

# Cardiovascular Research

## Heart Regeneration – beyond new muscle and vessels

--Manuscript Draft--

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<b>Abstract:</b>	<p>The most striking consequence of a heart attack is the loss of billions of heart muscle cells, alongside damage to the associated vasculature. The lost cardiovascular tissue is replaced by scar formation, which is non-functional and results in pathological remodelling of the heart and ultimately heart failure. It is, therefore, unsurprising that the heart regeneration field has centred efforts to generate new muscle and blood vessels through targeting cardiomyocyte proliferation and angiogenesis following injury. However, combined insights from embryological studies and regenerative models, alongside the adoption of -omics technology, highlight the extensive heterogeneity of cell types within the forming or re-forming heart and the significant crosstalk arising from non-muscle and non-vessel cell types. In this review, we focus on the roles of fibroblasts, immune cells, conduction system and nervous system cell populations during heart development and we consider the latest evidence supporting a function for these diverse lineages in contributing to regeneration following heart injury. We suggest that the emerging picture of neurologically, immunologically and electrically coupled cell function calls for a wider-ranging combinatorial approach to heart regeneration.</p>

## Abstract

The most striking consequence of a heart attack is the loss of billions of heart muscle cells, alongside damage to the associated vasculature. The lost cardiovascular tissue is replaced by scar formation, which is non-functional and results in pathological remodelling of the heart and ultimately heart failure. It is, therefore, unsurprising that the heart regeneration field has centred efforts to generate new muscle and blood vessels through targeting cardiomyocyte proliferation and angiogenesis following injury. However, combined insights from embryological studies and regenerative models, alongside the adoption of -omics technology, highlight the extensive heterogeneity of cell types within the forming or re-forming heart and the significant crosstalk arising from non-muscle and non-vessel cell types. In this review, we focus on the roles of fibroblasts, immune cells, conduction system and nervous system cell populations during heart development and we consider the latest evidence supporting a function for these diverse lineages in contributing to regeneration following heart injury. We suggest that the emerging picture of neurologically, immunologically and electrically coupled cell function calls for a wider-ranging combinatorial approach to heart regeneration.

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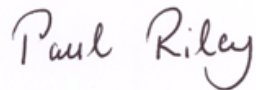
15<sup>th</sup> October 2020

Dear Dr Marian and Dr Guzik,

Thank you for your email, dated 13<sup>th</sup> October 2020, informing us of the decision on our revised manuscript/review article: "*Heart Regeneration – beyond new muscle and vessels*", reference: **CVR-2020-1205**.

Please find accompanying this cover letter the newly revised manuscript and response-to-reviewers.

Yours sincerely,

A handwritten signature in purple ink that reads 'Paul Riley'.

Professor Paul R. Riley

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Thank you for your email, dated 13<sup>th</sup> October, informing us of the decision on our revised manuscript/review article: "*Heart Regeneration – beyond new muscle and vessels*", reference: **CVR-2020-1205**.

We thank the reviewers and editorial for their informed comments and are pleased to have an opportunity to further revise the manuscript.

**In terms of addressing the editor's notes:**

*The Reviewers and the Editors are enthusiastic about publication of your manuscript in Cardiovascular Research. However, Reviewer 3 raises excellent points, pertaining to the paucity of the data on myocyte regeneration and contributions of the non-myocyte cells to this process in the adult mammalian hearts. Please address the points raised by the Reviewer and submit a revised manuscript. A responsive revision will be handled by the Editors only.*

We thank editorial for their recommendation and have now further revised the manuscript in order to address Reviewer 3's remaining comments. In particular, we have expanded our introduction (pages 6-7) in order to address the points raised by the Reviewer, as detailed below.

**In terms of addressing the reviewer's comments:**

**Reviewer #1:**

*The review further improved through this revision. My comments were all addressed.*

**Reviewer #2:**

*The authors made a commendable work in considering and replying to all the reviewers' comments.*

**Reviewer #3:**

*The authors have elegantly avoided to seriously tackle my comments in their revised manuscript. Most of their answers missed my points. First, they did not comment on the absence of evidence that myocardial regeneration recapitulate myocardial development.*

We have added a section to the Introduction in order to emphasise the absence of conclusive evidence for regeneration recapitulating development, and to explain that the extent to which transcriptional networks used in development are reactivated in regeneration remains an open question; please see pages 6-7, lines 134-138.

*Second, no comment on the limits of studying neonatal cardiac regeneration to translate it to adult regeneration.*

We have included comment on the limits of studying neonatal cardiac regeneration in translating to adult regeneration more directly; please see page 6, lines 115 -124.

*Nevertheless, they at least tried to be more open-minded including adult progenitors as potential targets for myocardial regeneration.*

*It is disappointing that the authors failed to take my comment to be more open-minded in projecting these cells on potential targets of myocardial regeneration. Indeed, the idea of regulating and supportive cells squarely fits with the presence of a tissue resident endogenous stem/progenitor cell population whose niche, their regenerative unit, could be indeed regulated by the actions of cardiac fibroblasts, immune cells, etc etc.*

We thank the reviewer for this comment. We have further amended our discussion of progenitor cells to mention the possibility that non-cardiomyocyte signalling may affect an endogenous regenerative “niche”; please see page 7, lines 146-148.

**Reviewer #4:**

*This is a timely and interesting review and there are no further suggestions*

Yours sincerely,

A handwritten signature in dark ink that reads "Paul Riley". The signature is written in a cursive, slightly slanted style.

Professor Paul R. Riley

## Heart Regeneration – beyond new muscle and vessels

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# Heart Regeneration – beyond new muscle and vessels

## Abstract

The most striking consequence of a heart attack is the loss of billions of heart muscle cells, alongside damage to the associated vasculature. The lost cardiovascular tissue is replaced by scar formation, which is non-functional and results in pathological remodelling of the heart and ultimately heart failure. It is, therefore, unsurprising that the heart regeneration field has centred efforts to generate new muscle and blood vessels through targeting cardiomyocyte proliferation and angiogenesis following injury. However, combined insights from embryological studies and regenerative models, alongside the adoption of -omics technology, highlight the extensive heterogeneity of cell types within the forming or re-forming heart and the significant crosstalk arising from non-muscle and non-vessel cell types. In this review, we focus on the roles of fibroblasts, immune cells, conduction system and nervous system cell populations during heart development and we consider the latest evidence supporting a function for these diverse lineages in contributing to regeneration following heart injury. We suggest that the emerging picture of neurologically, immunologically and electrically coupled cell function calls for a wider-ranging combinatorial approach to heart regeneration.

## Introduction

The heart is the earliest organ to form and begin to function in the developing embryo proper. As the contractile pump responsible for ensuring blood flow, oxygenation and concurrent nutrient delivery around the developing organism, maintaining heart function throughout development is essential. This must be achieved in the face of significant morphological change, as the vertebrate heart grows and remodels itself from a linear heart tube, through looping and septation, to form the four-chambered mature organ.

During development, the heart must do more than just continue to beat; it must become vascularised, a necessary event in order to deliver oxygen and nutrients to the developing cardiomyocytes, to maintain contractile activity. Concurrently, heart growth and expansion require extracellular matrix deposition and turnover. The heart must become integrated with the developing immune system, such that tissue-resident immune populations localise to the forming organ and contribute to its continued development and homeostatic function<sup>1</sup>. The heart must restrict its pacemaker activity so that it establishes a circuit of electrical control, to ensure coordinated, rhythmic contraction<sup>2</sup>. Lastly, the heart must become innervated by both afferent and efferent branches of the peripheral nervous system, to provide precise fine-tuning of heart function according to physiological needs<sup>3</sup>. Deciphering the spatiotemporal control of these various processes is key to forming a comprehensive picture of cardiac development.

Growth of the heart occurs during both foetal and postnatal stages of heart development. While foetal heart growth occurs through cardiomyocyte proliferation, postnatal stages of heart development involve hypertrophic growth of individual muscle cells without additional cell division, as the cardiomyocytes lose their capacity to divide<sup>4</sup>. During early post-natal development coincident with retained proliferative capacity, the neonatal mammalian heart is principally formed of mono-nucleated, diploid cardiomyocytes; as development proceeds and proliferative capacity is lost, this composition shifts as many cardiomyocytes become bi- or multinucleated and polyploid<sup>5</sup>. The extent to which mature cardiomyocytes are multinucleate and polyploid differs extensively between mammalian species, however: while human adult cardiomyocytes are generally uninucleate and often tetraploid, almost 80% of mouse and over 90% of pig adult cardiomyocytes are multinucleate<sup>5</sup>.

At the earliest stages of development, the heart forms as an avascular heart tube<sup>6</sup>. Vascularisation occurs first through angiogenesis, and then through a combination of



angiogenesis and vasculogenesis. While the former involves de novo vessel growth from angioblasts, the latter occurs through sprouting and branching of existing vasculature. The developing coronary plexus integrates with the myocardial layer<sup>6</sup>. Once the coronary artery reaches the root of the aorta, blood flow begins, and the associated shear stress helps to direct further vasculogenesis<sup>7</sup>. The principle cell types involved in these processes include endothelial populations that form the vessel walls, and mural/perivascular populations that line the abluminal vessel surface to help direct vascular development and maturation<sup>8</sup>. In addition to contributing to the vasculature, endothelial cells line the lymphatics, and specialised endothelial cells form the endocardium, which constitutes the innermost surface of the heart. This endocardial population acts as a signalling centre in development and disease, but also contributes to forming valves and to cardiac fibroblast populations through endothelial to mesenchymal transition (EndoMT) (reviewed in<sup>9</sup>). Emerging evidence highlights the importance of intercellular communication between diverse endocardial and cardiomyocyte populations in development and disease<sup>10</sup>.

