Decoding spatial heterogeneity in the regenerating heart

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Myocardial infarction (MI) causes permanent heart tissue loss in adult mammals. Following an MI, the injured heart recruits a significant number of monocyte-derived macrophages, essential immune cells that play critical roles in both scar formation and tissue regeneration. However, we still have limited understanding of the specific factors influencing distinct macrophage functions within the damaged heart. Our work aims to shed light on how macrophage heterogeneity is linked to their spatial distribution across the heart and how, in turn, this distribution shapes macrophage identity and function. Despite their vital roles in cardiac repair, the intricate local environmental cues and cell-cell interactions governing the diverse roles of macrophages in this context have not been fully deciphered. By comprehending the regenerative microenvironment, where the innate immune response persists while still supporting regeneration, and by targeting macrophage-induced pro-fibrotic pathways, we may develop therapeutic strategies that harness pro-regenerative responses in the injured mammalian heart.
Defining immune cell populations in the zebrafish heart

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Macrophages of the innate immune system are increasingly recognised as highly plastic, multifunctional cells that can influence multiple physiological and pathophysiological states. In the heart they support homeostatic functions, contribute to disease progression and play multiple roles in reparative and regenerative processes following tissue damage. Understanding the heterogeneous populations of cells that contribute to these diverse functions is crucial to facilitating beneficial, and limiting adverse, cardiac outcomes. Characterisation of precise populations of cardiac macrophages and related cells remains incomplete in vertebrate models capable of endogenous regeneration, such as adult zebrafish, and yet the ability to identify sub-populations of cells and understand their localisation in the heart would greatly facilitate the study of their precise functions. We have used a combination of transgenic lines to identify four distinct immune cell populations in the zebrafish heart. Macrophages are present in developing cardiac tissue from 2 days post fertilisation and are likely seeded from both primitive and definitive derived cells and correct seeding requires csf1ra. In adult hearts, macrophages and dendritic cells are differently distributed and can be distinguished via expression level of the perforin mpeg1.1. Following injury, tissue resident macrophages rapidly proliferate and exhibit limited pro-inflammatory activation, similar to what has been observed in neonatal mice. This new understanding of innate immune cell populations in the heart of regenerative adult zebrafish sheds light on the composition of a pro-regenerative cardiac microenvironment.
Genetic modifiers of myocardial regeneration and fibrosis in zebrafish

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Many forms of heart disease are associated with the loss of cardiomyocytes (CMs) and their replacement by fibrotic tissue. Cardiac fibrosis is central to the pathology of heart failure, a deadly syndrome and a leading cause of mortality in the US. Unfortunately, there are no effective therapies to prevent the progression of cardiac fibrosis. Recent findings suggest that the way the heart responds to injury, including the extent of fibrosis, is a variable trait influenced by several genes. One of the potential regulators of this outcome is the CM-specific kinase Tnni3k. Mutations that abolish Tnni3k expression confer resistance to injury, and high Tnni3k levels are associated with rapid functional decline and pathological remodeling. Elevated Tnni3k has also been associated with increased ploidy and reduced proliferation in the zebrafish heart after injury. Despite its multiple links to disease, Tnni3k is an understudied kinase, and its downstream targets and specific mechanisms by which it defines injury outcomes are unknown.

To identify the molecular mechanisms by which Tnni3k induces cardiac diseases, we have established several new genetic models in zebrafish, an animal model with the unparalleled ability to regenerate its heart after cryoinjury through myocardial regeneration and progressive fibrosis regression. We found that elevated expression of Tnni3k in cardiomyocytes is sufficient to induce cardiomyopathy-like phenotypes in zebrafish, including fibrosis and leukocyte infiltration at baseline. While CM ploidy and proliferation after injury were unaffected, these animals developed an extensive fibrotic response that failed to regress. Conversely, we found reduced fibrosis and robust regeneration in a newly generated tnni3k mutant. Transcriptomic profiling revealed that Tnni3k induces an exacerbated inflammatory response after injury and the upregulation of several genes involved in stabilizing the scar. Using new whole locus deletions lines, we discovered that some of these responses are protective and that preventing these adaptations worsens the response to cardiac injury. Finally, modulation of Tnni3k levels using a Tnni3k-Switch allele demonstrates that decreasing the expression of this gene post-injury reduces inflammation and fibrosis, improving overall myocardial recovery. Our findings suggest that Tnni3k acts as a modifier of regenerative competence not by affecting cardiomyocyte proliferation but by inducing a pro-inflammatory and pro-fibrotic environment in the heart. Our work exemplifies the power of zebrafish genetics to serve as the springboard for the rapid discovery of new targets to treat cardiac fibrosis in the injured heart.
Impact of cardiac ageing on regeneration

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Aging imposes a barrier for tissue regeneration. In the heart, aging leads to severe changes in cellular functions, which include increased cell death and hypertrophy of cardiomyocytes, fibroblast activation, impaired endothelial cell function and reduced innervation. Functionally, these cellular alterations result in impaired diastolic and later systolic function. The presentation will discuss how the altered cellular functions and cellular cross-talks during aging may contribute to a decline in repair and regeneration.

Further reading:


Wagner et al, Ageing impairs the neuro-vascular interface in the heart. *Science* 2023

Tzahor E, Dimmeler, S. A coalition to heal—the impact of the cardiac microenvironment. *Science* 2022
Tackling myocardial repair from both sides: Roles for the epicardium and endocardium

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Our research extrapolates our understanding of embryonic heart development to inform strategies to enhance the inadequate regenerative capacity of the adult mammalian heart following myocardial infarction, with a particular focus on stimulating neovascularisation. Following injury, there is a partial recapitulation of embryonic processes that drive coronary vessel growth, yet fundamental differences in the regulatory pathways limit the efficacy of the adult response. Comparative analyses allow us to identify key mechanisms that may be targeted in the adult mammalian heart to enhance repair. Our research in murine models is complemented with the use of human iPSC-derived models of coronary vascular development.
The Role of the Cardiac Lymphatics during Neonatal Heart Regeneration

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Heart attack or myocardial infarction (MI) triggers an immune response, whereby phagocytic cells remove dead tissue and assist with the subsequent remodelling and repair of the infarcted heart. High load and persistence of immune cells, however, contributes to further fibrosis and pathological remodelling and ultimately progression to heart failure.

