

Regeneration of the Cardiac Conduction System



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*This thesis is dedicated to my parents,
Nick and Yvonne:
for their unwavering support
throughout my studies.*

Table of Contents

LIST OF NON-STANDARD ABBREVIATIONS	7
ACKNOWLEDGEMENTS.....	10
SUMMARY OF DPHIL ACTIVITY	12
I MEETINGS ATTENDED.....	12
I.i Oral presentations	12
I.ii Poster presentations	12
II PRIZES	13
II.i Research Awards.....	13
II.ii Presentation Prizes.....	13
II.iii Imaging Prizes	13
II.iv Travel Awards	14
III PUBLICATIONS	14
IV TEACHING	15
V TIMING AND LOCATION OF RESEARCH	15
VI FUNDING.....	16
ABSTRACT.....	17
1 INTRODUCTION.....	19
1.1 THE CARDIAC CONDUCTION SYSTEM	19
1.1.1 Overview of the cardiac conduction system	19
1.1.2 Development of the cardiac conduction system	22
1.1.3 The ventricular conduction system in injured, stressed and diseased hearts.....	23
1.2 HEART REGENERATION	27
1.2.1 Fibrotic repair in the adult heart after myocardial infarction.....	27
1.2.2 The neonatal mouse model for heart regeneration.....	28
1.3 THESIS RATIONALE	32
2 MATERIALS AND METHODS.....	34
2.1 MOUSE STRAINS AND HUSBANDRY	34
2.2 ANIMAL GENOTYPING	34
2.2.1 Genomic DNA extraction	34
2.2.2 Polymerase chain reaction	35
2.2.3 Gel electrophoresis.....	36
2.3 NEONATAL MYOCARDIAL INFARCTION BY LAD CORONARY ARTERY LIGATION	37

2.4	IMAGING SAMPLE PREPARATION, ACQUISITION AND ANALYSIS	38
2.4.1	Neonatal heart dissection	38
2.4.2	Tissue clearing with immunostaining	38
2.4.3	Immunofluorescence on cryosections	41
2.4.4	Imaging the dissected ventricular conduction system	42
2.4.5	Image analysis	45
2.5	NEONATAL HEART DISSOCIATION	46
2.6	SINGLE-CELL RNA-SEQUENCING	47
2.6.1	Sample processing for single-cell RNA-sequencing	47
2.6.2	Pre-processing and quality control of single-cell RNA-sequencing data	48
2.6.3	Unsupervised clustering analysis and dataset integration	49
2.6.4	Differential abundance and differential expression analyses	50
2.7	SINGLE-NUCLEAR RNA-SEQUENCING ANALYSIS	50
2.7.1	Pre-processing and quality control of single-nuclear RNA-sequencing data	50
2.7.2	Clustering analysis and dataset integration	50
2.7.3	Re-clustering and identification of ventricular conduction system cells	51
2.8	ELECTROPHYSIOLOGY	51
2.8.1	Micro-electrode array recordings	51
2.8.2	Electrocardiogram recordings	52
2.9	STATISTICAL ANALYSIS	53
2.10	MODELLING AND SIMULATION	53
2.11	CLINICAL DATA	54

3 THE THREE-DIMENSIONAL ANATOMY OF THE MURINE VENTRICULAR CONDUCTION SYSTEM THROUGH POST-NATAL DEVELOPMENT 56

3.1	BACKGROUND	56
3.1.1	Tissue clearing for whole-organ imaging	56
3.1.2	Application of confocal and lightsheet microscopy to large tissue samples	59
3.1.3	Imaging the cardiac conduction system	60
3.1.4	The Nkx2-5 ^{cre/+} haploinsufficient mouse model	65
3.2	AIMS AND OBJECTIVES	66
3.3	RESULTS	67
3.3.1	A methodology to image the intact ventricular conduction system	67
3.3.2	The conduction system during late embryonic and post-natal development	72
3.3.3	Modelling the ventricular conduction system network	80
3.4	DISCUSSION	91
3.4.1	Tissue-cleared whole-organ imaging as a methodology to study the biology of the cardiac conduction system	92
3.4.2	Growth and maturation of the ventricular conduction system	93
3.4.3	His/Purkinje network complexity	94
3.4.4	Interactions between the conduction system and other tissue types during post-natal development	95
3.4.5	The role of NKX2-5 in determining the conduction system lineage	96
3.5	CONCLUSIONS	99

4	MORPHOLOGICAL AND NETWORK-LEVEL CHANGES TO THE CONDUCTION SYSTEM FOLLOWING MYOCARDIAL INFARCTION TO REGENERATIVE AND NON-REGENERATIVE HEARTS.....	100
4.1	BACKGROUND	100
4.1.1	The ventricular conduction system in infarcted hearts.....	100
4.1.2	Surgical LAD ligation in the neonatal mouse as a model for heart regeneration.....	101
4.2	AIMS AND OBJECTIVES	104
4.3	RESULTS.....	105
4.3.1	The His/Purkinje network is disrupted following myocardial infarction	105
4.3.2	The bundling of conduction fibres is altered by myocardial infarction with differences in regenerative versus non-regenerative hearts	107
4.3.3	A full His/Purkinje network is reconstituted during neonatal heart regeneration.....	110
4.3.4	A full connexin-40-positive His/Purkinje network is not restored following myocardial infarction in non-regenerative hearts	110
4.3.5	The His/Purkinje network remains intact but connexin-40 is downregulated regionally following myocardial infarction beyond the regenerative window	113
4.3.6	The pace-maker ion channel HCN4 is ectopically expressed in the infarct area after myocardial infarction and is dysregulated in non-regenerative hearts.....	115
4.4	DISCUSSION.....	117
4.4.1	Regeneration of a largely normal conduction system following myocardial infarction in neonatal mice	118
4.4.2	Normal and abnormal bundling of Purkinje fibres.....	120
4.4.3	Connexin remodelling in the conduction system of non-regenerative hearts	122
4.5	CONCLUSIONS.....	125
5	ELUCIDATION OF THE MOLECULAR PATHWAYS ASSOCIATED WITH CONDUCTION SYSTEM RESPONSE TO INJURY, (RE-) GROWTH AND FUNCTION	126
5.1	BACKGROUND	126
5.1.1	Single nuclear and single cell RNA sequencing in the context of heart regeneration...	126
5.1.2	Gene expression in the ventricular conduction system	128
5.2	AIMS AND OBJECTIVES.....	131
5.3	RESULTS.....	132
5.3.1	Transcriptomic identification of a ventricular conduction system cluster in a cardiomyocyte-enriched single-nuclear RNA-sequencing dataset	132
5.3.2	Enriched single-cell RNA-sequencing of the neonatal ventricular conduction system following myocardial infarction.....	137
5.4	DISCUSSION.....	165
5.4.1	Transcriptional heterogeneity in the ventricular conduction system	166
5.4.2	The distinct transcriptional response of regenerating versus non-regenerating conduction cells in the infarcted heart	168
5.4.3	Changes in expression of ion channels and calcium handling machinery in the non-regenerative heart.....	170
5.4.4	The implications of spatially dysregulated expression of NCX1	171
5.4.5	The gene expression changes underpinning increased incidence of conduction block following myocardial infarction in female versus male hearts.....	173

5.5	CONCLUSIONS.....	175
6	THE CONSEQUENCES OF HIS/PURKINJE ELECTRICAL REMODELLING FOR ARRHYTHMOGENESIS	176
6.1	BACKGROUND	176
6.1.1	The murine and human electrocardiogram.....	176
6.1.2	Computational approaches to modelling and simulation of the infarcted human heart: from ionic currents to electrocardiographic signal	179
6.2	AIMS AND OBJECTIVES.....	181
6.3	RESULTS.....	181
6.3.1	Electrophysiological analysis following neonatal heart regeneration.....	181
6.3.2	Modelling and simulation of electrical remodelling in the His/Purkinje network of the human heart following myocardial infarction.....	187
6.4	DISCUSSION.....	190
6.4.1	Functional electrical regeneration during neonatal heart regeneration.....	191
6.4.2	Reduced Purkinje conductivity outside of the infarct area as a causative mechanism for conduction delays following myocardial infarction	192
6.5	CONCLUSIONS.....	193
7	FINAL CONCLUSIONS AND FUTURE WORK.....	194
7.1	MAIN FINDINGS	194
7.2	FUTURE WORK	197
7.2.1	Elucidating the fast-conductance gene regulatory programme and origin of left-right differences during conduction system maturation	197
7.2.2	Characterisation of the adult murine cardiac conduction system following myocardial infarction	198
7.2.3	Exploring candidate targets for repairing the infarcted conduction system	198
7.2.4	Comprehensive spatial analysis of changes across the infarcted post-natal day 1 versus post-natal day 7 His/Purkinje network	200
7.2.5	Dissection of the mechanism driving connexin and ion channel remodelling.....	201
7.2.6	Electrophysiological follow-up to assess conduction system pathology following myocardial infarction.....	202
7.2.7	Investigation of the His-Purkinje niche in regeneration versus fibrotic repair	203
7.3	FINAL REMARKS.....	205
8	SUPPLEMENTARY MATERIALS	206
9	REFERENCES.....	219

