAMPS) and pathogen-associated molecular patterns (PAMPS) as well as release of cytokines lead to the accumulation of neutrophils and monocytes. Monocytes further differentiate into macrophages that can be polarized to more pro-inflammatory or more anti-inflammatory phenotypes. Furthermore, tissue-resident macrophages have also been shown to play an essential role in the control of

organ regeneration (Mescher, 2017; Pinto et al., 2014; Wynn and Vannella, 2016). Macrophage depletion by clodronate liposomes treatment leads to the blockage of several regenerative processes such as limb regeneration in the axolotl (Godwin et al., 2013) as well as heart regeneration in the zebrafish, axolotl and neonatal mice (Aurora et al., 2014; Godwin et al., 2017a; Lai et al., 2017).

There is still very little information on macrophage subtypes and macrophage polarization in the zebrafish. Given the accumulated evidence of macrophages in organ regeneration, and that the zebrafish poses such a good model to study regenerative capacity, we sought to investigate the role of macrophages in the cryoinjured heart (Figure 8). We made an initial interesting observation when imaging the epicardium. We found that labelling with the epicardial marker wilms' tumor 1 b (wt1b) also stained a subset of macrophages in the cryoinjured zebrafish heart. We decided to characterize this population more in detail and found that *wt1b*-positive macrophages were transcriptionally distinct from the rest of macrophages (Sanz-Morejon et al., 2019). They differed in the expression of genes related to leukocyte migration, TNF-alpha responsiveness as well as the expression of genes promoting vasculogenesis. Given that migration was one hallmark enriched in *wt1b*-positive macrophages we decided to analyze their migratory capacity in vivo. For this, we switched from the cardiac injury model to the larval fin amputation model, that readily allows in vivo imaging over long time periods at cellular resolution. We found that indeed, *wt1b*-positive macrophages migrated at a lower speed and accumulated more at the site of injury. We also found that *wt1b*-positive macrophages arrived only around 48 h after amputation, while the first macrophages arrive very fast, already within minutes following amputation. This observation is opposite to what has been described for pro-inflammatory *tnf-alpha*-positive macrophages (Nguyen-Chi et al., 2015), suggesting that *wt1b*-positive macrophages represent a population of anti-inflammatory more pro-regenerative macrophages, a hypothesis which is in line with the transcriptome analysis. Next, we were curious to understand if wt1b itself represents only a marker for this macrophage population or, if the gene plays a role in determining part of their phenotype. Firstly, we generated transgenic lines to specifically overexpress a wt1b dominant negative form in macrophages. We found that abrogation