We have shown that in adult mice, MI activates the cardiac lymphatics, which undergo sprouting angiogenesis (lymphangiogenesis) and function to drain the build-up of interstitial fluid (oedema) and traffic immune cells, including macrophages, to mediastinal lymph nodes (MLNs), reducing inflammatory/fibrotic immune cell content and improving cardiac output. Which functional subsets of immune cells/macrophages that are trafficked, versus retained in the heart to elicit improved outcome remains unknown and is the subject of ongoing studies via unbiased single cell transcriptional profiling.

Given the important role of the adult cardiac lymphatics in trafficking macrophages post-MI, we further investigated their role across the so-called regenerative window in neonatal mice (post-natal days 1-7; P1-P7). Mice at P1 fully regenerate their heart following MI in a pro-regenerative macrophage-dependent manner, whereas equivalent injury at P7 leads to scarring driven by pro-fibrotic macrophages. We hypothesised that lymphatics respond and function differently following MI during this regenerative window, to clear macrophage specific subtypes depending upon their requirement for regeneration (P1) or fibrotic repair (P7). Normal lymphatic growth and sprouting is evident in intact neonatal hearts until P16, with strain-dependent developmental differences. Importantly, the maturation status of lymphatic endothelial cell junctions, is altered across the neonatal period via transition from “zipper” (impermeable) to “button”-type (permeable) junctions. Moreover, the response to injury is significantly altered, with limited lymphangiogenesis and decreased clearance of macrophages in P1 compared to P7 mice 7-days post-MI, as determined by adoptive transfer experiments. To gain molecular insight into the mechanisms underpinning lymphatic endothelium-macrophage interactions in P1 versus P7, we have generated unbiased single cell RNA sequencing datasets from samples collected at different time-points after MI. Finally, in mice lacking lymphatic endothelial receptor-1 (Lyve1) that exhibit impaired transmigration of interstitial macrophages to lymphatic vessels, magnetic resonance imaging (MRI) revealed a surprising impaired functional outcome in P1 mice 28 days post-MI. Given our observations that pro-regenerative macrophages at P1 are not trafficked, this suggests a distinct role for Lyve-1 in tissue resident macrophages, consistent with its expression pattern in developing and post-natal hearts: investigation of macrophages-specific deletion of Lyve-1 during neonatal heart regeneration is ongoing.

Collectively, we have revealed that the cardiac lymphatics are essential in clearing immune cells to orchestrate optimal cardiac repair in injured adult mice, whereas they are developmentally compromised for clearance in early neonates which enables retention of pro-regenerative tissue resident macrophages. Further molecular studies to uncover functional subsets of macrophages that are required to be cleared versus retained following adult heart injury and to uncover the molecular mechanisms that lead to the differential response across the neonatal
regenerative window may provide therapeutic insights into lymphatic-based immunomodulation of the infarcted heart.
A small molecule PI3Kα activator in cardioprotection and neuroregeneration

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Although patient outcome following acute myocardial infarction (AMI) has improved, methods to limit ischaemia and reperfusion (IR) injury in AMI patients remain an important unmet clinical need. Treatment has improved, but long-term outcome is still poor with many patients going on to develop heart failure. Experiments over the past ~20 years have identified the PI3-kinase / AKT signalling pathway (referred to as the "Reperfusion Injury Salvage Kinase" pathway) as centrally involved in many cardioprotective drugs able to reduce myocardial infarct size after IR. The PI3Kalpha isoform is essential for many cardioprotective strategies in mice. We recently developed a novel small molecule called UCL-TRO-1938 or 1938, which is able to enter cells and directly bind to and allosterically activate PI3Kalpha, without affecting other isoforms. By thereby activating the RISK pathway, 1938 can protect the hearts of mice and rats from injury. It also stimulates cell proliferation and neurite outgrowth in vitro. After local in vivo administration, it enhances nerve regeneration following nerve crush injury. This novel chemical tool allows direct probing of the PI3Kα signalling pathway and a new approach to modulate PI3K activity, widening the therapeutic potential of targeting these enzymes through short-term activation for tissue protection and regeneration.
Role of vagus nerve, incretin hormone GLP-1 and cardiac pericytes in cardioprotection against ischaemia/reperfusion injury

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Vagus nerve is crucially important in protecting the heart in myocardial infarction, preserving cardiac function in heart failure, and improving exercise capacity (Mastitskaya et al., 2012; Machhada et al., 2020). The signaling pathway from the vagus to the heart is complex and comprises a brain-gut-heart communication axis. The majority of vagal efferent fibers project to the gut where they control digestion and secretion of several gut hormones, including incretin hormone glucagon-like peptide-1 (GLP-1). GLP-1 exhibits substantial positive effects on the cardiovascular system – vasodilation, reduced inflammation, improved myocardial blood flow, and cardiomyocyte survival. Our research has shown that vagus nerve stimulation's cardioprotective action on the heart is mediated, at least in part, by an increased release of GLP-1 from the gut (Basalay et al., 2016; Mastitskaya et al., 2016). Stimulation of GLP-1 receptors in the heart leads to the activation of pro-survival signaling pathways (Basalay et al., 2016) and reduces myocardial susceptibility to ventricular arrhythmias (Ang et al., 2018). Additionally, vagus nerve stimulation prevents no-reflow after myocardial ischemia. No-reflow is a phenomenon when, after reopening of the culprit artery in myocardial infarction, blood flow at the microvascular level is not fully restored, heavily contributing to poor healing of the infarct, development of heart failure, malignant arrhythmias, and even cardiac rupture. Microvascular no-reflow is mediated by pericytes – contractile cells wrapped around capillaries. In ischemia, pericytes contract and constrict the underlying capillaries (O’Farrell et al., 2017). Our most recent data suggest that this constriction can be prevented by the stimulation of GLP-1Rs on microvasculature. The downstream molecular mechanism of GLP-1R activation involves the opening of K_ATP channels. GLP-1R agonists and K_ATP channel openers are therefore novel therapeutic targets to prevent no-reflow, reduce infarct size and decrease the incidence of ventricular arrhythmias in patients with ischaemic heart disease.