List of non-standard abbreviations

2D – two-dimensional

3D – three-dimensional

AP – action potential

AV – atrioventricular

AVB – atrioventricular bundle

AVC – atrioventricular canal

AVN – atrioventricular node

BABB – benzyl alcohol and benzyl benzoate

BB – bundle branch

BBB – bundle branch block

BMP – bone morphogenetic protein

CCS – cardiac conduction system

CM – cardiomyocyte

CMO – cell multiplexing oligo

CmVCS – cardiomyocyte-like conduction cells

CNTN2 – contactin-2

CUBIC – clear, unobstructed brain/body imaging cocktails and computational analysis

Cx – connexin

DISCO – three-dimensional imaging of solvent-cleared organs

Dpi – days post injury

E – embryonic day

ECG – electrocardiogram

ECi – ethyl cinnamate

ECM – extracellular matrix

EF – ejection fraction

Endo – endocardium

Epi – epicardium

ETV – ETS transcription factor

FACS – fluorescence activated cell sorting

Fb – fibroblast

FbVCS – fibroblast-like conduction cells

eGFP – enhanced green fluorescent protein

GJA5 – gap junction protein 5

H&E – haematoxylin and eosin

HCN – hyperpolarised activated cyclic nucleotide gated potassium channel

HCR – hybridisation chain reaction

ISL1 – insulin gene enhancer protein

iPSC – induced pluripotent stem cell

IRX – iroquois homeobox protein

LA – left atrium

LAD – left anterior descending coronary artery

LBB – left bundle branch

LBBB – left bundle branch block

LV – left ventricle

MEA – microelectrode array

MI – myocardial infarction

MRI – magnetic resonance imaging

NCX – sodium-calcium exchanger

NSIVCD – non-specific intraventricular conduction delay

P – post-natal day

PBS – phosphate buffered saline

PBST – phosphate-buffered saline with tween

PCA – principal component analysis

PCR – polymerase chain reaction

PF – Purkinje fibres

PFA – paraformaldehyde

pS - picosiemens

RA – right atrium

RBB – right bundle branch

RBBB – right bundle branch block

RNA-seq – RNA sequencing

RV – right ventricle

SAN – sinoatrial node

scRNA-seq – single cell RNA sequencing

SH – sinus horn

SMC – smooth muscle cell

snRNA-seq – single nuclear RNA sequencing

SSCT – sodium chloride sodium citrate with tween

SUMIC – simple ultrafast multicolour immunolabelling and clearing

TBX - T-box transcription factor

UMAP – uniform manifold approximation and projection

VCS – ventricular conduction system

VF – ventricular fibrillation

VT – ventricular tachycardia

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Summary of DPhil activity

I Meetings attended

I.i Oral presentations

Department of Physiology, Anatomy and Genetics Sherrington Talks

Oxford, UK, July 2023.

Weinstein Conference for Cardiovascular Development and Regeneration

San Diego, USA, May 2023.

Institute of Developmental and Regenerative Medicine Cardio-floor Seminar

Oxford, UK, April 2023.

Jenkinson Oxford Developmental Biology Symposium

Oxford, UK, December 2022.

Royal Microscopy Society: Frontiers in Bioimaging

Birmingham, UK, July 2022.

I.ii Poster presentations

19th Congress of the International Society for Developmental Biology (ISDB)

Albufeira, Portugal, October 2022.

Oxford Medical Sciences Division Scientific Symposium

Oxford, UK, July 2022.

Oxford MRC-DTP Symposium

Oxford, UK, June 2022.

Weinstein Conference for Cardiovascular Development and Regeneration

Marseille, France, May 2022.

Department of Physiology, Anatomy and Genetics Graduate Poster Day

Oxford, UK, February 2022.

II Prizes

II.i Research Awards

G A Paul Memorial Scholarship for Research Excellence

University College Oxford, 2022-2023

MRC Supplementary Funding: £6000 Award for Advanced R, Python and Linux Training

Medical Research Council, 2022

II.ii Presentation Prizes

Oxford Medical Sciences Division Scientific Symposium Poster Competition: 1st Prize

Oxford, UK, July 2022

Oxford MRC-DTP Symposium Poster Competition: 1st Prize

Oxford, UK, June 2022

Department of Physiology, Anatomy and Genetics Graduate Poster Day: 1st Prize

Oxford, UK, February 2022

II.iii Imaging Prizes

British Heart Foundation Centre of Research Excellence Image Competition: 1st Prize

Oxford, UK, November 2023

British Heart Foundation *Reflections on Research* National Competition: Supporters' Favourite Image

UK, July 2023

Department of Physiology, Anatomy and Genetics Imaging Competition: Joint 1st Prize

Oxford, UK, July 2023

Oxford Biochemistry Department Centenary Scientific Imaging Competition: 1st Prize (video category) and 3rd prize (image category)

Oxford, UK, April 2023

Department of Physiology, Anatomy and Genetics Imaging Competition: 3rd Prize

Oxford, UK, July 2022

II.iv Travel Awards

BHF Centre of Research Excellence Travel Award

May 2023

University College Research Training Grant

May 2023

University College Old Members' Trust Graduate Conference Award

October 2022

The 11th Congress of the International Society for Developmental Biology Travel Award

October 2022

Company for Biologists Travel Grant

October 2022

III Publications

Cardiac conduction system regeneration prevents arrhythmias after myocardial infarction

Sayers, J. R., Riley, P. R. [and 10 others] (2023). *In submission*.

Stabilisation of HIF signalling in the epicardium extends embryonic potential and neonatal heart regeneration

Gamen, E., Riley, P. R. [and 12 others, including **Sayers, J. R.**] (2023). *Under revision*.

Centriole growth is limited by the Cdk1/Cyclin-dependent phosphorylation of Ana2/STIL.

Steinacker, T. L., Raff, J. W. [and 7 others, including **Sayers, J. R.**] (2022). *Journal of Cell Biology*, 221(9), p.e202205058

Heart Regeneration: Beyond New Muscle or Vessels

Sayers, J. R., Riley, P. R. (2021). *Cardiovascular Research*, doi.org/10.1093/cvr/cvaa320

IV Teaching

Lecturer in Biochemistry

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Tutor in Cellular and Molecular Biochemistry

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Led the teaching of two core undergraduate modules on Cellular Biochemistry and Genetics; 80% of my 2020-2021 cohort of undergraduate students received distinctions in their prelim examinations.

V Timing and Location of Research

The work presented in this thesis was undertaken between August 2020 and November 2023. The research was initially performed in the Sherrington Building of the Department of Physiology, Anatomy and Genetics, but following a laboratory move during June 2022, the second half of the work was resumed at the Institute of Developmental & Regenerative Medicine (IDRM) at the University of Oxford. I performed imaging studies at the Oxford Zeiss Centre for Research Excellence in the IDRM and the Kennedy Research Institute (Ultramicroscope light sheet imaging, Zeiss 980 confocal imaging, image analysis) and at the Dunn School of Pathology and Micron imaging centre in the Department of Biochemistry (Z1 light sheet imaging, Olympus confocal imaging) at the University of Oxford.

I performed FACS and single cell RNA-sequencing studies at the Weatherall Institute of Molecular Medicine at the University of Oxford. Paul Riley supervised this research throughout the duration of the project.

VI Funding

This research was funded by a 4-year Oxford MRC-DTP studentship from the Medical Research Council (MR/N013468/1), and an Oxford Graduate Scholarship from the University of Oxford Medical Sciences Division. Additionally, I benefited greatly from being part of the Wellcome Trust Chromosome and Developmental Biology PhD programme and am grateful to the Wellcome Trust for providing top-up funding to support the final months of my thesis work.

Abstract

Arrhythmias are a hallmark of myocardial infarction (MI) and increase mortality risk for patients. The cardiac conduction system is increasingly implicated in arrhythmias but how it is altered following MI is not well understood. I hypothesised that there is an impairment of the conduction system in adult infarcted hearts whereas it is rapidly restored in regenerative neonatal hearts to maintain normal rhythm.