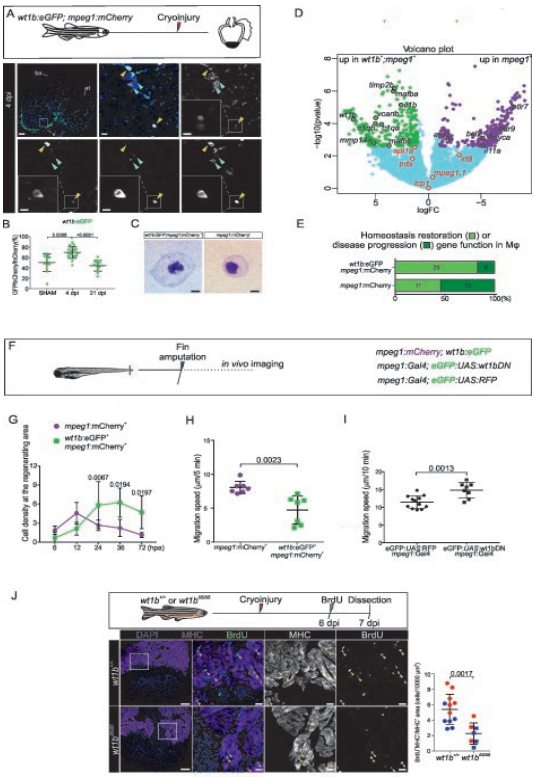

Figure 8. **wt1b-*positive macrophages are involved in heart regeneration in the zebrafish. A–C,*** *Detection by immunofluorescence staining and morphological characterization of* wt1b*:GFP;*mpeg1*:mCherry double positive cells in the uninjured and regenerating zebrafish heart.* ***D–E,*** *RNA-seq analysis of* wt1b*:GFP;*mpeg1*:mCherry compared to* mpeg1*:mCherry positive cells in the injured zebrafish hearts at 4 dpi. Shown is Volcano plot as well as literature search results.* ***F–H,*** *Macrophage migration assay in zebrafish amputated larval fin.* wt1b*:GFP;*mpeg1*:mCherry double positive cells migrate differently and accumulate at later time points at amputation site compared to compared* mpeg1*:mCherry positive cells.* ***I****, Macrophage migration in* wt1b *dominant negative gain of function model using Gal4/UAS system. Compared to a control line, macrophages migrate faster in the transgenic line impairing wt1b function.* ***J****, Analysis of cardiomyocyte proliferation upon cryoinjury of the cardiac ventricle. Shown are immunostaining of heart sections. Proliferation is assessed by BrdU incorporation in Myosin Heavy Chain (MHC)-positive cardiomyocytes. Figure adapted from (Sanz-Morejon et al., 2019).*

of wt1 function affected macrophages migration: They now migrated faster and did not accumulate any longer at the site of injury. Secondly, we studied heart regeneration in *wt1b* null mutant zebrafish. Indeed, we found reduced cardiomyocyte proliferation at 7 dpi compared to control siblings. The drop in proliferation was concomitant with a change in macrophage accumulation at the regeneration front, suggesting that changes in macrophage migration controlled by wt1b function influences proliferative capacity of cardiomyocytes. Overall, together with other recent studies (Bevan et al., 2020; Simoes et al., 2020), we contributed to better understand the role of macrophages during heart regeneration.

Cardiomyocyte subpopulations: can all do the same?

An essential difference between the adult human heart and the zebrafish heart is that the myocardium lost after damage can be replaced. Therefore, a central question is: Where are the new cardiomyocytes coming from? Cardiomyocytes are highly specialized cells, with their cytoplasm nearly completely filled with sarcomeres that allow cell contraction. To draw parallelisms to the skeletal muscle, there, new myofibres are formed, also in humans, through a stem cell pool of satellite cells. One hypothesis therefore was that myocardial regeneration could be contributed by a specific stem cell pool. So far, however, all accumulated evidence goes against this hypothesis. A seminal study was performed using Cre/lox fate mapping of cardiomyocytes using the *myosin light chain 7 (myl7)* promoter. Tracing of cells descendant of cardiomyocytes present in the uninjured heart revealed that the regenerated myocardium derived from pre-existent cardiomyocytes (Jopling et al., 2010; Kikuchi et al., 2010).

These results represented a convincing evidence that zebrafish regenerated the myocardium through re-entry into the cell cycle of differentiated cardiomyocytes upon injury. However, further questions remained. For example, can all cardiomyocytes contribute equally to the regenerated heart, or is there a subset of cardiomyocytes that preferentially proliferated in response to damage? Coincidentally, we observed that a subpopulation of *sox10*-derived cells accumulated at the injury border sites (Figure 9). We were investigating the fate of *sox10*-positive cells as we were interested in studying glia cells in the heart. However, the pattern ob-

served suggested that *sox10*-derived cells were indeed cardiomyocytes. We were able to confirm this by restricting the fate mapping to the myocardial lineage, using a lox reporter line with a promoter specific for ventricular cardiomyocytes. Again, in injured hearts, *sox10*-derived cardiomyocytes accumulated at the injury area. Interestingly, a large proportion of the regenerated myocardium was *sox10*-derived at 60 dpi. So, when are these *sox10*-positive cardiomyocytes emerging? Are they pre-existent in the adult zebrafish heart or do cardiomyocytes at the injury site upregulate *sox10* in response to injury and then contribute to regeneration? We found support for both hypotheses. Using the tamoxifen inducible CreERT2 system, we labelled *sox10*-derived cardiomyocytes well before injuring the heart. We found that in adult zebrafish heart, there was a very small population (less than 1%) of cardiomyocytes that were *sox10*-derived. Our results suggest that upon injury, this population expands 20-fold. We also characterized the transcriptional profile and found that *sox10*-derived cardiomyocytes revealed a distinct gene expression pro-