Cardiomyocytes and endothelial cells are the major cell types in the embryonic and adult heart. Consequently, studies have focused extensively on their development. Other important cell types have received less attention; these include the cardiac fibroblasts which regulate the structural organisation of the heart; tissue-resident immune cells which are emerging important contributors to heart development, including via remodelling of the developing vasculature; cardiac conduction system (CCS) specialist cardiomyocytes which ensure the propagation of rhythmic contraction; and nervous innervation to ensure the development of “fight or flight” responses<sup>11, 12</sup> (Figure 1).

The human adult heart is one of the least regenerative organs in the body. The heart shows a general inability to regenerate spontaneously following myocardial infarction (MI) or other pathological events such as viral infection (myocarditis). The adult heart instead undergoes

fibrosis post-injury, whereby non-contractile scar tissue is deposited without replacement of the lost cardiomyocytes or supporting vasculature<sup>13</sup>. A further round of pathological heart remodelling generally follows initial scarring, involving associated interstitial fibrosis. These events severely impact the overall contractility and mechanical environment of the heart tissue, and therefore impair cardiac function<sup>14</sup>. This is a major contributing factor to long-term heart disease and onset of heart failure, for which no curative treatments currently exist. The heart regeneration field, which seeks to regenerate cardiac tissue to restore full functionality to the damaged heart, provides great promise but has historically been a victim of hype, bias and fraudulence (as recently reviewed in <sup>15, 16</sup>). Any regenerative strategy must overcome two central hurdles to restore function: it must replace lost cardiomyocytes and coronary vessels, and remove persistent fibrotic tissue to relieve stiffening strain on the contractile cells. While focusing on restoring heart muscle and replenishing lost coronary vessels is clearly important, mounting evidence suggests that achieving full regeneration will require fibroblast, immune, conductive and nervous cell contributions to the regenerative process.

While the adult mammalian heart shows very limited regenerative capacity<sup>17</sup>, model systems exist which exhibit a full regenerative response. Notably, the zebrafish heart retains its ability to regenerate the heart to full functionality throughout adulthood<sup>18</sup>; the same is true of species with general regenerative ability such as the axolotl and salamander, which are able to regenerate whole limbs as well as their hearts<sup>19, 20</sup>. The neonatal mouse and pig both retain cardiac regenerative capacity immediately following birth, but this is lost by postnatal day 3 in porcine neonates and similarly is lost around day 4 in mouse neonates, following which point cardiomyocytes are generally thought to be unable to proliferate<sup>21-24</sup>. In humans, clinical case reports have indicated functional regeneration and an absence of scarring after cardiac surgery to treat congenital heart disease<sup>25, 26</sup> and in infants born with MI who have

107 blood flow restored<sup>27</sup>. Evidence of regeneration has also been reported in post-mortem  
108 analyses of infant hearts<sup>28</sup>.

109  
110 Together this indicates that humans have neonatal regenerative capacity, which is lost in  
111 transition to adulthood. However, <sup>14</sup>C dating and stereology analysis have identified low-level  
112 cardiomyocyte exchange that continues into adulthood<sup>17</sup>; the existence of such innate cell  
113 turnover provides a promising target for amplification to increase the endogenous  
114 regenerative capacity of the mature heart. Differences exist between the neonatal and adult  
115 environment, particularly in terms of cell ploidy, and the mono-, bi- or multi-nucleated state  
116 of cardiomyocytes<sup>4, 5</sup>. While neonatal regeneration depends on cardiomyocyte proliferation,  
117 evidence as to whether adult cardiomyocytes can re-enter the cell cycle and proliferate is only  
118 just emerging and remains somewhat inconclusive. This suggests that a straightforward  
119 parallel between neonatal regenerative capacity and the adult injury setting may be  
120 challenging, especially in terms of translating neonatal findings directly into regenerative  
121 therapeutics. However, increased understanding of the non-cardiomyocyte cell types and  
122 their signalling interactions in neonates may help to identify the supportive parameters  
123 promoting neonatal regeneration and to develop fundamental understanding with relevance  
124 to the mature heart.

125  
126 Through deepening our understanding of the changes triggered during naturally occurring  
127 heart regeneration across model organisms, the hope is that this knowledge will help inform  
128 intervention strategies to bring about similar regeneration in injured human adult hearts.  
129 What appears to be fundamental to this understanding is an appreciation that regeneration,  
130 at least in part, recapitulates many of the cellular and molecular mechanisms involved in heart  
131 development. Although several fundamental differences exist between the embryonic and  
132 post-injury landscapes – including injury-induced inflammatory responses and the presence  
133 of fibrotic scar tissue – many of the transcriptional networks used in development seem to be

reactivated following heart damage<sup>29</sup>. The extent to which transcriptional networks from development are “reactivated” following injury, versus whether changes in chromatin structure between developmental and adult cell types result in different enhancer availability driving related - but different - mature genetic regulatory networks, remains an open question<sup>30</sup>. The focus to-date has been on promoting cardiomyocyte proliferation through targeting so-called adult cardiac progenitors, or through restoring cell-cycle activity, re-instating embryonic or neonatal potential and stimulating developmental angiogenesis<sup>31</sup>. Regenerative strategies targeting endogenous mechanisms have centred around reactivating proliferation of the existing cardiomyocyte pool, identifying and targeting adult cardiac progenitor populations or reprogramming fibroblasts into cardiomyocytes in vivo, and reactivating the epicardium to support revascularisation. Increasingly, however, it is becoming evident that other key cardiovascular cell types need to be reconstituted from development to optimise cardiac repair and promote tissue regeneration. Extensive signalling from these cell types may alter the local cardiomyocyte “niche” to determine both whether the cells proliferate, and the balance between fibrotic repair versus regeneration.

This review will consider recent advances in developmental studies on the heart, combined with insight from regenerative studies across multiple organisms. In so doing, we hope to identify unifying themes from constituent cell types, besides the cardiomyocyte and vasculature cell populations, that will facilitate further insights into promoting complete heart regeneration.

## **1. Cardiac fibroblasts**

Fibroblasts are the most numerous cardiac cell type besides those that form the heart muscle and vasculature. Fibroblasts constitute approximately 20% of the mouse heart by cell number<sup>29</sup>, laying down the heart’s scaffold and forming interstitial populations that connect directly with cardiomyocyte and endothelial cells. Although fibroblasts have historically been

thought of merely as biological “glue”, extensive evidence (aided by the availability of new mouse strains) now indicates that fibroblasts across organs including the heart play central signalling, inflammatory and mechanical roles in development and disease<sup>32</sup>. Although an abundant cell type, defining the cardiac fibroblasts has proved contentious due to a lack of definitive markers and specific fate-mapping studies. Cardiac fibroblasts are considered a specific cell type of the heart, as opposed to a general mesenchymal population. Comparisons between transcriptomic datasets from the mouse heart and tail reveal that cardiac fibroblasts are transcriptionally more similar to cardiomyocytes than to fibroblasts resident in other organs<sup>33-35</sup>. Classically defined as mesenchymal cells that can secrete type I collagen, fibroblasts are additionally now grouped by embryonic origin, anatomical location and transcriptional signature<sup>11</sup>. Identifying fibroblasts definitively is confounded by the extensive heterogeneity and plasticity of fibroblast subtypes<sup>11, 36</sup>. Pericytes – fibroblast-like cells that associate with the cardiac vasculature – are distinct from cardiac fibroblasts but overlap via expression of several fibroblast markers. Pericytes and their roles in supporting vasculature have been reviewed extensively elsewhere and will not be considered further here.

### **1.1 Developmental origins of cardiac fibroblasts**

The cardiac fibroblasts derive principally from a combination of epithelial and endothelial sources (Figure 2). The former contribution results from a mesothelial pro-epicardial cell population that migrates over the looped heart tube, directed by Transforming Growth Factor beta (TGF- $\beta$ ), Wnt, Retinoic Acid (RA) and Bone Morphogenic Protein (BMP) signalling<sup>37</sup>. These pro-epicardial cells first form the epicardium, the outer mesothelial layer of the heart; subsequently, a cell population residing within the epicardium undergoes epithelial to mesenchymal transition (EMT) to form cardiac fibroblasts. Newly-formed cardiac fibroblasts migrate into the underlying ventricular wall and become embedded within the forming myocardium or constitute interstitial connective tissue at mouse stage E14.5<sup>38</sup>. Epicardial-derived fibroblasts are defined by the expression of Transcription factor 21 (TCF21), Wilms’

tumour protein (WT1) and T-box 18 (TBX18), and comprise approximately 85% of cardiac fibroblasts<sup>39</sup>. The other major fibroblast population originates from an endothelial population in the outflow tract, that undergoes endothelial to mesenchymal transition (EndoMT) from E11.5 and becomes enriched in the intraventricular septum<sup>38</sup>. Endothelially-derived fibroblasts are identified by expression of the transcription factor TIE2<sup>39</sup>. Finally, lineage tracing reveals a much smaller subset of cardiac fibroblasts of neural crest (NC) origin which mainly reside in the right atrial myocardium and seem to persist into adulthood<sup>40</sup>.