C01

Crosstalk between circadian rhythm and fish heart regeneration

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Zebrafish are able to fully regenerate their hearts through cardiomyocyte (CM) proliferation. Daily circadian rhythms regulate many processes including cell proliferation. As zebrafish differ from mammals in their ability to detect light with all cells in their body, this raises the question if there is a link between this difference in circadian rhythm and CM proliferation after injury. However, nothing is known in either species about a possible interplay.

To test this hypothesis, we characterised the circadian regulation of heart regeneration using zebrafish as animal models. As this is a completely unexplored field of research, we first confirmed if there is a clock oscillator in the fish heart by examining the rhythmic expression of a set of core clock genes including cry1a, per2, clock1, per1b and nr1d1 by qRT-PCR, confirming robust rhythmic expression of all these genes in the uninjured zebrafish heart. To test the importance of this circadian clock on heart regeneration, we performed cardiac cryo-injury on a mutant zebrafish line in which three core clock genes are knocked out (cry1a\textsuperscript{−/−}; per2\textsuperscript{−/−}; cry2a\textsuperscript{−/−} triple knock-out zebrafish), called TKO zebrafish. Importantly, light-dependent synchronisation of cellular clocks is impaired in the TKO. Wound size quantification at 7 and 21 days post cryo-injury (dpci) in TKO and wild-type (WT) control hearts showed reduced regeneration in the TKO. Moreover, CM proliferation was significantly reduced in the TKO consistent with inhibited regeneration. These data indicate that perturbing circadian rhythm negatively affects heart regeneration. To identify regeneration-dependent changes in circadian gene expression, we then performed bulk RNAseq on WT zebrafish control uninjured ventricles and at 7-8 days post cryo-injury (dpci), corresponding to the peak of CM proliferation. Ventricles were collected at 4-hour intervals for a total of 48 hours starting at 7dpci. Using the DryR algorithm we found that 322 gene exhibit a rhythmic expression pattern only after injury. Strikingly, gene ontology enrichment analysis revealed that this subset of genes is strongly enriched in functions linked to the cell cycle, indicating that the cell cycle is clock controlled during regeneration. As we know that fish heart regeneration is driven predominantly by proliferation of pre-existing CMs, we next investigated if CM proliferation is rhythmic. 7dpci WT zebrafish ventricles were collected at 6-hour intervals over 24 hours and analysed for CM proliferation. We found that CM proliferation seems significantly increased near the wound at night (zt23) compared with the other time-points analysed. The rhythmicity of the CMs was further confirmed by analysis of p21 expression. P21 (cdkn1a) is most strongly upregulated in the proliferating wound border myocardium at zt17, while almost absent at zt5. In summary, our data has uncovered that proliferation of zebrafish CMs is controlled by circadian rhythm, moreover, the disruption of the circadian rhythm impacts on the cardiac regenerative capacity in zebrafish. In the future, we would like to investigate if a distinct regulation of the circadian clock mechanism underlies the difference in proliferative capacity between species. Investigating these differences, we could identify novel targets for inducing CM proliferation after injury in patients.
Understanding the role of prolonged immune response to cardiac injury in regenerating Astyanax mexicanus heart

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Introduction

Myocardial infarction (MI) is the most common cause of cardiac injury in humans and results in acute loss of heart tissue, which becomes replaced with a fibrous scar that later might lead to post-MI heart failure. Unlike humans, some fish can regenerate their hearts after injury. Astyanax mexicanus is a single fish species comprising two different forms: a blind cave-dwelling and an eyed surface-dwelling form. Over several millions of years, geological events that occurred in Northern Mexico caused surface fish living in rivers to enter caves. Subsequently, with retreating river waters, river fish got trapped in numerous caves and evolved into different cavefish populations. Our laboratory has discovered that surface fish are able to fully regenerate their hearts, while cavefish from the Pachón cave form a permanent fibrotic scar like humans and also identified key differences in their immune responses (Stockdale et al., 2018).

Objectives

Here, we characterized the immune response to cardiac injury in A. mexicanus and hypothesized that the differences in immune response underlie the differential regenerative response between surface fish and cavefish.

Methods

To induce cardiac injury, cryoinjury was performed on the surface and cavefish. Hearts were isolated at timepoints from 0 to 60 days post-cryoinjury (dpci, n=10-18 per timepoint) and processed for scRNA-seq (0-14dpci), in situ hybridisation (0-60dpci) and (immuno-) histochemistry (0-60dpci). Pharmacological inhibition was performed by intraperitoneal injection between 7 to 11dpci (n=10 per group) or water immersion from 7 to 14dpci daily (n=15 per group).

Results

Using scRNA-seq, we found that immediately after injury, Pachón showed a strong innate immune response peaking at 1 and 3dpci whereas surface fish had a prolonged response throughout at least 14 days after injury. Additionally, surface fish showed a stronger adaptive immune response after 7dpci which was absent in Pachón. Both the adaptive and innate surface fish response was transcriptionally different from Pachón. In situ hybridization experiments validated the scRNA-seq findings, Pachón showed an immediate substantial influx
of innate cells into the wound, but this response was decreased and resolved by 7dpci (P<0.05). Surface fish innate immune response was prolonged throughout 60 days after injury whereas Pachón innate cell numbers remained lower from 3 to 60dpci (P<0.005, P<0.0001). Moreover, surface fish showed a significant increase in adaptive cells starting at 7dpci and peaking at 30dpci (P<0.0001), whereas Pachón adaptive response was at negligible levels at all time points from 0 to 60dpci. Suppressing the late immune response pharmacologically using either clodronate liposomes or dexamethasone significantly inhibited the leukocyte response at 30dpci and resulted in larger wound sizes (P<0.05) at both 30 and 60dpci suggesting that the prolonged leukocyte response might be essential for surface fish regeneration.

Conclusions

Prolonged innate and adaptive responses might be one of the key regulators of successful heart regeneration. Understanding the signals and mechanisms that govern regeneration and scarring in A. mexicanus could be useful to create new therapeutic targets to stimulate the regeneration of the human heart.

Erbb2 could promote heart regeneration in zebrafish by regulating cardiomyocyte dedifferentiation and proliferation

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Cardiac diseases are a leading cause of death worldwide as mammals cannot regenerate their heart effectively. One reason for this lack of regenerative capacity is insufficient cardiomyocyte (CM) renewal, by which CMs dedifferentiate, proliferate and redifferentiate to replace lost CMs.