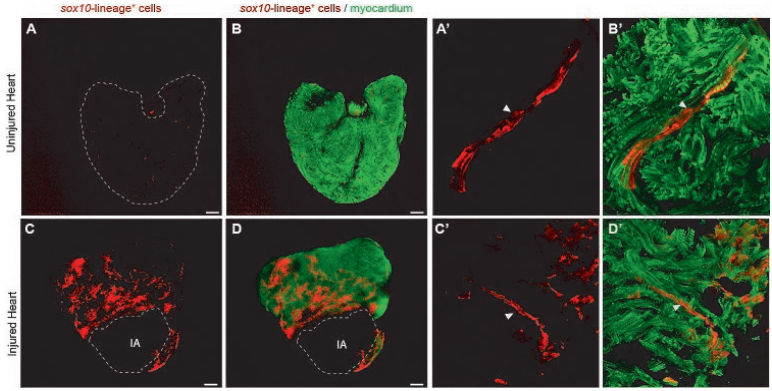

Figure 9. A subset of* sox10*-derived cardiomyocytes contributes to the regenerating myocardium. sox10:CreERT2 *lineage tracing was performed in* sox10:CreERT2; ubb:loxP-GFP-loxP-mCherry (ubi.Switch) *adult zebrafish by 4-Hydroytamoxifen administration 2 weeks before collection of control hearts and 2 weeks before cryoinjury.* ***A–D,*** *Whole mount views of an uninjured heart and a heart at 14 dpi. Note that the uninjured heart reveals few mCherry-positive cells, while upon injury, many mCherry-positive cells are close to the injury area (IA)* ***A'–D',*** *close up view of immunostainings in uninjured and injured heart showing that mCherry colocalizes with the myocardial marker Myosin Heavy Chain (green). Figure adapted from (Sande-Melon et al., 2019).*

file both in uninjured zebrafish heart, but in particular in response to injury, when compared with the rest of ventricular cardiomyocytes. To assess the importance of this population to heart regeneration we performed genetic ablation of *sox10*-derived cells and found that regeneration was impaired. Altogether, the data suggest that a subset of cardiomyocytes might be contributing preferentially to rebuild the injured heart (Sande-Melon et al., 2019). *Sox10* is a neural crest and neural crest derivative marker. Indeed, neural crest cells had been suggested to contribute to the heart tube during embryogenesis and also to contribute to regeneration of the adult heart (Abdul-Wajid et al., 2018; Tang et al., 2019). Whether neural crest progenitors actually contribute to the zebrafish myocardium or if a subset of cardiomyocytes upregulates a neural crest specific program will require further investigation.

The ventricular myocardium can be divided into three main layers, an inner trabecular layer, that fills most of the ventricular cavity, a single layered primordial layer, and an outer cortical layer. We learned from experiments listed above, that it seems that not all cardiomyocytes are equally capable to enter cell cycle and contribute to regenerate the lost myocardium. We also wondered whether cardiomyocytes from one layer are able to regenerate cardiomyocytes from other layers, or if they and their progeny are determined to a particular cardiomyocyte subtype. To answer this question, we first had to identify a reporter line that allows us to distinguish cardiomyocytes from particular myocardial layers. We identified the gene *tbx5a* to be expressed specifically in the trabecular layer and absent from the cortical layer (Figure 10). Therefore, we generated a *tbx5a:CreERT2* line as well as a *tbx5a:mCherry-P2A-CreERT2,* and used it in combination with loxP reporter lines to trace the fate of trabecular cardiomyocytes during regeneration. We found that indeed, when trabecular cardiomyocytes were recombined before injury, we could observe their descendants in the regenerated hearts not only in the trabecular layer, but also in the cortical layer. Interestingly, these *tbx5a*-derived cells were now not expressing trabecular markers any longer, but adopted not only a cortical position, but also expressed cortical marker genes. These results were showing that trabecular cardiomyocytes can undergo a phenotypic switch from trabecular to cortical myocardium in response to injury (Sanchez-Iranzo et al., 2018a). This cellular plasticity might be fundamental to allow rebuilding a heart in an efficient manner.