The distinct developmental origins of cardiac fibroblast populations have recently been implicated in distinct fibroblast activation post-injury. For example, lineage tracing finds that under 1% of fibroblasts of endocardial (or endothelial) origins express Periostin (Postn) following MI, a small extracellular matrix protein with baseline expression in homeostatic but heightened expression in injured hearts and well-studied roles in cardiac remodelling<sup>41</sup>. Contrastingly, almost all epicardial-derived fibroblasts express Postn in the same injury setting. This suggests that understanding the differences between epicardial, endocardial and NC fibroblast subpopulations will inform our understanding of fibroblast activation following cardiac injury.

## **1.2 Roles of cardiac fibroblasts during development**

Cardiac fibroblasts play several important roles in mediating normal heart development. Indeed, disrupting cardiac fibroblasts genetically results in the development of a heart with reduced ventricular chamber volume and myocardial thickness, and septation defects<sup>34</sup>. Cardiac fibroblasts signal via  $\beta$ 1-integrins to ensure correct myocardial growth occurs over development<sup>42</sup>, the absence of which results in ventricular cardiomyocyte hypertrophy and impaired compaction.

Cardiac fibroblasts are interspersed with cardiomyocytes in the developing heart and are thought to electrically couple with neighbouring cardiomyocytes to assist with contractile synchrony, although the existence and extent of fibroblast-cardiomyocyte coupling in native myocardium remains controversial<sup>43</sup>. While in vivo evidence is lacking, fibroblasts can directly modify neonatal cardiomyocyte excitability in rat cardiac myocyte: fibroblast co-cultures<sup>44</sup> and in linearly structured cell cultures fibroblasts have been found to electrically couple groups of cardiomyocytes over distances of up to 300µm<sup>45</sup>.

Cardiac fibroblasts are the principle cell type responsible for deposition of the cardiac extracellular matrix (ECM) during development<sup>46</sup>. The cardiac ECM is the structural scaffold of the heart, responsible both for mechanical support and for coordinating signal transduction. The cardiac ECM consists of structural elements including fibrillar collagens and fibronectin, and matricellular proteins including Postn that mediate local signal transduction<sup>46</sup>. Deposition of ECM is required to support cellular adhesion, to direct forming myocardial tissue architecture and to promote developmental signalling in a spatiotemporally controlled manner.

### **1.3 Fibroblasts mediate post-injury scar formation**

In the homeostatic adult heart, fibroblasts are a largely quiescent, non-proliferative cell type; although important to aid cardiac contraction, they contribute little to ECM turnover or signalling activity<sup>47</sup>. However, cardiac stress, induced by injury and local hypoxia, can lead to fibroblast activation<sup>46</sup>. Activated fibroblasts are characterised by increased proliferation, increased matrix deposition and secretion of cytokines and growth factors<sup>11</sup>. Activation is mediated by proteoglycans and their interactions with cytokines, including TGF-β<sup>48</sup> and osteopontin, a matricellular protein that is cleaved by thrombin and actively regulates the transition towards the fibroblast activated state by driving collagen production<sup>49</sup>. Proteoglycan Syndecan-4 protects osteopontin from thrombin-mediated cleavage; loss of

Sydecin-4 in later cardiac remodelling stages is suggested to support fibrotic progression by enabling osteopontin-driven fibroblast activation<sup>49</sup>.

Historically, the origin(s) and identity of activated fibroblasts post-injury have been controversial. Lineage tracing suggests that the tissue-resident cardiac fibroblasts of the homeostatic heart are the major source of activated fibroblasts following injury<sup>48</sup>. Disease-activated fibroblasts have generally been thought to differentiate into myofibroblasts, a cell type characterised by expression of Postn and of contractile proteins, especially  $\alpha$ -SMA<sup>41</sup>. It now seems in the heart that alongside myofibroblasts, a subset of fibroblasts become activated and upregulate Postn without taking on the contractile myofibroblast phenotype<sup>41</sup>.

While excessive scar deposition results in stiffening of the local heart tissue, with resultant loss of contractility and pathological pressure overload, initially scarring is essential to avoid ventricular rupture or cardiomyocyte hypertrophy. The need to encourage a certain level of fibroblast activation is evident from a recent study in which the primary cilium was disrupted in cardiac fibroblasts by knocking out Polycystin-1 (PC1) encoding gene *Pkd1*. Ciliary depletion resulted in impaired TGF- $\beta$  signalling and reduced ECM production; in vivo, PC1 depletion impaired remodelling following MI<sup>50</sup>. The precise balance of signalling crosstalk in the immediate post-injury microenvironment, therefore, seems to be crucial in determining the extent of beneficial versus pathological fibrosis and remodelling that ensues. A distinction can be drawn between replacement fibrosis, in which collagenous scar tissue is deposited in place of dead cardiomyocytes, and interstitial fibrosis, which often follows pressure overload or infection as opposed to acute injury and involves ECM deposition in the interstitial space<sup>36</sup>. While the former is initially cardioprotective, preventing ventricular rupture following largescale cell loss, the latter seems to be overwhelmingly detrimental to cardiac function.



Fibrosis is not incompatible with cardiac regeneration. In zebrafish, following ventricular cryo-injury, scar tissue is initially laid down and then later resolved following cardiomyocyte proliferation. Pre-existing cardiac fibroblasts together with a few newly formed endocardial fibroblasts synthesise collagen immediately following zebrafish cryo-injury<sup>51</sup>, which supports subsequent cardiomyocyte proliferation. After proliferating cardiomyocytes replace the dead cells of the infarct, the neighbouring fibroblasts return to their quiescent state, revealing expression profiles that closely mirror the uninjured cell population, although some collagen differences persist<sup>51</sup>. Single cell sequencing shows that cardiac fibroblast gene expression profiles in zebrafish closely match mammalian signatures following injury<sup>51, 52</sup>, suggesting conserved fibrotic pathways that are compatible with regeneration<sup>46</sup>. Furthermore, following ventricular resection and regeneration in the newt, initially matrix metalloproteinases (MMPs), ECM genes, Fibronectin and Tenascin C are the most upregulated factors after injury<sup>53</sup>, suggesting ECM deposition is also important in the newt pro-regenerative environment. Fibrillar type V collagen has recently been implicated in regulating scar size in the infarct zone following ischaemic injury in mice: *Col5a1* conditional knockout mice have modulated mechanosensitive feedback post-injury<sup>54</sup>. In the absence of Collagen V, altered integrin-dependent signalling leads to increased myofibroblast differentiation and ECM deposition, suggesting that the collagen make-up of the scar influences extent of fibroblast activation and subsequent matrix remodelling.

ECM deposition affects cardiomyocyte proliferative and migratory ability. Increasing ECM stiffness in in vitro cultures of neonatal mouse or rat cardiomyocytes results in altered myoskeletal organisation and promotes cardiomyocyte cell cycle arrest<sup>55</sup>. Co-culturing primary rat cardiomyocytes with postnatal cardiac fibroblasts, but not with fibroblast-conditioned medium alone, increases cardiomyocyte binucleation suggesting that it is the direct ECM matrix modification activity of cardiac fibroblasts that inhibits cardiomyocyte cytokinesis<sup>56</sup>. Comparing the make-up of the cytokinesis-permissive embryonic versus

cytokinesis-restrictive postnatal fibroblast ECM identifies embryonic SLIT2 and Nephronectin as ECM proteins responsible for discrepancies in cardiomyocyte proliferative capability as development progresses. Developing a deeper understanding of the signalling and microenvironmental mechanical differences during the second round of fibrosis, that result in pathogenic remodelling in the injured adult mammalian heart, but regeneration in zebrafish, newts and mammalian neonates, will aid in paving the way towards regeneration (Figure 3).

#### **1.4 Non-canonical roles for (myo)fibroblasts post-injury**

Several non-canonical roles for (myo)fibroblasts in the injured heart have recently been identified. Notably, in mice myofibroblasts engulf dead and apoptotic cells in the infarct region, following which they acquire anti-inflammatory properties. Knocking out milk fat globule-epidermal growth factor 8 (*MFGE8*), the growth factor secreted by myofibroblasts to promote phagocytosis, caused apoptotic cells to be cleared less effectively, with an associated increase in mortality<sup>57</sup>. Moreover, analysis of the fibroblast secretome at 1, 3 or 7 days post-MI finds that day 3 fibroblasts promote angiogenesis through cytokine production, while day 7 fibroblasts repress angiogenesis through Thrombosin 1 (Thbs1) signalling, implicating fibroblasts as temporal mediators of inflammation and angiogenesis<sup>58</sup>. (Myo)fibroblasts also mediate inflammatory recruitment of neutrophils and monocytes to the injured heart<sup>59, 60</sup>. Together, this suggests that myofibroblasts are playing cardioprotective roles besides scar deposition following injury, acting in close concert with the immune system.