Erbb2, a receptor tyrosine kinase, has been implicated in CM regeneration. Overexpression of constitutively active ERBB2 in mouse CMs promotes both their dedifferentiation and proliferation after myocardial infarction. In zebrafish, a regenerative model, overexpression of the Erbb2 ligand Nrg1 enhances dedifferentiation and proliferation. Nevertheless, it is not clear whether erbb2 is essential for CM dedifferentiation and proliferation during zebrafish heart regeneration.

Here, both an inducible dominant negative (DN) erbb2 line and a floxed erbb2 line are being established in zebrafish, as erbb2 mutants are not viable. Expression of the DN-erbb2 is temporally controlled by heatshock and tamoxifen treatment and CMs expressing the DN-erbb2 can be identified by mScarlet expression. This strategy should indicate whether mScarlet-expressing CMs can dedifferentiate and proliferate. In the floxed erbb2 line, recombination will produce a truncated protein at the tyrosine kinase domain. Therefore recombination should abrogate Erbb2-mediated signal transduction. While these tools are established and validated, CM dedifferentiation and proliferation are being assessed after treatment with an Erbb inhibitor. Preliminary data suggest that inhibiting Erbb signalling during regeneration leads to a decrease in a CM dedifferentiation marker.

On the whole, Nrg/Erbb2 signalling has been implicated in CM dedifferentiation and proliferation from gain-of-function experiments but more investigation is needed to delineate its role in zebrafish heart regeneration, which could open the door to new therapeutic strategies for patients.

Induction of Cytokinesis in Multinucleated Cardiomyocytes promotes Cardiac Regeneration

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Introduction: Following cardiac injury, neonatal mouse hearts are able to completely regenerate. However, a few days after birth in mouse cardiomyocytes, DNA synthesis occurs without cytokinesis leading to most cardiomyocytes becoming binuclear instead of generating two daughter cells with one nucleus each. This results in cell cycle arrest of cardiomyocytes and the mouse heart is no longer able to regenerate. A long-standing unanswered question in the field is whether multinucleation of cardiomyocytes is a result of cytokinesis failure.

Methods and Results: To address this, we generated several cardiomyocyte-specific transgenic mouse models to determine whether forced induction of cardiomyocyte cytokinesis generates mononuclear cardiomyocytes and restores the endogenous regenerative properties of the myocardium. We focused on two complementary regulators of cytokinesis, namely Polo-like kinase 1 (Plk1) and epithelial cell-transformation sequence 2 (Ect2). Here we report that cardiomyocyte-specific overexpression of constitutively active Plk1(T210D) alone [αMHC-Plk1(T210D) mice] promotes mitosis and cytokinesis in adult hearts, while overexpression of Ect2 alone (αMHC-Ect2 mice) promotes cytokinesis. Intriguingly, cardiomyocyte-specific overexpression of both Plk1(T210D) and Ect2 concomitantly [αMHC-Plk1(T210D); αMHC-Ect2 mice] prevents binucleation of cardiomyocytes postnatally and results in widespread cardiomyocyte mitosis, cardiac enlargement, contractile failure, and death before two weeks of age.

To assess the effect of inducing Plk1(T210D) and Ect2 in the adult heart (a stage when the majority of cardiomyocytes in mice are already binucleated), we generated a transgenic mouse model [TRE-Plk1(T210D)-T2A-Ect2; αMHC-rtTA] of doxycycline inducible cardiomyocyte-specific overexpression of both Plk1(T210D) and Ect2 proteins. High-dose doxycycline inducible cardiomyocyte-specific overexpression of both Plk1(T210D) and Ect2 proteins in the adult heart results in reversible widespread cardiomyocyte mitosis, cardiac enlargement, and contractile failure, while low-dose transient induction also results in significant cardiomyocyte proliferation and lower ejection fraction that is reversible after doxycycline is removed. Finally, we show that transient low-dose induction of both Plk1(T210D) and Ect2 in adult cardiomyocytes improves left ventricular systolic function following myocardial infarction.

Conclusion: Collectively, these results demonstrate that cytokinesis failure mediates cardiomyocyte multinucleation and cell cycle exit of postnatal cardiomyocytes but may be a protective mechanism to preserve the contractile function of the myocardium. After myocardial infarction, transient overexpression of Plk1(T210D) and Ect2 improves heart function.
Impact of Reactive Oxygen species on cardiac regeneration and fibrotic elimination in myocardial injury (cryo injury) of adult Zebrafish (Danio rerio)

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Aim: The objective is to study the role of reactive oxygen species on cardiac regeneration and fibrotic elimination in adult zebra fish after cryo-injured model of myocardial damage. The entair experiment was carried out after the approval of institutional animal Ethical committee, Institute of Basic Medical Science, University of Madras, Chennai, India. Materials and methods: Wild type zebra fish were subjected to induction of myocardial injury by surgical method according to the methods of (González-Rosa & Mercader, 2012). After the surgery the animals were subjected to ECG analysis and the animals which showed alteration in the ST segments were included in the study otherwise were excluded. After the confirmation of ECG the animals were grouped into 4 ei., group I- sham (subjected to surgical procedure except myocardial injury), group II - (cryoinjury: induction of myocardial injury by placing pre-cooled cryo-probe (0.8 mm) for 20 seconds, group III – cryoinjury + Quercetin 50mg/kg B/W (Lara-Guzmán et al., 2012) once in a day for the periods of 60 days and group IV- sham + Quercetin 50mg/kg B/W. once in a day for the periods of 60 days. The animals were sacrificed at 0, 3, 7, 15, 30,45and 60 days of post operative periods by over dose of anesthesia (Tricaine) and ECG analysis were done at every time point before the sacrifice. The heart tissues were subjected to various analysis like morphology, histology, fibrosis analysis by picrosirius red staining, cell cycle analysis by BrdU staining, estimation of ROS production, estimation of levels of antioxidants, expression of genes involved in fibrosis (fibrin, collagen, TGFb, SMAD, αSMA, FSP1 and PU.1) and cell cycle (ccne1) were studied by immunohistochemistry and Real-time PCR. Results: The data showed that significant increase in the ROS (p<0.001) production, reduced antioxidant levels (p<0.001), accumulation of fibrin followed by collagen replacement, significant increased in the BrdU positive cell in all the three layers of the heart (endo, epi and pericardium). Gene expression data showed increased expression of fibrotic gene (fibrinogen P<0.001, collagen 1a1 p<0.01, TGFb p<0.001, SMAD p<0.001, dSMA p<0.01, FSP1- p<0.001, and PU.1- p<0.001) and cell cycle (ccne1- p<0.001) genes in time dependent manner. But inhibition of ROS significantly represses the fibrin formation (p<0.001) in the initial days of post injury and the collagen replacement (p<0.01) also was found to be delayed. In the histological study showed more inhibition of ROS markedly increased the accumulation of inflammatory cells in the injury site when compared to cryo-injured groups. Further, the inhibition of ROS significantly altered the expression of both fibrotic and cell cycle genes (fibrinogen p<0.001, collagen 1a1 p<0.01, TGFb p<0.001, SMAD p<0.001, dSMA p<0.01, FSP1- p<0.001, and PU.1- p<0.001 and cell cycle ccne1- p<0.001). Conclusion: Taken together the present data clearly emphasized that the ROS in the regenerating adult zebra fish heart after the cryoinjury is essential for regulating the reentry of adult cells in to cell division and also for PU.1 dependent phenotypic switch of fibroblast from fibrogenesis to fibrolytic and vice versa.