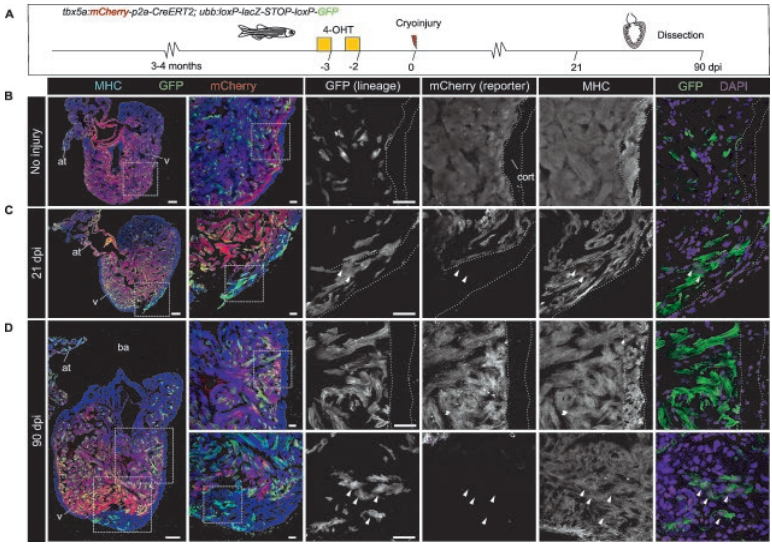

Figure 10. Contribution of trabecular cardiomyocytes to regeneration of the cortical myocardium. A, tbx5a:Cherry-p2A-CreER^{T2} *transgenic zebrafish were crossed into* ubb:loxP-lacZ-STOP-loxP-GFP. *4-OHT was added 2 and 3 days before cryoinjury to induce recombination of* loxP *sites. Hearts were fixed at 21 and 90 days postinjury (dpi) and sectioned for immunofluorescent detection of GFP*$^{+}$ tbx5a*-derived cells and mCherry*$^{+}$ tbx5a*-expressing cells. Nuclei were counterstained with DAPI.* ***B,*** *In the uninjured heart, mCherry expression was homogeneous in the trabecular myocardium and absent in the cortical layer. GFP+ cells were found in the trabecular layer. Single channels of boxed area are also shown.* ***C–D,*** *Section of a heart- at 21 and 90 dpi. Upon cryoinjury to the ventricular apex,* tbx5a^{+} *cardiomyocytes in general were restricted to the trabecular myocardium, but* tbx5a*-derived cardiomyocytes were present also in the cortical layer, particularly at the site of injury. Nuclear counterstaining revealed GFP*$^{+}$ *cell bodies in the cortical layer (arrowheads). at, atrium, v, ventricle. Scale bars, 100 µm (whole heart section), 25 µm (zoomed views). Figure adapted from (Sanchez-Iranzo et al., 2018a).*

While *tbx5a* expression was found throughout most of the trabecular myocardium of the ventricle, there was a region close to the outflow tract of the heart that remained *tbx5a*-negative. This region was first visible already in the embryonic heart. At 24 hours postfertilization, the whole heart tube is *tbx5a*-positive but after this, a *tbx5a*-negative domain starts to appear at the cranial pole of the heart. This is the region where new

progenitors from the second heart field (SHF) are entering the cardiac ventricle. Thus, the *tbx5a*-negative cardiomyocytes seemed to represent SHF-derived cardiomyocytes. It was well-known that, as in mammals, the zebrafish heart is build up from first heart field (FHF) precursors that make up the primordial heart tube and that cells from the SHF then are added to the venous and cranial pole of the embryonic heart to allow fur-