(Myo)fibroblasts can also modify cardiomyocyte electrical excitability. While both quiescent and activated fibroblasts post-injury express Connexins, Cx43 expression levels have been found to increase in (myo)fibroblasts in rats immediately following MI, as compared to sham controls<sup>44, 61</sup>. Changes in Connexin expression were observed in both myocyte and fibroblast populations following MI in sheep: within 12 hours post-injury, myocyte Cx43 spatial

patterning changes included redistribution of Cx43 from intercalated discs to the lateral domain of damaged myocytes; within 24 hours fibroblasts expressing Cx45 infiltrated the infarct zone; Cx43 fibroblast expression then increased and remained high for several weeks<sup>62</sup>. Moreover, patch clamp measurements from (myo)fibroblast: myocyte co-cultures of fibroblasts from normal or infarcted hearts found that following treatment with media conditioned by infarct-derived fibroblasts, a lower conduction velocity was measured in myocytes. Fibroblasts from infarcted hearts showed more hyperpolarised resting potentials and heightened coupling with neighbouring myocytes<sup>61</sup>. Collectively, these studies suggest that changes to fibroblast: cardiomyocyte coupling may directly affect myocyte excitability in the injured heart.

Controversy surrounds whether cardiac fibroblasts can transdifferentiate to form other cell types – and whether on activation they are ‘primed’ to do so. Transdifferentiation of human fibroblasts to endothelial cells has been achieved in vitro by innate immune activation to promote so-called mesenchymal to endothelial transition (MEndT)<sup>63</sup>, although lineage tracing has failed to identify a cardiac fibroblast contribution to regenerating endothelium in vivo<sup>64</sup>. Moreover, lineage tracing and in vivo transplantation experiments have identified partial transdifferentiation to a calcium depositing cell type resembling osteoblasts<sup>65</sup>. Considerable hype surrounds the potential transdifferentiation of fibroblasts to cardiomyocytes<sup>33, 66, 67</sup>. While lineage tracing finds that epicardial-derived fibroblasts do not transdifferentiate to form new cardiomyocytes<sup>39, 40</sup>, the equivalent analysis has not yet been performed for endocardial-derived fibroblasts, which are sometimes considered more “primed” for transdifferentiation given their transcriptional profiles<sup>36</sup>.

## **1.5 Manipulating fibroblast biology as a therapeutic approach**

Attempts to alter fibrosis by ablating cardiac fibroblasts have proven detrimental to cardiac function<sup>41, 52</sup>; this is unsurprising in hindsight given the identified cardioprotective roles of

fibroblasts in initial scar deposition and signalling. However, significant promise remains via therapeutic attempts to alter fibroblast transcriptional signatures or reprogramme fibroblasts into other cardiac lineages.

Initial attempts have focused on converting cardiac fibroblasts directly into cardiomyocytes: this could serve both to replace the dead cardiomyocytes lost during injury, and to limit the quantity of scar tissue deposited, to prevent pathogenic cardiac remodelling. Ieda et al. demonstrated that direct reprogramming of postnatal cardiac or dermal fibroblasts into cardiomyocytes is achievable in vitro using just three transcription factors, Gata4, Mef2c and Tbx5<sup>68</sup>. Further, human cardiac fibroblasts have been directly reprogrammed to cardiomyocytes<sup>33, 67</sup>. Subsequent studies have improved the speed and efficiency of direct reprogramming, most notably through using a tissue-engineered 3D hydrogel environment<sup>69</sup> and there is in vivo evidence for reprogramming of fibroblasts to form cardiomyocytes<sup>70, 71</sup>. Deriving a means to target cardiac fibroblasts specifically, without affecting general fibroblasts or neighbouring non-fibroblast cell types, will be important therapeutically. Moreover, given the stiffness and complexity of the mature mammalian cardiac ECM, it is increasingly clear that an effective regenerative approach should also manipulate the cellular microenvironment to support pro-regenerative ECM formation and anti-inflammatory, anti-fibrotic fibroblast-macrophage activity.

## **2. The immune system in the developing and regenerating heart**

Since the relatively recent identification of tissue-resident macrophage populations in the developing heart<sup>72, 73</sup>, the role of immune cells in cardiac development has received significant renewed interest. Recent evidence hints at extensive crosstalk between immune populations – especially macrophages – and tissue-resident fibroblasts, conductive cells and nerve fibres in heart development, homeostasis and injury.

## 2.1 Tissue-resident immune cells in development and homeostasis

Tissue-resident immune cells in the mouse heart include mast cells, macrophages, and smaller populations of T and B cells<sup>29</sup>. Mast cells, originally derived from precursor cells in the bone marrow, become seeded in the perivascular space and connective tissue of the heart and mature upon receipt of the c-Kit ligand, stem cell factor (SCF) in the local microenvironment<sup>74</sup> to become an integral part of the cardiac tissue. Tissue-resident macrophages are a heterogeneous cell population that make up 7-8% of non-cardiomyocytes by cell number in the mouse heart<sup>29, 75</sup>. Genetic fate mapping and parabiosis studies have traced the developmental origins of tissue-resident macrophages in the homeostatic adult mouse heart to the foetal yolk sac (Figure 2)<sup>72, 75</sup>. These cells seed the forming heart through epicardial-derived instructive signalling<sup>76</sup> and while it was initially suggested that bone marrow monocyte populations replenish this tissue-resident macrophage population with age, the growing consensus is that this population self-renews, with minimal input from monocytes, although this remains an area of some debate<sup>72, 75, 77</sup>.

Traditionally, cardiac macrophages have been categorised as CCR2-MHC II(low), CCR2-MHC II(high) or CCR2+MHC II(low). The CCR2- populations represent those with embryonic origins, whereas the CCR2+ contribution derives from circulating blood monocytes and may contribute 5-15% of the macrophage population during homeostasis<sup>78</sup>. The MHC II(low) subpopulations are responsible for removal of debris exocytosed by cardiomyocytes or fibroblasts, and remodelling in development and adulthood to reduce tissue strain and support long-term organ homeostasis<sup>78</sup>.

The extent of integration between tissue-resident macrophages and neighbouring cardiac cells in homeostasis is exemplified by the recent finding that macrophages couple directly with cardiomyocytes via Cx43 to facilitate electrical conduction through the heart<sup>79</sup>. Macrophages are especially enriched in the atrioventricular node and surrounding tissue, and

photo-stimulation of macrophages expressing channelrhodopsin-2 improved atrioventricular conduction, while conditional knockout of Cx43 in macrophages resulted in delayed conduction. These findings collectively highlight the relevance of intimate coupling of macrophage and cardiomyocyte/ cardiac conduction cell populations to maintain normal contractile function. It will be important to discern how changes in the macrophage population impact electrical conduction through the injured heart in regenerative versus non-regenerative models.

Finally, T and B cells form relatively minor tissue-resident cardiac populations. By cell number, leukocytes represent approximately 9% of cardiac cells, of which the majority are myeloid cells: T and B cell lymphocytes collectively constitute approximately 12% of all leukocytes<sup>29</sup>. Specific functions for tissue-resident T and B cells in the developing and homeostatic heart have not been identified to-date.

## **2.2 Macrophages and neutrophils in cardiac injury**

A well-documented cascade of innate immunity events occurs following cardiac injury, involving an initial inflammatory phase, a proliferative phase and a maturation phase<sup>80</sup>. Cell death at the injury site triggers an inflammatory response mediated by cytokine and chemokine signalling, which recruits leukocytes to the damaged heart. During the subsequent proliferative phase neutrophils and monocytes increase in number. Neutrophils, and then monocytes that differentiate to form macrophages, together with T cells, eosinophils and platelets, infiltrate the cardiac tissue, remove cellular debris and mediate the injury signalling environment. Finally, during the maturation phase macrophages adopt a reparative anti-inflammatory phenotype and signal to activate fibroblasts and to promote angiogenesis, resulting in fibrosis, neovascularisation and localised injury resolution (Figure 3)<sup>80</sup>.

Neutrophils are the first cell population to be recruited to the heart in large numbers following initial inflammation, attracted by cytokines and apoptotic signals released by dying cardiomyocytes<sup>81</sup>. Historically, neutrophil contributions in acute injury have been considered detrimental to heart function, involving secretion of MMPs that degrade cardiac ECM, release of high levels of Reactive Oxygen Species (ROS) and ultimately neutrophil apoptosis<sup>81, 82</sup>. However, recruited neutrophils are a heterogeneous population, with varying roles in the early and intermediate phases of cardiac repair, as indicated by transcriptional changes in neutrophil populations over the first few days post-MI, suggesting certain populations play a more cardioprotective role<sup>83</sup>.