• González-Rosa JM, Mercader N. Cryoinjury as a myocardial infarction model for the study of cardiac regeneration in the zebrafish. Nat Protoc. 2012 Mar 29;7(4):782-8 • Lara-Guzmán,
Oxidative phosphorylation is required for cardiomyocyte re-differentiation and long-term fish heart regeneration

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Introduction

In contrast to humans, zebrafish maintain the capacity to regenerate lost cardiac tissue throughout their adult life. This has been attributed to differences in metabolism, with the zebrafish relying mostly on glycolysis, allowing for cardiomyocytes to proliferate (Honkoop et al., 2019). In contrast, the mammalian heart mostly uses oxidative phosphorylation to maintain a high cardiac output. This results in the production of reactive oxygen species which can inhibit cardiomyocyte proliferation and is thought to block heart regeneration. However, using an inter- and intra-species comparative approach, we have found that OXPHOS is essential for long-term heart regeneration by promoting cardiomyocyte maturation following proliferation.

Methods

We cryoinjured the ventricles of seven wild-type zebrafish strains (AB, NA, SAT, TL, TU, WIK KCL) and Astyanax mexicanus and measured their regenerative characteristics at 1-, 7-, 21- and 90 days post-cryoinjury (Dpci). Additionally, we utilised bulk and single-cell (sc) RNAseq to identify the pathways and timeline of events during heart regeneration in our fish.

Results

Our data suggests that inter-strain differences exist in zebrafish heart regeneration with some strains (NA, TL) being capable of complete regeneration while others (SAT, TU) being regeneration-incompetent at 90Dpci. Surprisingly, correlating this data with our bulk RNAseq analysis identified OXPHOS as the top pathway which promotes regeneration. Furthermore, by re-analysing previously published scRNAseq datasets, we identified that it is the border zone cardiomyocytes that upregulate OXPHOS. Differential gene expression analysis identified that this upregulation might be fuelled by the malate aspartate shuttle (MAS) which was enhanced in the regeneration-competent strains. Indeed, pharmacological inhibition of both OXPHOS and
the MAS ablated the regenerative potential of zebrafish but did not affect cardiomyocyte proliferation.

Instead of the expected role for proliferation, we identified that it was the expression of embryonic myosins in the border zone cardiomyocytes that correlated with better regenerative outcome. This response appeared to be driven by increased OXPHOS gene expression. Indeed, pharmacological inhibition of both OXPHOS and the MAS led to reduced embcmhc expression but not proliferation, indicating that OXPHOS is necessary for cardiomyocyte re-differentiation. Our scRNAsq confirmed that OXPHOS is temporally separated from cardiomyocyte proliferation and is associated with the cardiomyocyte re-differentiation phase. Finally, we were able to identify that the beneficial role of OXPHOS is conserved and highly enriched in the regenerating surface fish hearts, whereas downregulated in the non-regenerative cavefish hearts, which show reduced cardiomyocyte re-differentiation.

**Conclusion**

We identified a surprising and unexpected beneficial role of OXPHOS and the MAS during heart regeneration. Our data suggests that OXPHOS is necessary to complete heart regeneration through promoting border zone cardiomyocyte re-differentiation by providing the energy required to sustain sarcomeric re-assembly. Importantly, this process is temporally distinct from cardiomyocyte proliferation, illustrating the existence of two phases in heart regeneration, a proliferative and maturation phase.

Introduction:
During development, the heart transforms from a linear tube to a multichambered organ. This process requires orchestration of both mechanical and chemical cues between primordial cardiac tissues and the surrounding environment. Errors in these early stages of human cardiogenesis are known to cause congenital heart defects, however, existing in vitro models are insufficient to address the morphometric abnormalities that occur in vivo.

Aims:
Our was to utilise novel bioengineering approaches to reverse engineer an in vitro model of the earliest stage of human heart development, the linear heart tube.

Methods:
Utilising a recently developed bioprinting technology that enables the photo-crosslinking of biopolymers within hydrogels combined with hiPSC, we have created a novel 3D tissue-engineered in vitro model of the embryonic heart tube.

Results:
We are able to finely tune the stiffness of the hydrogel over biologically matched ranges by modulating both the laser power and the number of printing cycles. This enables patterning of mechanical properties with micrometric resolution allowing us to generate regional domains with specific elastic modules. The bioprinted scaffold enables robust cardiomyocyte differentiation from human iPSCs with the formation of a single cell layer around the hydrogel scaffold with correct polarisation and organisation with representative morphology and geometry to what is observed in vivo. The mechanical properties of the tubes can be designed to be compliant with cardiomyocyte contraction with corresponding changes in the luminal cross-section depending on scaffold stiffness. We now envision the creation of controlled small molecule concentration gradients across the different axis of the linear heart tube in an attempt to recreate the patterning process that occurs in vivo.