In this thesis, I demonstrate complete conduction system restoration during neonatal mouse heart regeneration, versus pathological remodelling at non-regenerative stages that map onto the adult heart wound-healing response. By developing tissue-cleared whole-organ imaging and analysis approaches, I initially performed the first description and quantitative characterisation of the intact 3D architecture of the post-natal murine ventricular conduction system. Next, following MI injury, I identified global disruption to the His/Purkinje network, disorganised bundling of conduction fibres and regional loss of connexin-40 one week after birth which contrasted with regeneration of the network and restoration of fibres at immediate post-natal stages. Enriched single-cell RNA-sequencing revealed distinct transcriptional signatures of infarcted regenerating versus non-regenerating conduction cells. I identified that conduction cells undergo transition at a transcriptional and protein level to regenerate the network, as compared to sustained electrical alterations during fibrotic repair. I found that this manifests functionally via transition from normal rhythm to pathological conduction delay beyond the regenerative window. Finally, modelling the non-regenerative phenotype in the infarcted human heart implicated these changes as causative for bundle branch block and increased dyssynchrony of ventricular contraction, as is observed in MI patients.

Collectively, the findings of this thesis characterise conduction system growth and maturation following birth, identify pathophysiological electrical remodelling in the His/Purkinje network after MI, elucidate the cellular and molecular profiles of the regenerating conduction system, and demonstrate the consequence of MI-induced conduction system damage for clinical arrhythmogenesis.

1 Introduction

The introductory text in sections 1.1 and 1.2 of this Chapter was published as part of a review article in the 3rd issue of the 117th volume of *Cardiovascular Research* in March 2021:

Sayers, J. R., Riley, P. R. (2021). “Heart Regeneration: Beyond New Muscle or Vessels”. *Cardiovascular Research*, doi.org/10.1093/cvr/cvaa320³.

I wrote the full article. I benefited from the support of my supervisor, Paul Riley, who helped to conceptualise the subject for the review and who edited the manuscript. The text inclusions are taken verbatim from my published manuscript with permission from Oxford University Press, but I have updated and expanded upon several sub-sections for the purpose of this thesis.

1.1 The cardiac conduction system

1.1.1 Overview of the cardiac conduction system

The cardiac conduction system (CCS) is the dedicated electrical system of the heart. The 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 cardiac 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 (**Figure 1.1**). The BBs and Purkinje fibres are rapid-conducting peripheral components of the CCS, composed of free-running and subendocardial fibres 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 artery⁴.

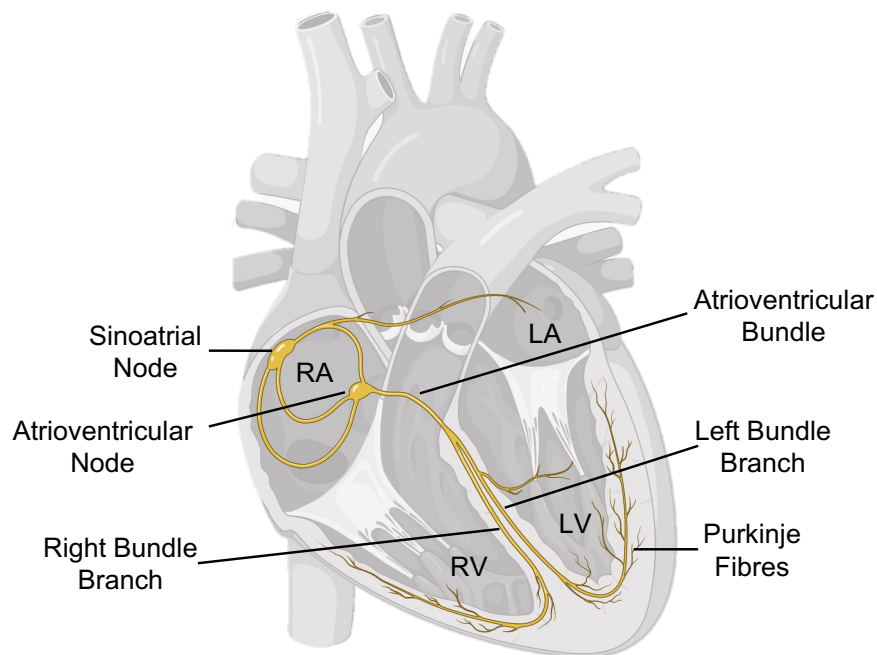


Figure 1.1: The cardiac conduction system

Schematic to illustrate the main components of the cardiac conduction system. The electrical impulse is generated in the sinoatrial node and travels across the atria to the atrioventricular node, where the signal is delayed. The signal then propagates from atria to ventricles via the atrioventricular bundle, bundle branches and Purkinje fibres of the ventricular conduction system. Labels indicate left atrium (LA), right atrium (RA), left ventricle (LV), right ventricle (RV). *Schematic created using Biorender.com.*

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 conduction system (VCS), composed of the AVB, BBs and Purkinje fibres, 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- (200 pS), medium- (60-100 pS), small- (20-40 pS) and ultra-small- (9 pS) conductance gap junction channels respectively⁵. Each gap junction channel forms from two hemi-channels of six Cx proteins each, where each hemi-channel derives from one of two participating cells; hemi-channels may contain uniform or diverse Cx isoforms⁶. 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 myocardium⁷; 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 myocardium⁸.

The differences in ion channel expression and calcium handling between conductive versus cardiomyocyte cells, results in marked differences in their cellular action potentials (APs). VCS cells have characteristically long APs, with a particularly rapid depolarisation and more negative plateau phase than the APs of working ventricular cardiomyocytes⁹. While this gives rise to the specialised electrophysiological properties of the conductive network, the longer AP duration of VCS cells is also thought to be a major contributing factor driving increased Purkinje pro-arrhythmic susceptibility. This can manifest via greater likelihood of

developing either early or delayed after-depolarisations¹⁰. Understanding of these VCS-specific electrical properties at steady state is, therefore, necessary to assess Purkinje-derived ventricular arrhythmias.

1.1.2 Development of the cardiac conduction system

The CCS develops concomitantly with the heart chambers. At the earliest stages in the primitive heart tube, the entirety of the sinus venosus, upstream of the right atrium, acts as pacemaker, characterised by HCN4 channel expression⁴. With heart tube elongation, cells added at the inflow tract assume dominant pacemaker activity, while rapidly proliferating progenitors joining the heart tube form cardiac muscle. Extensive lineage tracing indicates that there is no single CCS lineage; rather, the lineages of the various CCS components are segregated early on in development. These lineages remain interconnected despite having been subject to early differential cell fate decisions¹¹. Thus, the SAN forms from early segregated Insulin gene enhancer protein (*Isl1*)⁺ mesodermal progenitor cells and becomes morphologically distinct by embryonic day (E) 10 in the mouse¹¹. Pacemaker activity is restricted to the SAN during and following ballooning of the primitive atria. This occurs through repression of *HCN4* and T-box Transcription Factor 3 (*TBX3*) expression in the chambers, ensuring that in the SAN only, *TBX3* expression represses atrial myocardial genes while indirectly activating SAN specific genes including *HCN4*^{12,13}.

A *TBX2*⁺, *cGATA6*⁺ posteriorly-located cardiogenic mesodermal population that initially forms the atrioventricular canal (AVC) is probably the only lineage that forms the atrioventricular node (AVN) and the atrioventricular ring bundles. *BMP2* expression in the AVC activates expression of *TBX3* and *TBX2*, which act together with Msh Homeobox 2 (*MSX2*) to repress the working myocardial gene programme and stimulate the genetic programmes required for pacemaker and AV cushion formation^{14,15}. Finally, the origins of the VCS trace to the embryonic trabeculae and interventricular ring. The VCS shares common

ancestry with the ventricular working cardiomyocytes, both deriving from a myocardial progenitor pool present in the looped heart tube¹⁶. The VCS develops from this pool by specification of a Gap Junction protein 5 (*GJA5*)⁺ (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 form¹⁷.

Except for the sinus node, which derives from a mesodermal population located just beyond the heart field, CCS cells share their origins with the heart's cardiomyocytes. The CCS is, therefore, sometimes considered a rare subpopulation of cardiomyocytes, thought to comprise approximately 2% of the overall cardiomyocyte population^{2,11}. CCS cells become genetically and phenotypically distinct from cardiomyocytes; they undergo characteristic calcium cycling and have distinctive electrical properties allowing action potentials to spread through Purkinje tissue much faster than through working myocardium¹⁸. However, the CCS must integrate electrically with the working myocardium to form a synchronized contractile system. The finding that macrophages and fibroblasts also couple electrically to cardiomyocytes hints at the extent of integration in the conductive system^{19,20}. This seems especially true in the SAN, where extensive heterogeneous cell coupling with fibroblast populations has been observed²¹. 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.