Prolonged retention of neutrophil populations at the site of injury impairs zebrafish heart regeneration following cryoinjury, suggesting that in regenerative contexts neutrophil activity is very time-limited following cardiac stress<sup>84</sup>. Comparative transcriptomics between the zebrafish and another teleost, the non-regenerative medaka, identifies failure to clear neutrophils in a timely manner in the medaka, along with delayed and reduced macrophage recruitment, as determining factors in preventing medaka heart regeneration<sup>85</sup>. Moreover, analysis of mice mutant for Reg3 $\beta$ , a factor released by cardiomyocytes that directs immune cell recruitment to the injured heart, finds that absence of Reg3 $\beta$  leads to failure of neutrophil clearance with consequent scar instability and increased likelihood of rupture<sup>86</sup>. Circadian oscillations in neutrophil recruitment influence the extent of cardiac healing post-MI, with higher mortality from MIs early in the morning<sup>87</sup>: limiting neutrophil-mediated inflammation during the active oscillatory phase resulted in better post-MI cardiac function. Although the precise interactions of neutrophils with the injury microenvironment over time require further study in regenerative and non-regenerative contexts, neutrophils clearly play important roles with both protective and detrimental effects on cardiac repair.

Following initial neutrophil infiltration, macrophages are recruited to the heart and perform diverse signalling roles during injury. Pro-inflammatory cytokine signalling results in rapid deployment of splenic reservoir and bone marrow-derived monocytes to the injury site<sup>88</sup>. Tissue-resident macrophage populations play an active role in mediating monocyte recruitment: CCR2+ macrophages promote monocyte recruitment via MYD88, whereas CCR2- macrophages block monocyte recruitment<sup>89</sup>. Controversy remains as to whether tissue-resident macrophages die post-injury and are replaced by circulating monocytes long-term, or whether tissue-resident macrophages self-renew after injury<sup>72, 75, 77</sup>. Collectively, tissue-resident and recruited macrophages signal via TGF- $\beta$ , Vascular Endothelial Growth Factor (VEGF) and Interleukin 10 (IL-10); and have well-documented roles in promoting angiogenesis<sup>90, 91</sup> and in activating fibroblasts<sup>92</sup>.

An injury classification system distinguishes between early M1 (inflammatory, Ly6C(high) expression, remove necrotic debris) and later M2 (alternatively activated, Ly6C(low) expression, promote fibrotic response/ angiogenesis) macrophages. However, the M1/M2 categorization is an over-simplification: single cell sequencing has recently highlighted significant heterogeneity amongst macrophage subpopulations during development and following injury. An unbiased view of macrophage subtypes as correlates with function, therefore, seems more appropriate than the traditional biphasic M1/2 distinction<sup>78, 93</sup>. Despite substantial macrophage heterogeneity, general functions attributed to macrophages involve early roles in phagocytosis of apoptotic and necrotic debris from cardiomyocytes and neutrophils; like neutrophils, early macrophage populations release high levels of ROS and pro-inflammatory cytokines locally, but over time macrophages that have engulfed apoptotic neutrophils switch to producing anti-inflammatory mediators including IL-10 and TGF- $\beta$ <sup>81</sup>. This is then followed by anti-inflammatory, fibrotic responses in association with activated (myo)fibroblasts.



Macrophages also play cardioprotective roles to limit adverse tissue remodelling at the infarct site: Toll-like Receptor 9 (TLR9)/Myeloid differentiation primary response protein (MYD88)/C-X-C motif chemokine 5 (CXCL5) mediated signalling from tissue-resident macrophages is found to promote initial neutrophil extravasation post-MI to clear necrotic debris from dying cardiomyocytes<sup>69</sup>. Moreover, following inducible genetic ablation of tissue-resident macrophages Dick et al. recently revealed increased adverse remodelling within the peri-infarct zone, and reduced cardiac function<sup>94</sup>; although mechanistic details remain unclear.

Injury studies highlight the importance of a balanced immune response. For example, elevated monocytosis in humans is pathogenic and leads to reduced ability for wound-healing post-MI; this is also true of ApoE-null mice with enhanced monocytosis<sup>95</sup>. Conversely, a reduced monocyte population is pathogenic: monocyte-depleted mice and patients on steroids that lower monocyte count have impaired wound-healing post-MI<sup>88, 96-98</sup>. A fine-tuned macrophage-fibroblast injury response is evidently important, whereby upsetting the balance of immune activity in a regenerative setting renders cardiomyocyte proliferation insufficient to repair heart tissue.

### **2.3 Tissue-resident Mast, T and B Cells in the injured heart**

Mast cells increase in number significantly in conditions of myocardial stress including post-MI, cardiomyopathy or sustained cardiac pressure; immature resident mast cells mature within hours of pressure overload or ischaemic injury<sup>99</sup>. Emerging evidence finds that mast cells are central mediators of ventricular remodelling<sup>74</sup>, acting in concert with neighbouring fibroblasts and macrophages. Mast cells also interact with other non-immune resident cardiac cell types: for example, they are stimulated by neuropeptide Substance P and neurotensin (released from cardiac nerves in the stellate ganglia) to secrete histamine and promote detrimental cardiac remodelling via an unknown mechanism<sup>100, 101</sup>. The mast cell secretome under injury conditions includes cytokines (notably TNF $\alpha$ ), growth factors (notably

TGF- $\beta$ ) and proteases, which together with histamine drive ventricular remodelling<sup>74</sup>. However, while mast cell products are known to activate MMPs that remodel the cardiac ECM<sup>102</sup>, it remains unclear how mast cells mediate fibrosis and whether they interact with fibroblasts and macrophages in so doing<sup>74</sup>.

T cells are important in supporting post-MI cardiac repair. Genetically ablating mouse T cells prior to inducing MI increases inflammation and lowers cardiac function post-injury<sup>103</sup>. T cells are thought to control the proliferative phase of the innate immune response through modulating monocyte infiltration and differentiation into macrophages<sup>104</sup>, and perhaps modulating fibroblast activation and thereby ECM deposition<sup>105, 106</sup>. Paracrine signalling by T cells has also been found to promote regeneration following MI by secretion of mitogens and signalling that supports cardiomyocyte proliferation<sup>107</sup>. B cell activity in the injured heart has not been extensively investigated to-date and this merits further study.

## **2.4 Cardiac macrophages in regeneration**

Macrophages are directly implicated in mediating the regenerative response of the neonatal mouse heart. Importantly, when macrophages were depleted using clodronate-loaded liposomes before inducing MI in postnatal day 1 (P1) neonatal mice, neonates lost their ability to regenerate and instead formed a permanent scar and had impaired neovascularisation, with reduced cardiac function<sup>108</sup>. Microarray analysis comparing macrophages from P1 versus P14 mice post-MI found that inflammatory, oxidative stress and angiogenesis related genes were upregulated in P1 post-MI, whereas in P14 the key anti-inflammatory factor *Il10* was upregulated. P1 macrophages did not show expression profiles corresponding with either M1 or M2 traditional (adult) groupings, and macrophage response was much more global in P1 than in P14. Contrastingly in P14, a sustained increase in M2 occurred locally to the infarct site<sup>108</sup>. Macrophage subpopulations of regenerative versus non-regenerative mice models therefore seem to have diverged.

535

536 These findings are supported by a study using a genetic ablation model of cardiac injury to  
537 show that macrophage populations from embryonic lineages are important in promoting  
538 tissue repair, whereas subpopulations from adult lineages, derived from splenic or blood  
539 monocyte reservoirs, promote inflammation and lack regenerative activity<sup>73</sup>. While depleting  
540 tissue-resident macrophages in mouse neonates led to reduced proliferation of  
541 cardiomyocytes and endothelial cells, and increased interstitial fibrosis, depleting adult  
542 lineages marked by *CCR2* expression resulted in improved reparative ability following  
543 cardiomyocyte ablation<sup>73</sup>. Given the adult heart relies on embryonic-derived macrophages  
544 during homeostasis, this suggests that promoting contributions by tissue-resident  
545 subpopulations and inhibiting or limiting monocyte recruitment therapeutically could help to  
546 reduce inflammation and optimise tissue repair.

547

548 Several follow-up studies have begun to elucidate the molecular nature of the macrophage-  
549 mediated signalling that promotes regeneration in the neonatal mouse heart. Transcriptomic  
550 and epigenomic comparisons of neonatal versus adult mice hearts following MI identified  
551 macrophage-dependent CCL24-mediated pro-regenerative signalling that is upregulated in  
552 neonates but not adults<sup>28</sup>. Acute inflammation can stimulate a regenerative response in  
553 neonatal mouse hearts, acting via the cytokine IL-6<sup>109</sup>, although the precise contribution of  
554 macrophages as opposed to other inflammatory leukocytes including neutrophils to this  
555 remains unclear. Interestingly, inflammation has been found to be sufficient to initiate  
556 regeneration of the adult zebrafish brain<sup>110</sup>. Although relatively poorly understood, it seems  
557 likely that acute inflammation is more generally involved in promoting early pro-regenerative  
558 cues in the immediate post-injury environment<sup>111</sup>.