Conclusion:
We are using this technology to investigate the process of linear heart tube looping, developmental asymmetry and trabeculation. Ultimately, an in vitro model of early-stage human
heart development will provide a powerful testbed to explore the mechanism of cardiogenesis and the possibility to develop novel therapies for congenital heart malformations.
Senolytics enhance cardiac recovery after isoproterenol-induced injury in adult mice

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Background: Senescent cells accumulate with ageing, including in the heart, and contribute to tissue deterioration. Due to their senescence-associated secretory phenotype (SASP) they negatively affect an organ’s microenvironment. Clearance of senescent cells, either genetically or pharmacologically using senolytics, has been shown to enhance cardiac function. Our group showed that eliminating senescent cells in aged mice using the senolytics, Dasatanib+Quercetin (D+Q) rejuvenated the heart’s regenerative potential, with progenitor cell activation, new cardiomyocyte formation, and improved cardiac function.

Aim: To investigate the effects of D+Q senolytics on cardiac recovery and remodelling in adult mice following isoproterenol-induced cardiac injury.

Methods: Male mice (~12 weeks) were subcutaneously administered with 150 mg kg⁻¹ isoproterenol (ISO; n=18) or saline (saline; n=18) for six consecutive days. Next, D (5 mg/kg) and Q (50 mg/kg) or vehicle (5% DMSO, 5% ethanol, 50%, polyethylene glycol, and 40% dH₂O) were administered for 5 consecutive days by oral gavage to each group. D+Q treatment started on day 5 after the last ISO or saline dose. The final groups were: ISO-D+Q, saline-D+Q, ISO-vehicle, saline-vehicle, n=9 per group. Echocardiography was performed at baseline, day 7 after ISO, and day 28 after the last D+Q dose. Excised hearts were analysed to assess the cardiac injury and fibrosis, cardiomyocyte hypertrophy, senescence, and SASP factors. ANOVA or Student's t-test was used to analyse the data between 4 groups or 2 groups, respectively.

Results: Ejection fraction and cardiac output decreased (p<0.05) in the ISO-treated group (n=18) compared to the saline group (n=18) on day 7. On day 28 after the last D+Q dose, ISO-D+Q treated mice had improved (p<0.05) ejection fraction and cardiac output (EF: 64.3 ± 5.4%; CO: 22.5 ± 3.9%; n=9) compared to the ISO-vehicle group (EF: 52.6 ± 5.7%; CO: 16.3 ± 3.4%; n=9). No changes were observed for cardiac function in the saline-vehicle (n=9) or the saline-D+Q (n=9) groups. D+Q treatment decreased (p<0.05) the expression of senescence marker, SA-β-gal in the ISO-D+Q group (0.5 ± 0.2%; n=3), compared to the ISO-vehicle group (2.8 ± 0.8%; n=3). Furthermore, the number of p21 cells decreased in the ISO-D+Q group (0.3 ± 0.1%; n=3), compared to the ISO-vehicle group (2.9 ± 0.4%; n=3). SA-β-gal and p21 were not detected in the saline-vehicle (n=3) or the saline-D+Q (n=3) groups. The mRNA expression of senescence markers, p16, p21 and SASP factors, including TGF-β2, IL-6, IL-1, and CXCL10 were decreased ~2-fold (p<0.05) in the ISO-D+Q group (n=3), compared to the ISO-vehicle group (n=3). Cardiomyocyte cross-sectional area was decreased (p<0.05) in the ISO-D+Q group (464.3 ± 59.9μm²; n=6) compared to the ISO-vehicle group (570 ± 69.9μm²; n=6). ISO-D+Q treatment did not significantly change fibrosis, compared to the ISO-vehicle group (ISO-D+Q:2.8 ± 1.9%; ISO-vehicle:3.9 ± 1.7%).

Conclusion: D+Q senolytics decreased senescence and SASP factors after ISO injury, which resulted in improved cardiac function. These findings support the use of senolytics as a potential...
therapeutic for cardiac injury and deterioration. Further pre-clinical and clinical studies are warranted.
Development of a percutaneous myocardial infarction model in 12-week-old rabbits: a platform for regenerative medicine research

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Regenerative medicine research offers promising therapies to regenerate the myocardium after a myocardial infarction (MI) but relevant small animal models are lacking. Rabbit heart has similar coronary circulation, MI scar structure and ventricular electrophysiology to humans and could therefore represent an important small animal model to study electromechanical graft-host coupling in vivo. The standard technique to induce MI in rabbits is surgical coronary ligation (CL) but is limited by procedural severity and thoracic adhesions. Percutaneous coronary occlusion avoids these shortcomings and is widely used in large animal models including pigs. However, it is currently only applicable to large rabbits (> 3.5kg) because of the limited availability of specialized catheters required for the coronary arteries (CA) of smaller animals.

Here, we describe a new approach to percutaneous coronary occlusion in 2.5-3.5kg rabbits. New Zealand White rabbits (n=15, male, 2.5-3.5 kg) were sedated using ketamine (s.c.; 15mg/kg) and medetomidine (s.c.; 0.25mg/kg), intubated and anaesthetized using isoflurane (inhalation; 1.5-3%). Cardiorespiratory output was closely monitored, including two ECG-leads, peripheral pulse pressure sensor, pulse oximeter and capnograph. Continuous ECG recordings were used to confirm the presence of ST-segment changes. The carotid artery was accessed through a surgical cut-down, with the direct insertion of a 4F vascular sheath. Anterior-posterior fluoroscopic cardiac projections were acquired to identify the vasculature. A 4F angiographic catheter was positioned near the coronary ostium using a 0.035” guidewire, followed by contrast injections used for angiography. Five mm of the microcatheter tip (≤1.5F), including radiopaque marker, was cut, and used for coronary occlusion. A 0.007/8” wire was then manoeuvred through the 4F catheter into the distal left CA, whereafter the catheter tip was pushed over the wire by the microcatheter. For sham operated animals (n=5), the CA was instrumented with only the wire. Blood samples were taken to measure troponin (cTnI) levels (detection range: 0.02-180 ng/ml). Ejection fraction (EF) was measured at 6-8 weeks post-occlusion. Hearts were then excised and processed for MRI and histology. Outcomes were compared to the CL model (n=18) or respective sham (n=17).