1.1.3 The ventricular conduction system in injured, stressed and diseased hearts

Arrhythmias contribute significantly to mortality risk following injury, including after myocardial infarction (MI). Ventricular tachycardia (VT) (characterised by an abnormally fast ventricular rate) or ventricular fibrillation (VF) (characterised by rapid and uncoordinated

ventricular contractions) often present in the early phase after acute MI and are a primary cause of sudden cardiac death, but arrhythmias including atrioventricular (AV) block and bundle branch block (BBB) also arise during and after the transition from acute to chronic MI stages²². This suggests that the CCS is depleted, CCS function is impaired, 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, downregulation of Cx43 in cardiomyocytes at the infarct border and death of cardiomyocytes that were directly coupled to CCS cells, combine to result in VT, with repercussions for cardiac output that can be fatal²³. 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 risk²⁴.

While there has been historic focus on the role of the scar in triggering arrhythmia following MI, the CCS and particularly the fast-conductance BBs and Purkinje network of the VCS are increasingly implicated²⁵⁻²⁹. Analysis of infarcted dog and pig hearts has found that the conduction cells may have a higher capacity to survive ischemia than working myocardium^{30,31}. However, while Purkinje cells can persist in infarcted regions where working myocardium is absent, they have been shown to undergo cellular changes in morphology and sub-cellular structure, and overall it seems that Purkinje cell populations decline³¹. Surviving but impaired Purkinje fibres in the ischaemic infarct area are believed to be a major source of automatic foci in ventricular arrhythmia; catheter ablation represents the current treatment for patients with recurrent VT/VF triggered by damaged Purkinje fibre activity³². Although Purkinje cells may have a greater propensity to survive ischemia than myocardial cells, to what extent Purkinje cells die or remodel post-MI, and how the heart repairs the VCS, is not well understood.

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 contraction²². 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 maintain VCS function to achieve electrical regeneration of the heart³³.

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 made up of a small number of cells, which are embedded within the heart; this complicates cellular and molecular analyses pre- and post-injury. The location of the CCS also presents significant imaging challenges, requiring tissue clearing in order to penetrate the tissue. Moreover, targeting the CCS deep within the heart presents a 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 laid down during injury and from direct coupling between fibroblasts and cardiomyocytes in the infarct area³⁴. Regenerative approaches must also consider 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*³⁵. 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 heart³⁶. Tbx18 was found to suppress transcription of Cx43 directly, resulting in partial electrical uncoupling and slow propagation of APs, reminiscent of SAN activity. The reprogrammed cardiomyocytes underwent calcium cycling and could depolarise spontaneously³⁶. 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 viable³⁷. 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' cells³⁸. Altering canonical Wnt signalling resulted in ectopic formation of AV-junction-like cells with altered electrophysiological properties³⁹ and Neuregulin-1 has been implicated in supporting CCS cell fate in development suggesting it might act to facilitate reprogramming^{35,40}. More recently, direct reprogramming of human ventricular myocytes towards a Purkinje fate *in vitro* was achieved through treatment with a cocktail of 11 small molecules including Neuregulin, Epinephrine and Retinoic Acid⁴¹.

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, conduction diseases may be treated by cardiac resynchronisation therapy, by anti-arrhythmic drugs, or by catheter or laser ablation of ectopic regions of conduction²². However, available anti-arrhythmic drugs are poorly understood mechanistically with potential side effects including exacerbated arrhythmia, dizziness, chest pain and affected vision, while ablation of ectopic areas represents a last resort, which can induce lethal refractory or repetitive CCS symptoms, and it can be difficult to determine the precise area to

target for ablation. Repairing the CCS, therefore, presents an outstanding clinical challenge. Moving forwards, future strategies may centre around reactivating embryonic CCS transcriptional networks to programme a “biological pacemaker”, to reconstitute a functional His/Purkinje system free from aberrant activity, and to restore control of integrated electrical conduction.

1.2 Heart regeneration

1.2.1 Fibrotic repair in the adult heart after myocardial infarction

During an MI event, commonly referred to as a “heart attack”, a coronary artery narrows significantly or becomes blocked. Blood flow along this artery is either substantially reduced or entirely impeded, and so the cardiac tissue usually supplied by the blocked vessel becomes ischaemic. Consequently, cardiomyocytes and other non-muscle cells in the vicinity die. The adult heart then undergoes fibrosis, whereby non-contractile scar is deposited without replacement of the lost cardiomyocytes or supporting vasculature⁴². The laying down of the fibrotic scar is a necessary event following MI to provide mechanical support to the de-muscularised heart wall, without which there is greater risk of ventricular rupture. However, 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 consequently impair cardiac function⁴³.

The permanent loss of cardiomyocytes and other cardiac cells, coupled with subsequent pathological fibrosis, is a major contributing factor to long-term heart disease and onset of heart failure. No curative treatments currently exist for either heart disease or heart failure. The main treatment option available for patients is heart transplant, which relies on availability of a donor organ and has associated issues including rejection, graft

dysfunction, and extra-cardiac complications⁴⁴. Development of treatments for heart attack survivors with downstream pathologies remains a major and outstanding unmet clinical need.

1.2.2 The neonatal mouse model for heart regeneration

The human adult heart is one of the least regenerative organs in the body; unable to regenerate spontaneously following MI or other pathological events such as viral infection (myocarditis). While the adult mammalian heart shows very limited regenerative capacity⁴⁵, model systems exist which exhibit a full regenerative response (**Figure 1.2**). Notably, the zebrafish heart retains an ability to regenerate to full functionality throughout adulthood⁴⁶; the same is true of species such as the axolotl and salamander, which are able to regenerate whole limbs as well as their hearts^{47,48}. The neonatal mouse and pig both retain cardiac regenerative capacity immediately following birth, but this is lost by post-natal day 3 in porcine neonates and around day 4 in mouse neonates, following which point cardiomyocytes are generally thought to exit the cell cycle and are unable to proliferate⁴⁹⁻⁵². In humans, clinical case reports have indicated functional regeneration and an absence of scarring after cardiac surgery to treat congenital heart disease^{53,54} and in infants born with MI who have blood flow restored⁵⁵. Evidence of regeneration has also been reported in post-mortem analyses of infant hearts⁵⁶. Together this indicates that humans have neonatal regenerative capacity, which is lost in transition to adulthood. However, ¹⁴C dating and stereology analysis have identified low-level cardiomyocyte exchange that continues into adulthood⁴⁵; the existence of such innate cell turnover provides a promising target for amplification to increase the endogenous regenerative capacity of the mature heart.