559

560 Mechanisms underpinning macrophage-directed regenerative responses are also beginning  
561 to be understood in axolotl and zebrafish hearts. Depletion of macrophages in the axolotl

cryo-injury model results in lower fibroblast activation, modified ECM synthesis and remodelling, and ultimately blocks regeneration despite successful cardiomyocyte proliferation<sup>112</sup>. In the zebrafish, cytokine signalling triggers pro-inflammatory (*tnfa*+) macrophage-mediated scar deposition immediately after injury, whereas *tnfa*- macrophages promote scar removal after cardiomyocyte proliferation has occurred to replace the dead cells<sup>113</sup>. Csf1a has been implicated in mediating the initial scar deposition, whereby loss of Csf1a led to fewer *tnfa*+ macrophages being recruited to the site of injury, and a reduction in collagen deposition<sup>113</sup>. Macrophages have been implicated not just in promoting fibrosis by fibroblast activation, but also directly in the laying down of collagen at the site of injury: through unbiased transcriptomics and adoptive transfer of macrophages with fluorescently-labelled collagen, direct contribution from macrophages as well as fibroblasts to the post-injury scar was demonstrated in both zebrafish and neonatal mouse hearts<sup>114</sup>. This highlights the extent of crosstalk and functional overlap between fibroblast and immune cells and opens the window to a much more integrated fibrotic environment (Figure 3).

### **3. The cardiac conduction system**

The cardiac conduction system (CCS) is responsible both for initiation and conduction of the electrical impulses that bring about rhythmic contractility of the heart muscle. Correct functionality of the CCS is required for the heart to contract in a coordinated sequence, to pump blood efficiently and unidirectionally through the chambers.

The CCS initiates the electrical impulse that organises cardiac contractility in the sinoatrial node (SAN). From here, the electrical impulse propagates through the atria to the atrioventricular node (AVN), the sole electrical connection between atria and ventricles. Following delay at the AVN, the impulse propagates through the atrioventricular bundle (AVB), which divides into left and right bundle branches (BBs) in the interventricular septum, and then through the Purkinje network. The BBs and Purkinje fibres are rapid-conducting

peripheral components of the CCS, that spread the electrical impulse across the ventricles. This organisation ensures the contraction of the atrial myocardium is the first event of the cardiac cycle. Due to electrical delay at the AVN, the ventricular myocardium only contracts once the atria have finished contracting, to complete the cycle by pumping the blood into the aorta and pulmonary artery<sup>115</sup>.

Tight regulation of the cardiac cycle relies upon careful control of the speed of electrical conduction through the CCS. While conduction of electrical impulses through the SAN and AVN is slow, the fast-conducting components of the ventricular conductive system (VCS) ensure that ventricular contraction is synchronous. These electrical properties result from CCS ion channel expression, including robust expression of Hyperpolarised Activated Cyclic Nucleotide Gated Potassium Channel (HCN) ion channels, decreased expression of voltage-gated sodium and inward rectifying potassium ion channels, and a careful balance of Connexin (Cx) isoforms. The heart expresses four Cx isoforms. Cx40, Cx43, Cx45 and Cx30.2 form large- (200pS), medium- (60-100pS), small- (20-40pS) and ultra-small- (9pS) conductance gap junction channels respectively<sup>116</sup>. Each gap junction channel forms from two hemichannels of six Cx proteins each, where each hemi-channels derives from one of two participating cells; hemi-channels may contain uniform or diverse Cx isoforms<sup>117</sup>. The Cx isoforms that each cell type expresses, as well as the number, size and distribution of gap junction channels, determine extent of cell-cell coupling and speed of signal propagation. Accordingly, faster-conductance Cx40 and Cx43 are highly expressed in the working atrial myocardium, His bundle, BBs and Purkinje fibres, but not in the transitional cells in the AVN; medium-conductance Cx43 but not fast-conductance Cx40 is strongly expressed in the working ventricular myocardium<sup>118</sup>; Cx43 gap junctions are concentrated at the intercalated discs between cardiomyocytes. Weak electrical coupling in the AVN is probably supported by low-level Cx45 expression, allowing (delayed) electrical propagation through to the ventricular myocardium<sup>119</sup>.

616

### 617 **3.1 Development of the CCS**

618 The CCS develops concomitantly with the heart chambers. At the earliest stages in the  
619 primitive heart tube, the entirety of the sinus venosus, upstream of the right atrium, acts as  
620 pacemaker, characterised by HCN4 channel expression<sup>115</sup>. With heart tube elongation, cells  
621 added at the inflow tract assume dominant pacemaker activity, while rapidly proliferating  
622 progenitors joining the heart tube form cardiac muscle. Extensive lineage tracing indicates  
623 that there is no single CCS lineage; rather, the lineages of the various CCS components are  
624 segregated early on in development. These lineages remain interconnected despite having  
625 been subject to early differential cell fate decisions<sup>120</sup>. Thus, the SAN forms from early  
626 segregated Insulin gene enhancer protein (*Isl1*)<sup>+</sup> mesodermal progenitor cells and becomes  
627 morphologically distinct by E10 in the mouse<sup>120</sup>. Pacemaker activity is restricted to the SAN  
628 during and following ballooning of the primitive atria. This occurs through repression of *HCN4*  
629 and T-box Transcription Factor 3 (*TBX3*) expression in the chambers, ensuring that in the SAN  
630 only, *TBX3* expression represses atrial myocardial genes while indirectly activating SAN  
631 specific genes including *HCN4*<sup>121, 122</sup>.

632

633 A *TBX2*<sup>+</sup>, *cGATA6*<sup>+</sup> posteriorly-located cardiogenic mesodermal population that initially forms  
634 the atrioventricular canal (AVC) is probably the only lineage that forms the atrioventricular  
635 node (AVN) and the atrioventricular ring bundles. *BMP2* expression in the AVC activates  
636 expression of *TBX3* and *TBX2*, which act together with Msh Homeobox 2 (*MSX2*) to repress  
637 the working myocardial gene programme and stimulate the genetic programmes required for  
638 pacemaker and AV cushion formation<sup>123, 124</sup>. Finally, the origins of the ventricular conduction  
639 system (VCS) trace to the embryonic trabeculae and interventricular ring. The VCS shares  
640 common ancestry with the ventricular working cardiomyocytes, both deriving from a  
641 myocardial progenitor pool present in the looped heart tube<sup>125</sup>. The VCS develops from this  
642 pool by specification of a Gap Junction protein 5 (*GJA5*)<sup>+</sup> (encoding Cx40) conductive

phenotype. Progressive restriction of the fate of progenitors to VCS fate occurs through until E16.5. A limited number of cell divisions then expands the VCS to its final form<sup>126</sup>.

Except for the sinus node, which derives from a mesenchymal population located just beyond the heart field, CCS cells share their origins with the heart's cardiomyocytes (Figure 2). The CCS is, therefore, sometimes considered a rare subpopulation of cardiomyocytes, comprising approximately 2% of the overall cardiomyocyte population<sup>1, 120</sup>. CCS cells become genetically and phenotypically distinct from cardiomyocytes; they undergo characteristic calcium cycling and have distinctive electrical properties. However, the CCS must integrate electrically with the working myocardium to form a synchronized contractile system. The recent finding that macrophages and fibroblasts also couple electrically to cardiomyocytes hints at the extent of integration in the conductive system<sup>61, 79</sup>. This seems especially true in the SAN, where extensive heterogeneous cell coupling with fibroblast populations has been observed<sup>127</sup>. The mechanistic details relating to how electrical coupling of fibroblasts and macrophages changes during injury in regenerative versus non-regenerative systems, and whether other non-cardiomyocyte cell types also couple electrically to neighbouring cell types, warrant further investigation.

### **3.2 Towards CCS repair and regeneration**

Disruption to the CCS results in arrhythmias that can cause heart failure and mortality. Abnormal ECG traces present following acute injury, and arrhythmias including bundle branch block and left anterior hemiblock are features of MI<sup>128</sup>. This suggests that the CCS is depleted and/or coupling between cardiomyocytes in the infarct zone is impaired in acute injury settings, and that both the CCS and coupling fail to be endogenously restored post- infarction due to subsequent scarring. Features of the injured heart, including the laying down of fibrotic collagen-rich scar tissue, downregulation of Cx43 in cardiomyocytes at the infarct border and death of cardiomyocytes that were directly coupled to CCS cells, combine to result in

ventricular tachycardia, characterised by an abnormally fast heart rate, with repercussions for cardiac output that can be fatal<sup>129</sup>. Re-entry arrhythmia, in which the electrical impulse forms a deviant circuit (generally near scar tissue) rather than following its normal course, often accompanies both acute and chronic injury and contributes to increased mortality risk (Figure 3)<sup>130</sup>.

It is well known that CCS failure is associated with aging, independently from MI-induced heart disease or tissue injury, and chronic heart failure is often characterised by an inability to maintain rhythmic contraction<sup>131</sup>. As such insertion of a cardiac pacemaker to correct natural pacemaker dysfunction is a routine operation amongst older patients. Given the apparent lack of regeneration of the CCS following acute injury, and the prevalence of age-associated conduction impairment, there is a need to understand biologically how to restore CCS integrity and function in these conditions. Developing an increased understanding of the differences between the developmental versus injured CCS will help inform strategies to establish a so-called “biological pacemaker” and achieve electrical regeneration of the heart<sup>132</sup>.