Blood markers were increased in procedural animals at 24 hr (cTnI range: percutaneous 46.8 to ≥180ng/ml; CL 37.1 to ≥180ng/ml) but was negligible in the sham-groups (≤0.02 to 1.6ng/ml). Left ventricular function was not significantly different between both MI groups (EF: percutaneous 51±10%; CL 49±8%; p=0.89) but was reduced compared to surgical sham (EF: 63±6% p=0.0063). MRI and histological assessment showed comparable scar volume between CL and percutaneous MI hearts. However, the percutaneous procedure resulted in hearts with clean epicardial surface with no adhesions.
The percutaneous MI model is a refinement over the current CL model and can be used to aid the development of post MI therapies including cardiac regeneration. It avoids: 1) post-surgical adhesions that complicate the integration of implanted tissues, 2) the need for a second thoracotomy if cardiac patch implantation is required and finally the procedures allow the option to deliver therapeutic cells or molecules into the coronary circulation at the time of the MI or subsequently.

Examining potential shared mechanisms that govern neovascularisation and lymphangiogenesis in the heart following acute myocardial infarction

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Introduction

Myocardial infarction (MI) is the leading cause of heart failure, affecting ~1–3% of the global adult population (1). Lymphangiogenesis and neovascularisation are key regenerative mechanisms in the heart post-MI and both are known to be augmented by vascular endothelial growth factor (VEGF-C) (2-4). However, clinical studies using VEGF-C have proven problematic, including requirements for repeated invasive dosing (5-6). Identifying dual regulatory mechanisms of both vascular and lymphatic activation, including those downstream of VEGF-C, presents an exciting approach for future therapeutic strategies post-MI.

Hypothesis

Pathways that regulate endogenous vascular and lymphatic coronary regeneration after MI are co-ordinated in a spatial and temporal fashion.

Aims

1. To study single nuclei and spatial transcriptomics data from the human heart to identify genes activated in both vascular and lymphatic EC post-MI.
2. To define the mechanisms through which VEGF-C signalling mediates both neovascularisation and lymphangiogenesis post-MI.
Methods and Results

**Aim (i).** Informatics analysis of single nuclei RNaseq (7) and spatial transcriptomics data (in-house and (5)) identified 14 differentially expressed genes upregulated in both vascular and lymphatic endothelial cells (EC) in patients with acute MI versus the healthy heart. **Aim (ii).** Mice were administered VEGF-C or PBS at 0-, 2-, 4- and 6-days post-MI and hearts collected at 7-days for bulk RNA-sequencing (2). Eighteen genes were identified as activated downstream of VEGF-C. For each aim, 3 genes were selected for experimental validation (ongoing) based on; average Log2 fold change, literature assessment of function, and reproducibility in human MI data (for Aim ii).

Conclusion and Future Studies

We have identified genes with a potential regenerative role in acute MI via co-regulation of lymphatic and vascular EC responses, or as downstream molecular regulators of VEGF-C-mediated regeneration. Validation using immunofluorescence staining in human control heart and acute MI (n=8-10) is ongoing and will be followed by functional assessments in vitro and in vivo. Identification of novel targets that regulate both coronary angiogenesis and lymphangiogenesis after MI may enhance myocardial repair and prevent progression to heart failure.

The Effects of Hypoxic Myocardium on Coronary Vessel Development

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The coronary vascular system is a dense network of arteries, veins and capillaries that provides the blood supply to the heart, and thus its formation and function are crucial for efficient cardiac function. Whilst hypoxia is a known stimulus for the growth of new vessels by sprouting angiogenesis in development and disease, the mechanisms underlying neovascular growth in response to developmental and injury-induced hypoxia in the heart remain poorly understood.

We previously identified three independent transcriptional pathways that regulate different aspects of coronary vessel growth during development and following neonatal injury, but are not reactivated following myocardial infarction (MI) in the adult murine heart. We are now investigating the specific consequences of myocardial hypoxia on these developmental pathways using a cardiomyocyte-specific knockdown of the Phd2 gene. In this model, the hypoxia-responsive HIF pathway is ectopically stabilised, resulting in the induction of the HIF-dependent transcriptional response in the myocardium. This hypoxic environment results in expansion of endocardial-derived angiogenic sprouting, whilst establishment of the sinus-venosus (SV) derived vascular plexus is largely unaffected. However, patterning of the SV-derived coronary veins and the transition of venous to arterial endothelial cells to form the coronary arteries appears to be disrupted. Single cell RNA sequencing on the developing coronary ECs is now being used to identify underlying transcriptional changes that are contributing to these phenotypes.
The role of IncRNA CARMN and its associated miRNAs during scar regression and cardiac repair

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Long non-coding RNAs (LncRNAs) are important regulators of gene expression and function. Their functional contribution to the pathophysiology of cardiovascular disease, including myocardial infarction (MI), is still relatively unknown. The IncRNA CARMN has cardiogenic traits and is known to regulate differentiation of cardiac progenitor cells (CPCs) into smooth muscle cells (SMCs) and cardiomyocytes (CM). However, its role in cardiac repair/regeneration, including the role of the host gene miRNAs miR-143 and miR-145, remains unknown. We found that CARMN is expressed primarily in CM and pericytes of the vertebrate heart and its expression is increased following cardiac injury in zebrafish. Further, CRISPR-generated CARMN zebrafish mutants show impaired cardiac regeneration following injury. In addition, CARMN-/- injured hearts display modified scar composition with impaired deposition of collagen at lesion site, suggesting its role in physiology of scar regression and efficient cardiac repair. Thus, the specific role of CARMN in CPC differentiation and our data suggest its potential implications in cardiac tissue repair in zebrafish, and ultimately in adult mammalian systems.
The cardiovascular impact of intermittent fasting in rats with genetic absence epilepsy in terms of gender difference

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A large number of studies have shown an association between cardiovascular diseases and neurodegenerative disorders. Moreover, seizures which are common conditions in epilepsy, may induce a variety of transient cardiac effects. Intermittent fasting (IF) is one of the dietary restriction protocol which have shown in recent studies that have potential benefits to lose weight and to improve on other heart disease risk factors. We aimed to elucidate the cardiac and aortic protective effects of IF in rats with epilepsy in terms of gender difference.