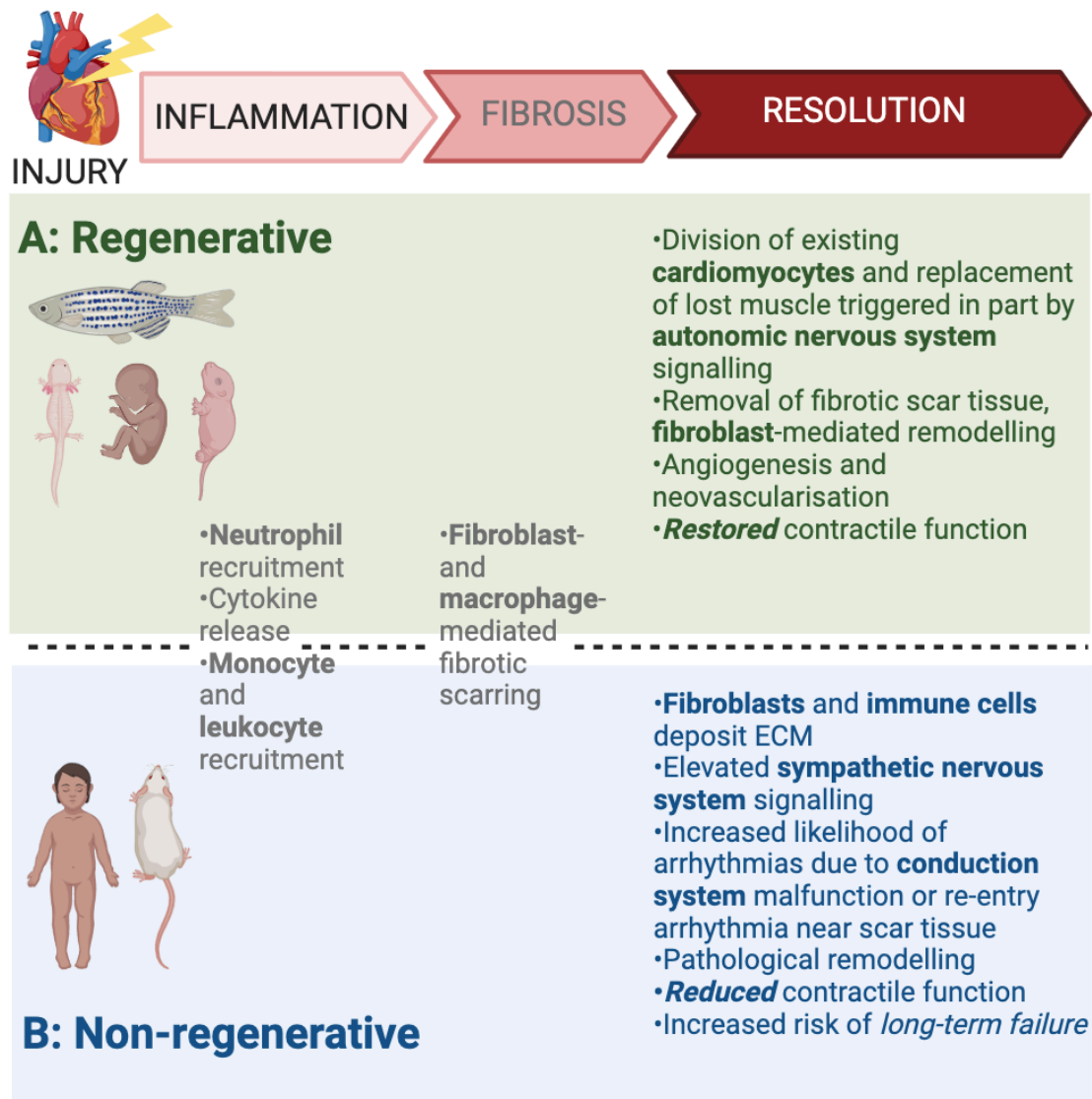


Figure 1.2: Diverse cellular contributions to regenerative versus non-regenerative cardiac responses following acute injury

a. Diverse cellular contributions promote a regenerative response to restore cardiac function in systems including zebrafish, axolotl and neonatal mammals. A pro-regenerative chemical and mechanical microenvironment, mediated principally by fibroblast, macrophage and nervous system signalling, result in removal of the scar, cardiomyocyte division to replace lost muscle tissue, and generation of new vasculature. **b.** In non-regenerative adult mammalian hearts, acute injury is resolved without restoration of cardiac function. Differences in cellular signalling and activity, including extent and nature of ECM deposition, fibroblast-macrophage crosstalk and sympathetic nervous system signalling, produce an anti-regenerative environment. Lost cardiomyocytes are not replaced, and new vessels do not grow. Instead, scar tissue is deposited long-term, resulting in lower contractility in the infarct region. Altered excitability of the fibrotic scar, and potential loss or migration of conduction system cells, increases the likelihood of arrhythmia. Pathological remodelling leads to heart failure which is a principal cause of mortality worldwide. *Schematic created using Biorender.com, reproduced with permission from Sayers and Riley 2021³.*

Differences exist between the neonatal and adult environment, particularly in terms of cell ploidy, and the mono-, bi-, or multi-nucleated state of cardiomyocytes⁵⁷. While neonatal regeneration depends on cardiomyocyte proliferation, evidence as to whether adult cardiomyocytes can re-enter the cell cycle and proliferate is emerging with evidence of induced proliferation in animal models, for example via gene therapy approaches⁵⁸⁻⁶¹. Furthermore, the neonatal environment is significantly hypoxic as compared to the adult heart, and there is a metabolic shift from reliance on glycolysis at birth, to fatty acid oxidation in the mature adult heart which affects cardiomyocyte proliferative capacity^{61,62}. Together, this suggests that a straightforward parallel between neonatal regenerative capacity and the adult injury setting may be challenging, especially in terms of translating neonatal findings directly into regenerative therapeutics. However, increased understanding of cellular behaviour and signalling in neonates may help to identify the supportive parameters promoting neonatal regeneration and to develop fundamental understanding with relevance to the mature heart.

Importantly, it should be noted that while adult cardiomyocytes are often bi- or multi-nucleated and polyploid, a recent study in the mouse found that VCS cells are more often diploid than working cardiomyocytes are (33%, versus 4% of ventricular working cardiomyocytes), because they escape cell cycle activity during post-natal maturation⁶³. This observation would support the hypothesis that the diploid state of Purkinje cells better enables them to re-enter the cell cycle to support conduction system regeneration following MI, and suggests that targeting VCS cells therapeutically to avoid post-MI arrhythmogenesis may be an especially promising strategy. However, when the same study investigated whether diploid VCS cells proliferate to replace lost cardiomyocytes, the authors found that despite VCS cells being highly diploid they did not preferentially proliferate following adult MI. While VCS cells seem overall not to have a special general regenerative competence, therefore, it remains to be determined whether VCS cells can divide to replenish damaged Purkinje cells and repair the conduction system in neonatal (regenerative) versus mature (non-regenerative) contexts.

Besides cardiomyocyte proliferation, heart regeneration additionally relies upon angiogenesis to vascularise the regenerated myocardium. Collateral artery growth in infarcted neonatal hearts supports regeneration, whereas artery regrowth is almost absent in adults⁶⁴. Increasing evidence from the neonatal mouse model is also implicating other cell types in heart regeneration³ (see **Figure 1.2**). Cardiac immune populations including macrophages are required for neonatal regeneration: when macrophages were depleted using clodronate-loaded liposomes before inducing MI at P1, neonates lost their regenerative ability and instead formed a permanent scar⁶⁵. Furthermore, cardiac innervation by the autonomic nervous system has been implicated as a regulator of heart regeneration, affecting cardiomyocyte proliferative capacity and the inflammatory immune response^{66,67}. However, the extent to which the CCS is damaged following MI, and whether it can regenerate and integrate with newly-formed cardiovascular tissue during neonatal heart regeneration, has not been systematically addressed.

1.3 Thesis rationale

The central hypothesis of this thesis is:

The cardiac conduction system can regenerate and integrate with the replenished cardiovascular tissue in early neonatal infarcted mouse hearts but is disrupted during the transition to fibrosis beyond the regenerative window.

The primary aims of this thesis are:

1. To map the morphology of the murine cardiac conduction system from birth, to provide detailed insights into conduction system development and 3D architecture.
2. To characterise changes to the conduction system following myocardial infarction at regenerative (P1) versus non-regenerative (P7) stages which recapitulate the mature injury response of fibrotic repair.
3. To define the molecular response of the regenerative and non-regenerative ventricular conduction system at single-cell resolution.
4. To establish whether and how molecular and cellular changes in the ventricular conduction system, lead to rhythmic disturbance following myocardial infarction.

These aims are realised in the following ways:

1. Development of bespoke tissue clearing, advanced high-resolution whole-organ imaging, and quantitative image analysis of the murine heart (**Chapter 3**). This is used to perform a systematic 3D-anatomical assessment, characterisation and quantification of the His/Purkinje network across late embryonic and post-natal mouse development.
2. Assessment of morphological and network-level disruption in the conduction system across the neonatal regenerative window following MI, including changes to bundle morphology, connexin and ion channel expression (**Chapter 4**).
3. Transcriptomic profiling using enriched single-cell RNA-sequencing of the infarcted P1 and P7 VCS (**Chapter 5**). This identifies previously unrecognised heterogeneity of cell types within the VCS (including a novel fibroblast-like conductive population), as well as a shift in cell type composition and electro-pathophysiological remodelling in the non-regenerative as compared to the regenerative VCS.
4. Electrophysiological analysis of mice following MI at P1 and P7 stages (**Chapter 6**). This establishes that there is functional electrical regeneration at P1 but sustained rhythmic disturbance after MI at P7.
5. Simulation of the human Purkinje network within a chronic infarcted heart, using a multiscale computational framework (**Chapter 6**). By modelling regional reduction of conduction velocity in the His/Purkinje network, the functional implications of the findings from the P7 non-regenerative network in Chapters 4-5 are assessed.

2 Materials and Methods

2.1 Mouse strains and husbandry

The *Cx40*^{EGFP/+} and *Nkx2.5*^{Cre/+} mouse strains used in this thesis have been described previously^{68,69}. Experimental litters in Chapters 3-5 were generated through crossing *Cx40*^{EGFP} homozygous males with CD1 females (Charles River Laboratories). Experimental litters in Chapter 6 were generated through crossing C57BL/6 males with CD1 females (Charles River Laboratories). Mice were housed by the Oxford University Biomedical Services. All animal experiments were carried out in accordance with UK home office project licenses PDDE89C84 and PP3194787, and were compliant with the UK Animals (Scientific Procedures) Act 1986.

2.2 Animal genotyping

2.2.1 Genomic DNA extraction

Ear biopsies were collected between 2-3 weeks of age and stored at -20°C. The genomic DNA was extracted using the REDExtract-N-Amp™ Tissue PCR Kit (Merck) according to manufacturer's instructions. In brief: thawed tissue was incubated in a mixture of 100µl extraction solution and 25µl tissue preparation solution for 2+ hours at room temperature. Samples were then heated to 95°C for 5 minutes before adding 100µl neutralisation solution, and vortexing the mixture.