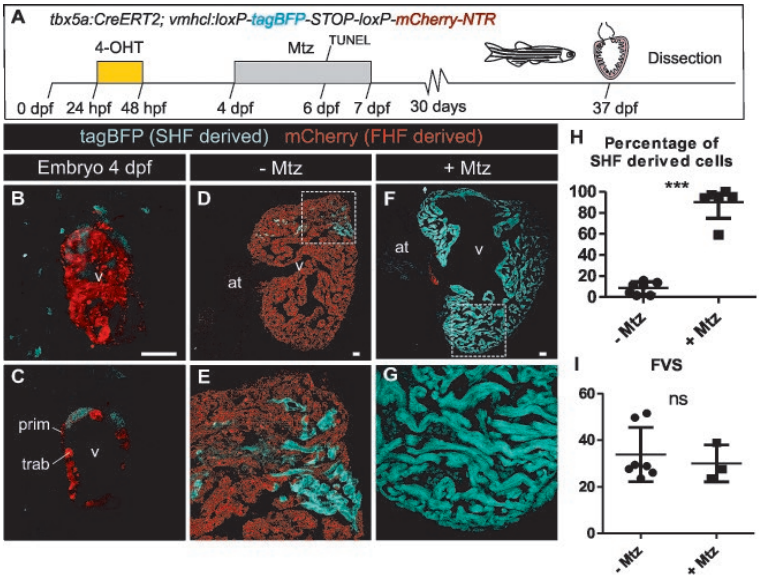

Figure 11. Cellular plasticity during development: second heart field progenitors can compensate for the loss of first heart field derived cardiomyocytes. A, tbx5a^{+} *ventricular cardiomyocytes were genetically ablated in* tbx5a:CreERT2;vmhcl:loxP-tagBFP-loxP-mCherry-NTR *double transgenic zebrafish. Recombination was induced by administration of 4-OHT. Cell ablation was induced by administration of Metronidazol (Mtz) from 4 to 7 dpf. Hearts were dissected 30 days later.* ***B–C,*** *Ventral views of larval hearts at 4 dpf. Anterior is to the top. Note that the proximal ventricle is completely mCherry^{+}, and that the distal ventricle is blue (tagBFP^{+}).* ***D–E,*** *Section of the ventricle of an adult recombined heart. Most cells are mCherry+. Only the* tbx5a- *region is tagBFP^{+}. (**F–G**) Sagittal section of an Mtz-treated fish. Most of the cardiomyocytes are BFP^{+}.* ***H,*** *Quantification of the percentage of myocardium that is tagBFP^{+} (SHF derived). I, Cardiac function is not affected by ablation of tbx5a-derived cells. FVS, fractional ventricular shortening (in %). mean±s.d; *** P<0.0001 by two-tailed unpaired t-test. at, atrium; prim, primordial; trab, trabecular; v, ventricle. Figure adapted from (Sanchez-Iranzo et al., 2018a).*

ther heart development (Knight and Yelon, 2016). Our genetic lines now allowed us to interrogate if FHF and SHF progenitors are interchangeable or in other words, if SHF progenitors can also give rise to FHF structures, if needed. To test this hypothesis, we ablated FHF derived ventricular cardiomyocytes using the nitroreductase (NTR) system (Figure 11). We crossed the line *tbx5a:CreERT2* into the line *vmhcl:loxP-BFP-loxP-mCherryp2A-NTR*. Upon recombination, these double transgenic animals express mCherry and NTR in *tbx5a*-derived FHF ventricular myocardium. Addition of the compound Metronidazol leads to cytotoxicity in NTR-expressing cells, and as such, eliminates the FHF-derived ventricle. With this experimental set up, we now could investigate if the FHF-derived myocardium can be regenerated from SHF-derived progenitor cells. Indeed, we found that while in control situation recombined animals have an mCherry-positive (red) and blue fluorescent protein (BFP)-positive (blue) ventricle, animals that underwent genetic ablation revealed some days later a fully BFP-positive ventricle. Interestingly, we did not observe neither major morphological alterations nor changes in cardiac function in these hearts, now comprised fully by SHF derived myocardium. In conclusion, while in a wildtype scenario the cardiac ventricle is built up from FHF and SHF precursors, SHF precursors can fully compensate the loss of FHF-derived myocardium (Sanchez-Iranzo et al., 2018a). This is a second example of the high plasticity that cardiomyocytes reveal during heart regeneration.