Female (n=5, in each group) and male (n=5, in each group) rats (10 weeks old) were randomly divided into 3 groups as control (Wistar Albino rats), GAERS (genetic absence epilepsy rats), and GAERS + IF. Rats were deprived of food for a full day, every other day and were fed ad libitum on the intervening day for 1 month in IF groups.

At the end of the experiment the passive avoidance test (PAT) was performed to investigate the 24 hours of retention. The cardiac and aortic tissues were obtained to measure the oxidative stress and antioxidant parameters. Animal care and procedures were followed in accordance with the guidelines of Institutional Animal Ethical Committee. The data were analysed using one-way ANOVA followed by post hoc Tukey test. The non-parametric Kruskal-Wallis test was applied when appropriate. P<0.05 was considered statistically significant.

Compared to control rats in both sexes, elevated cardiac myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels with decreased superoxide dismutase, glutathione and catalase levels in the GAERS groups (p<0.05, 0.01), which were reversed by IF not only in female rats but also in male rats in MPO, MDA and catalase levels (p<0.05). Also the aortic antioxidant levels also prevented with IF in GAERS rats as compared to GAERS group in both sexes. The GAERS group demonstrated a significant memory impairment in PAT in both sexes as compared to the control group (p<0.05). The PAT score was slightly increased in the GAERS+IF group, it was not statistically significant.

The IF application diminished the cardiovascular functions with a prevented oxidant and antioxidant balance, and also eased the memory loss. Taken together, more research is needed to elucidate how IF influences the metabolism of cardiac functions and the relationship between sexes.

Identification of FDA-Approved Drugs that Induce Heart Regeneration in Mammals

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The inability of the adult human heart to regenerate lost or damaged myocardial tissue has created one of the most pressing public health dilemmas due to the devastating impact of heart failure. Our group and others have outlined several regulators of cardiomyocyte mitosis that may impact the regenerative capacity of the adult myocardium in mammals. Recently, we reported that the TALE transcription factors Meis1 and Hoxb13 regulate postnatal cardiomyocyte cell cycle arrest, where concomitant deletion of both genes induced cardiomyocyte proliferation and myocardial regeneration following ischemic injury. These studies suggest that pharmacological targeting of Meis1 and Hoxb13 transcriptional activity could be a viable path toward heart regeneration. Therefore, we performed an in-silico screen to identify FDA-approved drugs that can inhibit Meis1 and Hoxb13 transcriptional activity based on the published crystal structure of Meis1 and Hoxb13 bound to DNA. Our screen yielded a number of top candidates based on binding profiles to either Meis1 DNA binding domain, Hoxb13 DNA binding domain, or the interface between Meis1 and Hoxb13, as well as safety and side effect profiles. Out of the shortlist of top hits, two antibiotics induced the proliferation of neonatal rat ventricular myocytes in vitro and displayed dose-dependent inhibition of Meis1 and Hoxb13 transcriptional activity. X-ray crystallography demonstrated that both antibiotics bind Meis1 at the Hoxb13 interaction domain. Intriguingly, the combination treatment of these antibiotics induced cardiomyocyte mitosis in vivo in adult mice, and improved left ventricular (LV) systolic function following experimental ischemia/reperfusion injury. Finally, intravenous administration of the antibiotics in pigs following ischemia/reperfusion injury induced cardiomyocyte proliferation, improved LV systolic function, and decreased scar formation. Collectively, these results identify the paromomycin-neomycin combination as FDA-approved drug with therapeutic potential for the induction of heart regeneration in humans.
Using single-cell techniques for evaluating the role of Endothelial Cells in matrix remodelling in context of cardiac regeneration in neonatal mice.

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Background:

Following Myocardial Infarction (MI), the myocardium heals through a process of scarring in which healthy heart muscle is replaced by non-contractile fibrous tissue causing high morbidity due to arrhythmia, ventricular remodelling, and heart failure¹. Remarkably, rather than forming a permanent fibrous scar, neonatal mice (P1) regenerate functional cardiomyocytes, and a provisional collagen 'patch' at the site of injury is actively resorbed². By post-natal day 7 (P7) this property is lost, and mice form permanent scars, as in humans. The understanding of important cell types and key processes involved in cardiac regeneration in neonatal mice up to P7 is limited.

Methods:

To identify the important cell types and candidates associated with neonatal heart regeneration, we performed single-cell RNA sequencing (scRNA-seq) experiments on regenerative (P1) and non-regenerative (P7) hearts in healthy, 1- and 7-days post induction of MI in mice (n=3 for each condition). We hypothesised that changes in chromatin accessibility during development is associated with the differential gene expression in the hearts of regenerative P1 and non-regenerative P7 mice. We performed single-cell ATAC-seq on P1 and P7 hearts (n=6 for each condition) to delineate the changes in chromatin accessibility in single-cell populations in hearts. The scRNA-seq and scATAC-seq data were generated using 10x genomics platform and analysed using R-based packages Seurat and Signac, respectively.

Result:

The study reveals dysregulation of functionally important genes implicated in the processes of ECM remodelling and angiogenesis in Endothelial cells. The most significant differentially regulated genes, upregulated in the regenerative groups was Timp4, a matrix metalloproteinase inhibitor that regulates matrix remodelling. Independent works have established that Timp4 plays a protective role in MI and mice lacking Timp4 have compromised ECM, increased accumulation of neutrophils and increased post-MI mortality³. Several animal and cell culture studies in the recent years have established the importance of ECM remodelling in promoting cardiac regeneration⁴. We also observed an upregulation of Plvap, an EC-specific gene associated with angiogenesis and is known to be upregulated in the ECs in the border zone in infarcted hearts⁵. We performed single-cell ATAC-seq to map the differences in chromatin landscape between regenerative and non-regenerative hearts and observed differential chromatin accessibility associated with the Timp4 and Plvap genes in the regenerative mice compared to the non-regenerative mice.

Conclusion:
Our preliminary results based on scRNA-seq and scATAC-seq from P1 and P7 hearts point towards an important role of Endothelial cells in the P1 hearts. We have identified upregulation of *Timp4* in regenerative hearts, and propose that Endothelial Cells in the hearts of regenerative neonatal (P1) mice regulate ECM remodelling and promote angiogenesis, both the processes being necessary (but not sufficient) in promoting regeneration of cardiomyocytes after MI.