2.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify the relevant DNA sequence for genotyping. Primer sequences are displayed in **Table 2.1**. Each PCR reaction tube contained 5µl REDExtraction-N-Amp PCR Reaction Mix, 0.5µM of each primer, 2µl template DNA and ultrapure Milli-Q water to a total reaction volume of 10µl. All PCRs were run using the programme displayed in **Table 2.2**.

Primer name	Sequence
Cx40, sense	5'-CTCCAATTAACCTTGTGAGCC-3'
Cx40, antisense	5'-AGGCTGAATGGTATCGCACC-3'
Neo, antisense	5'-CTTGCCGAATATCATGGTGG-3'
Cre, forward	5'-GTTCGCAAGAACCTGATGGACA-3'
Cre, reverse	5'-CTAGAGCCTGTTTTGCACGTTC-3'

Table 2.1: Polymerase chain reaction primer sequences used for genotyping

Step	Temperature (°C)	Duration (min:sec)	Number of cycles
Denaturation	94	2:00	1
Annealing	94	0:30	35
	60	0:30	
	72	0:45	
Extension	72	5:00	1
Hold	4	-	-

Table 2.2: Polymerase chain reaction thermal cycling programme used for genotyping

2.2.3 Gel electrophoresis

PCR products were resolved using agarose gel electrophoresis. 2.4g of agarose (Sigma Aldrich, UK) was dissolved in 120mL of 1X TBE buffer by heating the mixture in a microwave, for a 2% (w/v) agarose gel. 7µl of GelRed (VWR) was then added to the molten gel prior to setting. Each gel was run at an electric potential of 140V for 40 minutes to allow sufficient band separation. Gels were then visualised in a UV box. For Cre genotyping, the Cre formed a band at approximately 300 bp. For *Cx40^{EGFP}* genotyping, wild-type and recombined alleles formed bands at 380 and 450 bp, respectively.

2.3 Neonatal myocardial infarction by LAD coronary artery ligation

Neonatal MI was performed as described previously⁷⁰. In brief: litters were separated from their mother and placed in a 35°C incubator. General anaesthesia was induced (short exposure to 4% isoflurane in oxygen) followed by immersion on ice to induce hypothermal cardiorespiratory arrest. A horizontal incision was then made with surgical scissors at the left mid-thorax. Thoracotomy was achieved using sharp dissecting forceps applied to the 3rd intercostal space near the costochondral junction. The space was widened with blunt forceps and the heart extruded from the thoracic cavity by applying gentle pressure to thorax and diaphragm while holding the ribs open. An 8-0 Ethilon nylon suture was then passed through the heart muscle below the left atrium. Two throws of a surgeon's knot were tied to ligate the left anterior descending (LAD) coronary artery in the case of MI surgeries, but no knot was tied for sham procedures. The heart was then gently pressed back into the chest cavity, and the ribs and skin closed in layers using a 7-0 Prolene suture. The neonates were recovered under an infrared lamp, with administration of oxygen via the nose cone on return of respiration. Warmed pups, once motile and breathing independently, were cleaned and given a faecal bath to mask the surgical smell before returning them to the dam.

Sham and MI surgeries were performed by research technicians Carla de Villiers and Sarah Sigal; I performed the recovery from anaesthesia, returned pups to the dam and undertook animal monitoring post-surgery.

2.4 Imaging sample preparation, acquisition and analysis

2.4.1 Neonatal heart dissection

Neonatal mice were sacrificed by schedule I cervical dislocation. Hearts were removed using dissection scissors and forceps, and transferred to PBS immediately.

Hearts for tissue-cleared imaging were washed in PBS and the blunt end of the forceps used to apply pressure to the ventricles to pump out residual blood. After shaking in PBS for 30 minutes, hearts were fixed in 4% PFA at 4°C.

Hearts for histological imaging were washed in ice-cold PBS and then transferred to 4% PFA at 4°C immediately.

Hearts to be dissociated for fluorescence-activated cell sorting (FACS) and single-cell RNA-sequencing experiments were transferred to PBS. The atria, connective tissue and top of the major arteries were removed under a dissection microscope before proceeding immediately with enzymatic and gentle mechanical digestion.

2.4.2 Tissue clearing with immunostaining

2.4.2.1 Clear, unobstructed brain/body imaging cocktails and computational analysis (CUBIC) tissue clearing

Neonatal hearts were washed in PBS and the blunt end of the forceps used to apply pressure to the ventricles to pump out residual blood. After shaking in PBS for 30 minutes, hearts were fixed in 4% PFA at 4°C overnight. Hearts were washed in 1X PBS at room temperature before incubation in CUBIC-L (for delipidation and decolouring), shaking at 37°C. Hearts were incubated in CUBIC-L for at least 3 days, replacing CUBIC-L daily. Older postnatal hearts from P7 onwards remained in CUBIC-L for a further 8 days, replacing the CUBIC-L solution every other day. Hearts were then washed three times with PBS at room

temperature for 2 hours, and transferred to a blocking solution of 0.2% (v/v) Triton X-100, 2% (w/v) BSA, 10% serum in PBS overnight at 4°C. Primary antibodies (Table 2.3) were added for 3 days at 4°C. Following 10 wash steps in PBT, 0.1%, secondary antibodies (Table 2.4) were added for 2 days at 4°C. Following 7 wash steps in PBS, hearts were moved to CUBIC-R (for refractive index matching). Hearts remained in CUBIC-R for at least 4 days prior to imaging, shaking at room temperature, and CUBIC-R was replaced every other day. Hearts were imaged in CUBIC-R solution directly. Younger hearts up to P4 were imaged on an Olympus FV1000 inverted confocal in a LabTek 8-well imaging chamber. Older post-natal hearts were imaged in CUBIC-R on the Miltenyi-LaVision Bio Tec light sheet fluorescence microscope (Ultramicroscope II, Miltenyi Bio Tec, Bielefeld, Germany). Hearts were mounted and suspended in CUBIC-R mounting solution (RI 1.520), attached between two screws to the Miltenyi-LaVision mounting chamber.

2.4.2.2 Simple ultrafast multicolour immunolabelling and clearing (SUMIC) and CUBIC-ECi tissue clearing

Neonatal hearts were dissected and fixed as above, and then processed for SUMIC and CUBIC-ECi tissue clearing.

SUMIC: hearts were processed in accordance with Biswas et al.⁷¹. In brief: hearts were dehydrated through an ascending methanol gradient (50%, 80%, 100%, twice each for 20 min) at 4°C. Samples were immersed in H₂O₂ (5%) for 3 hours and then rehydrated through the reverse gradient as before. Samples were washed with PBS (3 x 20 min) and then incubated overnight in 25% urea, 15% glycerol, 7% Triton X-100 in PBS + 10% DMSO at 4°C. Samples were incubated in Collagenase A solution for 30 min, shaking at 37°C, and then washed 2 x 5 min PBS, 2% FBS. Samples were blocked in 10% Donkey serum, 10% DMSO, 0.5% Triton X-100 in PBS for 20 min at 37°C. Antibody staining was performed using 2% Donkey serum, 10% DMSO, 0.5% Triton X-100 in PBS. Primary antibodies (Table 2.3)

were added overnight, shaking at 37°C. Following washes in 2% Donkey serum, 0.5% Triton X-100, secondary antibodies (Table 2.4) were added for 6-8 hours, shaking at 37°C.

Following washes in 2% Donkey serum, 0.5% Triton X-100, samples were dehydrated in an ascending isopropanol gradient (30%, 50%, 80%, 100%, 100%) under gentle rotation at room temperature. Samples were then rinsed twice in Ethyl Cinnamate (ECi) (Sigma-Aldrich, cat. No 112372), cleared with 80% ECi, 20% PEGM under gentle rotation at room temperature for at least 30 min, and imaged in ECi on the Miltenyi-LaVision Bio Tec Ultramicroscope II.

CUBIC-ECi: samples were treated according to the CUBIC protocol detailed above (**Chapter 2.4.2.1**) through to the secondary antibody immunostaining step, and then were dehydrated through an ascending methanol gradient (20%, 40%, 60%, 80%, 100%, 100%) at 4°C and moved to Ethyl Cinnamate (ECi) (Sigma-Aldrich, cat. No 112372). Samples were imaged in ECi on the Miltenyi-LaVision Bio Tec Ultramicroscope II.

Due to Covid-19 access restrictions that prevented my entering the Kennedy Institute at the time of these experiments, the Ultramicroscope II imaging of the SUMIC samples was kindly performed by Zhangfan Ding of the Kusumbe Laboratory.