The cellular properties of the CCS present several unique difficulties to understanding the diseased CCS with a view towards regeneration. Firstly, the CCS core is formed of a small number of cells, which are embedded within the heart. This complicates cellular and molecular analyses of the CCS pre- and post-injury. The location of the CCS also presents severe challenges to imaging approaches, requiring tissue clearing in order to penetrate the tissue. Moreover, targeting the CCS deep within the heart presents an obvious barrier to any drug and/or cell delivery strategy aimed at regenerating the system. A second significant challenge relates to the extent of integration between CCS and working myocardium during development. The CCS is the control-room for a fully integrated electrical organ: in a sense, the whole heart forms part of the conductive system, since the electrical impulses generated



in the nodes and conveyed along the Bundle of His and Purkinje fibres must ultimately propagate through the working myocardium via gap junctions and patterned Cx channels. Regenerative strategies must factor in how to avoid rhythmic disruptions from any fibrotic scar tissue laid down during injury, and how to couple new cardiomyocytes electrically, whether these cardiomyocytes are generated in vivo via provision of small molecule drugs or gene therapy to promote proliferation of surviving cells, or whether these are derived from cell engraftment approaches.

Considerable recent progress has been made in attempts to reprogramme cardiomyocytes into CCS cells both in vivo and in vitro<sup>133</sup>. Notably, expression of *Tbx18* in ventricular cardiomyocytes is sufficient to induce a genetic and phenotypic shift towards the pacemaker SAN cell type both in vitro and following direct injection and adenoviral delivery into the left ventricle of the guinea pig heart<sup>134</sup>. *Tbx18* was found to suppress transcription of *Cx43* directly, resulting in partial electrical uncoupling and slow propagation of action potentials, reminiscent of SAN activity. The reprogrammed cardiomyocytes underwent calcium cycling and could depolarise spontaneously<sup>134</sup>. Similar efforts to use developmental TFs to reprogramme fibroblasts into CCS cells have not yet met with equivalent success, although generation of a mixed population of immature cardiomyocytes with partial CCS phenotypes suggests that with optimisation, this method may be viable<sup>66</sup>. Several canonical signalling pathways may provide alternative routes to reprogramming cardiomyocytes into CCS cell types. Activation of notch signalling in new-born cardiomyocytes in vitro reprogrammed the cells such that both transcriptionally and phenotypically they resembled 'Purkinje-like' cells<sup>135</sup>. Altering canonical Wnt signalling resulted in ectopic formation of AV-junction-like cells with altered electrophysiological properties<sup>136</sup> and Neurugulin-1 has been implicated in supporting CCS cell fate in development suggesting it might act to facilitate reprogramming<sup>133, 137</sup>.

Currently, the main CCS-associated treatment for heart disease involves insertion of an electronic pacemaker. Complications associated with this include infection, allergic reaction during the insertion procedure, inflammation and bleeding at the pacemaker site, exacerbated by blood thinning medication. Besides electronic pacemaker insertion, ventricular arrhythmias may be treated by anti-arrhythmic drugs, or by catheter or laser ablation of ectopic regions of conduction<sup>131</sup>. However, the former option is poorly understood mechanistically with potential side effects including exacerbated arrhythmia, dizziness, chest pain and affected vision, while the latter represents a last resort approach, which can induce lethal refractory or repetitive CCS symptoms. Targeting the CCS therefore presents an outstanding clinical challenge. Going forwards, future strategies are required to reactivate embryonic CCS transcriptional networks to programme a “biological pacemaker” and restore control of integrated electrical conduction.

#### **4. Cardiac innervation in development and regeneration**

Cardiac innervation provides stable control over heart rate: sympathetic innervation increases heart rate and contraction force, while parasympathetic innervation contrastingly lowers the heart rate. A balance of sympathetic versus parasympathetic activity must be maintained to meet physiological requirements, whereas appropriate imbalance must be achievable during extreme stress to instigate the so-called “fight or flight” response<sup>12</sup>.

The heart has both sensory and motor innervation. The nerves that innervate the heart meet in the cardiac plexus, which sits cranio-dorsally to the heart. Most autonomic neurons innervating the heart stretch from here along the coronary arteries running from aorta to pulmonary trunk, and then branch to innervate the chambers<sup>138</sup>. Cardiac innervation can be direct or facilitated by the cardiac ganglia distributed across the heart’s neural network, particularly concentrated in the subepicardial layer at the roots of the caval and pulmonary veins and within the infolded atrial walls. Each human cardiac ganglion is formed of between

200 to 1000 intracardiac neurons, with considerable variation between hearts of different maturity<sup>139, 140</sup>. Autonomic neurons that synapse in the cardiac ganglia excite intrinsic inter-neurons that stretch outwards and modulate the activity of cardiomyocytes, and the SAN and AVN of the CCS<sup>140</sup>. These cardiac ganglia regulate the autonomic inputs to left and right vagal branches to varying degrees, to provide interlinked pathways that control heart rate in a nuanced fashion. Recently, three-dimensional mapping has provided a single-cell-resolution overview of the neuroanatomy of the rodent cardiac nervous system<sup>141</sup>.

#### **4.1 Development of cardiac innervation**

Co-transmitters seem to be active in the heart before sympathetic innervation develops, and catecholamines are produced by the myocardium early in development. Although in many species onset of sympathetic innervation occurs in the neonatal heart after birth, tachycardiac (rapid heart rate) responses to catecholamines occur during gestation. Moreover, some myocardial cells express the enzymes responsible for conversion of L-tyrosine into norepinephrine in early development; these cells concentrate at the forming SAN and AVN from mid-gestation onwards<sup>3, 142</sup>. Whether there are ongoing roles for co-transmitter release from heart tissue itself during later development and into adulthood remains unclear.

The heart begins to be innervated relatively early in development. Innervation can broadly be divided into four developmental stages: i) migration of NC to the dorsal aorta; ii) differentiation to form neurons; iii) formation of cardiac ganglia and sympathetic chains; iv) axonal projections into the working myocardium<sup>139</sup>. Neurons are first seen in the dorsal mesocardium by E10.5 in mice. Using the neurofilament marker NF160D, the first neural elements arising between the separating aorta and pulmonary trunk to innervate the outflow tract are present from E11.5; well-developed nerve tracts extend through the mesocardium and reach the heart arterial pole by E12.5<sup>143</sup>. Most, but not all cardiac neurons, glia and inter-

neurons originate from NC derivatives (Figure 2)<sup>3</sup>. Lineage tracing and NF160D staining together show that the parasympathetic system consists of NC and non-NC derivatives of heterogeneous origin<sup>143</sup>. Moreover, non-NC precursor populations from the ectodermal plaque (known as the nodose placode) can form functional cholinergic neurons in embryos in which NC has been ablated<sup>144</sup>.

While pre-ganglionic and vagal post-ganglionic neurons are characterised by an exclusive dependence on the neurotransmitter acetylcholine, post-ganglionic neurons employ other neurotransmitters including norepinephrine and peptide cotransmitters, and neuromodulators act at the intersections of different nerve branches. While most sensory innervation links up with motor neurons in the central nervous system, in rarer cases some pathways are believed to be intrinsic to the heart itself, passing reflex impulses along sympathetic and parasympathetic neurons, and interneurons in the cardiac ganglia. The sensory (afferent) and motor (efferent) pathways are so deeply interconnected both locally within the organ, and within the central system brainstem and spinal cord as to render the traditional distinction between sensory and motor innervation relatively meaningless, except for their having spatiotemporally distinct developmental origins.

#### **4.2 Cardiac innervation in disease**

Neurohormonal changes, particularly elevated sympathetic signalling activity, are a hallmark of heart failure. Elevated sympathetic activity is required to maintain circulation during pathogenic remodelling of the heart immediately following injury, but this change is thought to trigger long-term fluid overload, myocardial hypertrophy and gradual death of cardiomyocytes<sup>145</sup>. Sympathetic innervation increases following MI in the mouse: CUBIC organ clearing followed by immunostaining finds over a two-fold increase in the density of nerves at the ischaemic border zone compared to non-injured hearts<sup>146</sup>. Studying patients with systolic heart failure reveals a direct correlation between cardiac sympathetic nerve

activity and epicardial adipose tissue thickness, which suggests that changes to cardiac innervation may affect the density of fatty heart tissue, particularly in the epicardium<sup>147</sup>. Furthermore, sympathetic denervation can actively induce heart injury: uninjured rats whose sympathetic nerves were ablated through 6-OHDA treatment (leaving the parasympathetic network intact) undergo MI and fibrosis<sup>148</sup>. The mechanisms responsible for altered sympathetic nerve activity negatively affecting other cardiac cell types during heart failure require further investigation.