Conclusions and Outlook

Cardiac regeneration is a complex process during which several cell types interact and communicate with each other to promote heart regrowth, including the re-establishment of cardiac function. Our own studies combined with those from others show that a first injury response including inflammation and fibrosis are key steps towards myocardial regeneration. Our studies also show that a regenerated heart is not completely equal to an uninjured heart, to a low degree, cellular composition and function is altered. The zebrafish remains a key model organism in the research of the cellular and molecular mechanisms of heart regeneration. Further technological improvements such as intra vital imaging methods in the adult zebrafish and genome editing approaches allowing the generation

of spatial and temporal control of gene expression will strongly contribute to provide further knowledge on heart regeneration. A very important central question will be, in my opinion, to understand the epigenetic control mechanisms underlying heart regeneration. Which cells are prone to contribute to regeneration and how is this "readiness" encoded?

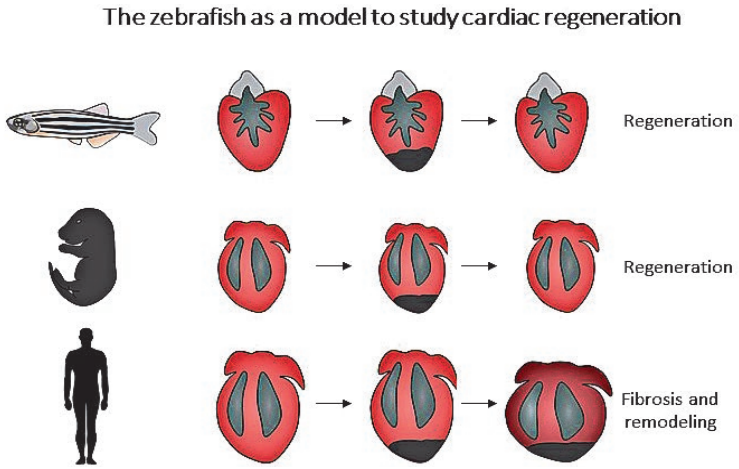

Figure 12. Zebrafish as a model to study heart regeneration. *Zebrafish as well as neonatal mice regenerate their hearts after sever injury. Adult human hearts undergo fibrosis and remodelling upon injury.*

The incorporation in the last decade of a mammalian model to study heart regeneration has been very important to the field (Figure 12). The groups of Eric Olson and Enzo Porrello described that during their first week after birth, mice can regenerate the heart with a similar efficiency as zebrafish (Porrello et al., 2011). During the first week of age, neonatal cardiomyocyte also re-enter the cell cycle and divide upon cardiac lesion. Indeed, several pathways and mechanisms of regeneration are conserved between mouse and zebrafish, supporting the possible translational impact of studies on heart regeneration in the zebrafish. While classically the human heart has been considered to be postmitotic, there is a physiological turnover of cardiomyocytes, which is particularly prominent in

the first two decades of life. Indeed, some reports estimate that the cardiomyocyte pool is replaced twice during human lifespan (Bergmann et al., 2009; Bergmann et al., 2015). While after cardiac injury, there is a statistically significant increase in cardiomyocytes undergoing cell cycle reentry, the numbers are far too small to support heart regeneration. The finding that some mammals have the capacity to regeneration early on in life, but that this program is repressed in the adult, might indicate that there is a therapeutic window to re-activate a naturally repressed mechanism. A close interaction of researchers working with different species and the combination of approaches ranging from basic to translational science hopefully pave the way not only to fully uncover the mechanisms of heart regeneration but also to design strategies to promote it.

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