2.4.2.3 Immunolabelling-enabled three-dimensional imaging of solvent cleared organs (iDISCO) tissue clearing

Neonatal hearts were dissected and fixed as above. Hearts were then processed in accordance with Coronado et al.⁷². Details of buffers and reagents used are listed at <https://idisco.info/idisco-protocol>. In brief: hearts were dehydrated through an ascending methanol gradient (20%, 40%, 60%, 80%, 100%, 100%) at room temperature. Following overnight incubation in 66% DCM: 33% methanol, samples were washed in 100% methanol and bleached in H₂O₂ (5% in methanol) rotating at 4°C overnight. Hearts were rehydrated through the reverse methanol gradient and then washed in PBS, 0.2% Triton X-100. Hearts

were permeabilised for 2 days at 37°C and blocked for a further 2 days at 37°C. Primary antibodies (Table 2.3) were added in PTwH buffer with 5% DMSO, 3% Donkey serum for 4 days, rotating at 37°C. Following washes in PTwH, secondary antibodies (Table 2.4) were added in PTwH with 3% Donkey serum for 4 days, rotating at 37°C. Following further PTwH washes, samples were embedded in 1% low-melting agarose in PBS and then dehydrated through a methanol gradient (20%, 40%, 60%, 80%, 100%, 100%) at room temperature. Samples were incubated in 66% DCM: 33% methanol, and then in 100% DCM (2 x 30 min), all at room temperature. Samples were then rinsed in ECI (Sigma-Aldrich, cat. No 112372) and incubated in ECI until ready for imaging. Hearts were imaged in ECI on an Olympus FV1000 inverted confocal or on the Miltenyi-LaVision Bio Tec Ultramicroscope II.

2.4.3 Immunofluorescence on cryosections

Hearts were fixed in 4% PFA at 4°C overnight, washed in 1X PBS and incubated in 30% sucrose solution overnight. Hearts were then moved to OCT (Optical Cutting Temperature Compound, Fischer Health Care, Catalogue:4585) for 3 hours before mounting in OCT and storing at -80°C. For immunostaining, cryosections were thawed at room temperature, washed in PBS, permeabilised in PBT,0.5%, washed in PBT,0.1% and then incubated in blocking solution for 1 hour at room temperature. Primary antibody in block was added (Table 2.3) and incubated at 4°C overnight. The following day, tissue sections were rinsed in PBS,0.1% and then incubated in AlexaFluor secondary antibody (Invitrogen, 1:200) for 1 hour at room temperature. Slides were then rinsed in PBS and washed in PBT,0.1%. Slides were mounted in 1:1 glycerol: PBS prior to imaging on an Olympus FV1000 inverted confocal. Negative controls (without primary antibody) were included for each secondary antibody in order to confirm signal specificity.

2.4.4 Imaging the dissected ventricular conduction system

2.4.4.1 Dissection of the ventricular conduction system

Cx40^{EGFP/+} hearts were harvested and dissecting forceps were used under a fluorescent Zeiss SteReo Discovery.V8 microscope to remove the left atrium. The forceps were inserted into the hole directly below where the atrium was removed from, and the ventricular chamber was prized open without disturbing the conduction network. Either side of the ventricle wall was folded outwards and pinned open at apex and base onto a 4% agarose plate, submerged in sterile PBS. The VCS was imaged and samples were then fixed in 4% PFA at 4°C for 5 hours.

2.4.4.2 Wholemount immunofluorescence

Following fixation as above, the dissected hearts were washed at room temperature in PBT, 0.3% and then blocked for 1 hour at room temperature in a solution of 0.3% (v/v) Triton X-100, 1% (w/v) BSA, 10% serum in PBS. Primary antibodies (Table 2.3) were added in blocking solution overnight at 4°C. Hearts were washed 5+ times in PBT, 0.3% and then 3+ times in PBS. Secondary antibodies (Table 2.4) were added in PBS overnight at 4°C, and then further incubated at room temperature for 30 minutes. Following PBS washes, the hearts were immersed in 1:1 glycerol: PBS. Hearts were imaged in the 1:1 glycerol: PBS solution in a Labtek 4-well imaging chamber. 4% agarose blocks were used as weights to press the dissected hearts flat from above, with the open chamber surface facing downwards. Hearts were imaged on a Zeiss LSM 980 inverted confocal microscope.

2.4.4.3 *In situ* Hybridisation Chain Reaction (HCR) RNA fluorescence in situ hybridisation

Whole mount *in situ* HCR RNA fluorescence in situ hybridisation (RNA-FISH) was performed using the commercially available methodology from Molecular Instruments. The HCR RNA-FISH protocol for whole-mount mouse embryos (revision number 9) was performed in general accordance with manufacturer's instructions, but with omission of the methanol dehydration steps to retain endogenous CX40-eGFP fluorescence.

In brief: cut-open hearts (dissected as per **Chapter 2.4.4.1**) were fixed in 4% PFA overnight at 4°C, and then rinsed and stored in sterile PBS at 4°C prior to commencing HCR. Samples were washed in PBST at room temperature (2 x 10 min) and immersed in 10ug/mL proteinase K solution at room temperature for 15 min. Following PBST washes, samples were post-fixed in 4% PFA, at room temperature for 20 min. Samples were washed in PBST and incubated in 1mL probe hybridisation buffer for 5 min at room temperature. Samples were then incubated in probe hybridisation buffer at 37°C for 30 min before adding them to 2pmol of each probe set in 500µL probe hybridisation buffer, and incubating them overnight at 37°C. The following custom probes were used: Pde4d-B3, Prkaca-B2, Cdk8-B4, Inpp5a-B1, Esrrg-B2, Nkx2-5-B1. Samples were next washed with probe wash buffer at 37°C and then with 5 x SSCT at room temperature before moving to the amplification stage.

Samples were pre-amplified with 1mL amplification buffer at room temperature for 5 min. 30pmol of snap-cooled hairpins h1 and h2 were then added to 500µL amplification buffer and the samples incubated in the dark at room temperature overnight. The following amplifiers were used: B1-647, B2-546, B3-546, B4-647. Excess hairpins were then removed in wash steps with 5 x SSCT at room temperature and samples were stored at 4°C before imaging. The dissected hearts were imaged in 1:1 glycerol: PBS solution on a Zeiss LSM 980 inverted confocal microscope (as per **Chapter 2.4.4.2**).

Antigen	Host Species	Company reference	Dilution (application)
GFP	Chick	Abcam ab13970	1:200 (IF-WM, IF-Fr)
HCN4	Rabbit	Alomone Labs APC-052	1:200 (IF-WM, IF-Fr)
CNTN2	Goat	R&D Systems AF4439	1:100 (IF-WM), 1:50 (IF-Fr)
Nav1.5	Rabbit	Alomone Labs ASC-005	1:50 (IF-Fr)
ER81	Rabbit	Biolegend 11050	1:50 (IF-Fr)
Connexin-43	Rabbit	Sigma Aldrich C6219	1:200 (IF-Fr)
ROBO1	Rabbit	Abcam ab7279	1:100 (IF-Fr)
SLIT3	Goat	R&D Systems AF3629	1:100 (IF-Fr)
CD68	Rat	Bio-Rad FA-11	1:200 (IF-WM, IF-Fr)

Table 2.3: Primary antibodies

Abbreviations: IF-WM: immunofluorescence of whole-mount tissue; IF-Fr: immunofluorescence of frozen/ cryo-sections

Antigen	Host Species	Company reference	Dilution
Chick-488	Goat	Invitrogen A32931	1:200
Rabbit-555	Donkey	Invitrogen A-31572	1:200
Goat-555	Donkey	Invitrogen A-21432	1:200
Rat-555	Goat	Invitrogen A-21434	1:200
Rabbit-647	Donkey	Invitrogen A-31573	1:200

Table 2.4: Secondary antibodies

2.4.5 Image analysis

2.4.5.1 *Analysis of the intact ventricular conduction system*

Whole-heart tiled confocal images were stitched in the Olympus software with 10% overlap. Lightsheet images were acquired as single-frame Z-stacks and so did not require stitching prior to analysis.

3D-rendering, background subtraction, segmentation and quantification of wholemount cleared hearts was performed in Imaris 10.0. Background subtraction was first performed on the 488-channel. Next, an Imaris surface element was built. A region of interest was first defined in XYZ planes to cover the full ventricular area within which Purkinje fibres existed. Segmentation was then performed: global thresholding was defined according to local contrast, applying a Gaussian filter to estimate and subtract the background intensity value per voxel; touching objects were split to allow for region growing around defined seed points; seed points were selected using a supervised machine-learning approach to differentiate His/Purkinje from artery signal. Once an Imaris surface element covering the full His/Purkinje network had been generated and artery surface elements subtracted, the resulting surface volume was masked to a new channel: original voxel values were retained within the surface element volume, but all other voxels were assigned a zero value. The 3D filament model of the network was then built using the Imaris 10.0 Filament Tracer module. The automated autopath algorithm was used to create a looped filament network based on local intensity contrast. Seed points were defined with a minimum diameter of 9.75µm. Supervised machine-learning training rounds were used to remove extraneous linkages between filaments. The left and right filament models were then duplicated to new channels to extract chamber-specific quantification statistics. Detailed statistics and quantitation were collated in Imaris and analysed using Prism 9.0 GraphPad software.