### 4.3 Cardiac innervation in regeneration

Two seminal papers have directly demonstrated the importance of innervation to cardiac regeneration. Using both adult zebrafish and neonatal mice, Mahmoud et al. find that chemical or mechanical cholinergic nerve inhibition impairs the heart's ability to regenerate post-injury<sup>149</sup>. Reduced expression of cell cycle genes, and of growth factors *NRG1* and *NGF* in the neonatal mouse following cholinergic nerve inhibition suggested that loss of cholinergic signalling reduces cardiomyocyte proliferation. Moreover, injection of recombinant *NRG1* and *NGF* partially rescued the regenerative response following cholinergic denervation<sup>149</sup>. Mechanical denervation disrupted inflammatory gene expression after apical resection in the neonatal mouse<sup>149</sup>, suggesting that innervation actively promotes the inflammatory immune response. White et al. also demonstrate the importance of innervation to regeneration through lineage tracing using *Wnt1-Cre-tdTom* to identify NC derivatives in the developing mouse embryo. NC-derived subepicardial autonomic nerves which innervate the ventricles showed robust re-growth after ventricular resection in P2 mice, but re-growth was inhibited when these nerves were specifically targeted using 6-OHDA to induce chemical sympathectomy<sup>150</sup>.

Collectively, these studies suggest that the autonomic nervous system controls cardiomyocyte proliferation and implicate innervation as a regulator of heart regeneration. It

remains unclear whether the autonomic nervous system can also impact fibrosis and ECM deposition and turnover. It will be important to determine whether cholinergic signalling is involved in the adult mammalian cardiac injury response, and whether and how autonomic innervation of the heart is important not just in regeneration following traumatic post-injury response but also in chronic disease contexts.

The extent of crosstalk between cardiac innervation and other cardiac-resident cell types remains relatively unexplored. The finding that the density of neurotensin-containing nerve fibres is lower in diseased hearts, and that these fibres sit in close contact with the CCS, intracardiac ganglia, cardiomyocytes and vasculature, suggests extensive integration between nervous control, the CCS and heart contraction<sup>151</sup>. Given that neuromodulators and co-transmitters are released from non-neuronal cell types over development, including from heart tissue itself, the cardiac ganglia may be acting as a local signalling hub between CCS, nervous system and potentially also immune subpopulations. The link between innervation and the CCS is inextricable: the intracardiac nervous system acts principally on nodal cells via  $\beta_1$  adrenergic receptors to fine-tune the heart rate set by the CCS's pacemaker and to alter conduction velocity through the AVN<sup>1, 12</sup>. There is also probably a wider link between innervation and immune response in regeneration. For example, increased sympathetic nervous system activity triggers leukocyte progenitor recruitment to the spleen and increases total production of inflammatory leukocytes, enabling a greater number of monocytes to reach the infarcted heart<sup>152</sup>.

While the mechanisms by which innervation acts locally to facilitate cardiac regeneration are unclear, this already represents an area of therapeutic potential. Early advances using vagal nerve stimulation as a bioelectronic treatment option for heart disease were recently reviewed elsewhere<sup>153</sup>; whether similar approaches can be combined with tissue restoration therapies to promote regeneration remains unexplored.

## **5. Intercellular communication and cross-talk in the damaged and regenerating heart**

Recent evidence indicates that not only the existence of many different cell types in the damaged heart, but also their interactions and integration through cell-cell signalling, will be important for restoring heart function and achieving regeneration<sup>58, 105, 107, 154</sup>. The non-myocyte cell types resident in the heart seem to be involved in extensive chemical, electrical and mechanical signalling, which together define the transcriptional profile of each cell population, their activity states and behaviour following injury.

The molecular network of non-myocyte chemical signalling in the damaged and regenerating heart remains relatively poorly understood. There is extensive paracrine signalling from the tissue-resident immune cells (especially the mast, macrophage and T cell populations), recruited neutrophils and monocytes, activated (myo)fibroblasts, endothelial cells, epicardium and endocardium<sup>28, 58, 69, 74, 81, 91, 100, 102, 105-107, 109, 154, 155</sup>. This paracrine signalling modulates contractility; regulates cell death, arrest, differentiation or activation; affects extent of inflammation and cell recruitment to the injury site; and determines fibrotic remodelling. Identified mediators of paracrine signalling in the heart include inflammatory cytokines and interleukins, nitric oxide, prostaglandins, angiotensin, endothelin, thyroid hormone and periostin<sup>154, 155</sup>. While signalling pathways including FGF, Wnt, RA and Nrg-1 are required for proper cardiac development, as described for the specific cell types above, their activity in the injured heart and potential to support regeneration warrants further study<sup>155</sup>.

Beyond traditional chemical signalling, electrical coupling involving not just CCS cells and myocytes, but also fibroblasts and macrophages, affects cardiac regenerative capacity and contractile function<sup>44, 61, 62, 79</sup>. Moreover, the stiffness profile of the ECM deposited during fibrosis affects cardiomyocyte proliferation versus cell cycle arrest, cell migration and

probably also fibroblast activation and any potential transdifferentiation, through poorly defined mechanical signalling cues<sup>54, 55</sup>.

## **6. Concluding thoughts**

The heterogenous mix of cell populations including cardiac fibroblasts, immune cells, conduction system cells and innervating nerves that come together with muscle and vasculature to form the heart, act as a functionally integrated adaptive system. Through cell fate mapping and single cell -omics, we are beginning to build a comprehensive picture of the different cell types of the developing heart, their extent of heterogeneity in terms of ontogeny and how they come together to establish complex cell-cell interactions essential for maintenance of heart function. Understanding how all these different cell types respond following injury in both regenerative and non-regenerative settings is a therapeutic priority which extends beyond a focus exclusively on cardiomyocytes and vascular cells. The balance of signalling from cardiac fibroblasts, immune cell types, the conduction and innervation systems defines critical parameters such as length of the inflammatory period post-injury, extent of ECM deposition and fibrosis, risk of arrhythmia and extent of cardiac remodelling. Through an increased understanding of the network of cell-cell communication during development and into adult heart injury, we may begin to integrate attempts to reactivate embryonic programmes not only to promote new cardiomyocytes and vasculature but to ensure appropriate mechanical, conductive and nervous support to progress towards complete heart regeneration.

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913

## 914 **Competing interests**

915 PRR is co-founder and equity holder in OxStem Cardio, an Oxford University spin-out that  
916 seeks to exploit therapeutic strategies stimulating endogenous repair in cardiovascular  
917 regenerative medicine.

918

## 919 **Authors Contributions**

920 JS wrote the manuscript. PRR conceptualised the subject for the review and edited the  
921 manuscript.

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## 1389 **Figure Legends**

### 1390 **Figure 1. The heterogeneous cell types that constitute the mature heart.**

1391 Cardiomyocytes and endothelial cells of the coronary vasculature are the major cell types in  
1392 the developing and adult heart. Consequently, studies have focused extensively on their  
1393 development. Other important cell types have received less attention; these include the  
1394 cardiac fibroblasts which regulate the structural organisation of the heart; cells of the tissue-  
1395 resident immune populations which are emerging important contributors to heart  
1396 development, including via remodelling of the developing vasculature; cardiac conduction  
1397 system (CCS) specialist cardiomyocytes which ensure the propagation of rhythmic  
1398 contraction; and nervous innervation to ensure the development of “fight or flight”  
1399 responses.

1400

### 1401 **Figure 2. The principle developmental origins of the mature cardiac cell types besides the** 1402 **cardiomyocytes and vasculature.**

1403 The mature cell types of the mammalian heart have heterogenous developmental origins.  
1404 Cardiac fibroblasts derive principally from a combination of epithelial and endothelial  
1405 sources, with a much smaller subset of cardiac fibroblasts of neural crest (NC) origin. Most,  
1406 but not all cardiac neurons, glia and inter-neurons originate from NC derivatives; non-NC  
1407 precursor populations from ectodermal plaque contribute minorly to cardiac innervation.  
1408 Immune cells derive from heterogenous pools: tissue-resident macrophages originate in the  
1409 foetal yolk sac, while mast cells originate from bone marrow precursor populations. Lastly,  
1410 the Purkinje fibres and nodal cells of the conduction system derive from a specialized pool of  
1411 cardiomyocytes segregated early on in development. These lineages remain connected to one  
1412 another despite having been subject to early differential cell fate decisions.

1413

1414 **Figure 3. Diverse cellular contributions to regenerative versus non-regenerative capacity**  
1415 **following injury.**

1416 Diverse cell types including cardiac fibroblasts, immune cells, conduction system and nervous  
1417 system cells mediate the cardiac post-injury response.

1418 **A:** Diverse cellular contributions promote a regenerative response to restore cardiac function  
1419 in systems including zebrafish, axolotl and neonatal mammals. A pro-regenerative chemical  
1420 and mechanical microenvironment, mediated principally by fibroblast, macrophage and  
1421 nervous system signalling, result in removal of scar tissue, cardiomyocyte division to replace  
1422 lost muscle tissue, and generation of new vasculature.

1423 **B:** In non-regenerative adult mammalian hearts, acute injury is resolved without restoration  
1424 of cardiac function. Differences in cellular signalling and activity, including extent and nature  
1425 of ECM deposition, fibroblast-macrophage crosstalk and sympathetic nervous system  
1426 signalling, produce an anti-regenerative environment. Lost cardiomyocytes are not replaced,  
1427 and new vessels do not grow. Instead, scar tissue is deposited long-term, resulting in lower  
1428 contractility in the infarct region. Altered excitability of the fibrotic scar tissue, and potential  
1429 loss or migration of conduction system cells, increases the likelihood of arrhythmia.  
1430 Pathological remodelling leads to heart failure which is a principle cause of mortality  
1431 worldwide.