2.4.5.2 Quantification of the dissected His/Purkinje network

For calculation of CX40 network proximal: distal intensity ratio, analysis was performed in Fiji/ImageJ using the 2D SteReo Discovery.V8 image. Each image was cropped to exclude regions beyond the VCS, and transformed to position the AVB centrally at the top of the image. Two equally-sized rectangular regions of interest were defined to cover the upper and lower half of the image, respectively. The ROI manager tool was used to calculate the sum of pixel intensity values within the defined regions. The ratio of upper: lower summed values was then calculated for each image. Statistics were analysed using Prism 9.0 GraphPad software.

2.4.5.3 Analysis of histological sections

Images were analysed in Fiji/ImageJ. Brightness and contrast were adjusted and settings applied across all channels without altering digital gain. Scalebars were applied prior to exporting the image, and corresponding scales are reported in all relevant figure legends.

2.5 Neonatal heart dissociation

Hearts were harvested, the atria and the top of major arteries were removed with forceps and the blunt end of the forceps used to apply pressure to the ventricles to pump out residual blood. Hearts were then immediately transferred to a gentleMACS™ C Tube (Miltenyi Biotec 130-093-237). Enzyme mixes were prepared and added in accordance with the manufacturer's instructions (Miltenyi gentleMACS Neonatal Heart Dissociation Kit

protocol) and the samples were dissociated into a single cell suspension by running the 37C_mr_NHDK_1 programme on a gentleMACS Octo Dissociator with Heaters.

Following completion of the Dissociator programme C tubes were detached. Sample resuspension and red blood cell lysis were performed in accordance with the Miltenyi Neonatal Heart Dissociation protocol. Centrifugation steps between washes were performed at 500 x G, 5 minutes, 4°C.

2.6 Single-cell RNA-sequencing

2.6.1 Sample processing for single-cell RNA-sequencing

Cx40^{EGFP/+} litters from *Cx40^{EGFP}* homozygous males crossed with CD1 females were used for surgeries. Half of each P1 and P7 litter was subjected to sham surgery and half to MI surgery so as to minimise variation in genetic background by using littermate controls. 8 P1 MI hearts, 6 P1 sham hearts, 5 P7 MI hearts and 5 P7 sham hearts were harvested 3 days post-injury for single-cell RNA-sequencing. Hearts were individually processed through dissociation, lysis and staining steps. Following neonatal heart dissociation as above, cell pellets were resuspended in 100µl FcX blocking buffer (1:50 in DMEM, 3% FBS) for 10 minutes on ice. Next, cells were stained for CD31-Biolegend BV421 (1:50 in 100µl DMEM, 3% FBS) for 20 minutes on ice, protected from light. 1mL cold DMEM, 3% FBS was then added to each sample and the samples were centrifuged. Cells were next resuspended in PBS, 10% FBS and then a second wash step performed, again resuspending in PBS, 10% FBS. Samples were centrifuged at 450 x G, 10 minutes, 4°C.

Samples were next labelled with Cell Multiplexing Oligos from 10X 3' CellPlex Kit Set A (PN-1000261). Before use, Cell Multiplexing Oligos were thawed at room temperature, vortexed for 5 seconds and centrifuged briefly for 5 seconds in accordance with manufacturer's instructions (CG000391 Demonstrated Protocol 3: Cell Multiplexing Oligo

Labelling). Supernatant was removed following the previous wash step and each cell pellet resuspended in 100µl Cell Multiplexing Oligo at room temperature. Following a 5 min room temperature incubation, 1.9mL cold PBS, 10% PBS was added per sample and the sample mixed gently by pipetting. Cells were then centrifuged at 450 x G, 10 min, 4 degrees. Following centrifugation, the supernatant was removed fully. Samples were then resuspended in PBS, 10% FBS and individual hearts were pooled across condition (i.e., all hearts of same stage and MI or sham treatment type were combined into one Eppendorf tube, giving 4 Eppendorf tubes overall) to give a total volume of 1mL per sample. Cells were kept on ice at all times from this stage onwards.

Viability staining was performed immediately before progressing to FACS by adding 1µl zombie NIR dye (1:1000 in PBS) per tube. Cells were then sorted on an Aria Fusion II cell sorter. Gates were drawn using single and combined controls and GFP+ CD31- viable singlets were collected into a 2mL low-bind Eppendorf tube.

Following FACS, cell concentration and viability were calculated using a Countess II cell counter. Cells were then centrifuged at 450 x G, 10 min, 4°C. The supernatant was removed and cells resuspended in the appropriate residual volume based on concentration. Samples were then immediately processed according to the 10X Chromium Next GEM Single Cell 3' v3.1 (Dual Index) with Feature Barcoding Technology for Cell Multiplexing protocol (CG000388 Rev C). Gene expression and cell multiplexing feature barcode libraries were constructed using the Single Cell 3' with Cell Multiplexing Reagent Kit v3.1. Paired-end sequencing was performed with an Illumina NovaSeq 6000 instrument.

2.6.2 Pre-processing and quality control of single-cell RNA-sequencing data

FASTQ sequencing data was processed using the 10X CellRanger multi pipeline (version 7.0.0), aligning reads to the mm10 genome. Only singlets correctly assigned to a single heart in demultiplexing were retained at this stage. Downstream analysis was

performed in R (version 4.3.0). Individual heart datasets were combined within conditions by merging Seurat objects, and quality filtering was performed using Seurat (version 4.3.0)⁷³. In brief: cells in the top 1% quantile, cells with fewer than 200 detected genes, and cells with a contribution of over 40% transcripts from mitochondrial genes were excluded from downstream analysis. The relatively high mitochondrial threshold of 40% was chosen due to the properties of conduction cells, since CCS cells are known to have a higher-than-average mitochondrial content⁷⁴. The data were normalised by total expression, multiplied by a scale factor of 10,000 and log-transformed. The scaled expression matrix was normalised using the Seurat ScaleData function and vars.to.regress set to nUMI and nGenes. 8778 P1 MI, 12431 P1 sham, 7646 P7 MI and 3922 P7 sham cells were kept for downstream analysis.

2.6.3 Unsupervised clustering analysis and dataset integration

Datasets were integrated across conditions in Seurat via the IntegrateData function. Principal component analysis (PCA) was applied using genes with the most variable expression across the integrated dataset, followed by non-supervised clustering and data visualisation using Uniform Manifold Approximation and Projection (UMAP). Cell type identities were assigned manually by identifying the most highly conserved genes for each cluster, and comparison of these with published datasets^{75,76}, known expression profiles (including via the Human Protein Atlas proteinatlas.org)⁷⁷, and reported function⁷⁸. Smooth muscle, immune and epicardial-like VCS clusters were then removed before re-running normalisation, PCA and UMAP analysis. Cell cycle analysis was performed using G2M and S Phase genes⁷⁹.

2.6.4 Differential abundance and differential expression analyses

Differential abundance testing using k-nearest neighbour graphs was performed using the Milo package in R⁸⁰. Differential gene expression analysis was performed using Seurat FindMarkers. Gene regulatory analysis was performed using the Single-Cell rEgulatory Network Inference and Clustering (SCENIC) workflow in R⁸¹.

2.7 Single-nuclear RNA-sequencing analysis

2.7.1 Pre-processing and quality control of single-nuclear RNA-sequencing data

The dataset of single nuclei expression matrices (<http://www.ncbi.nlm.nih.gov/geo/GSE130699>) was downloaded for re-analysis. Quality filtering was performed using Seurat⁷³ and in accordance with Cui et al¹. In brief: cells in the top 1% quantile, cells with fewer than 200 detected genes, and cells with a contribution of over 25% transcripts from mitochondrial genes were excluded from downstream analysis. The data were normalised by total expression, multiplied by a scale factor of 10,000 and log-transformed. Cell cycle analysis was performed in Seurat and differences were regressed out between G2M/S phases. The scaled expression matrix was normalised using the Seurat ScaleData function and vars.to.regress set to nUMI and nGenes. 10,276 cardiomyocyte nuclei from control hearts and 9165 cardiomyocyte nuclei from injured hearts were analysed overall.

2.7.2 Clustering analysis and dataset integration

Integration of datasets across conditions was performed in Seurat via the IntegrateData function, in accordance with standard process and with Cui et al^{1,82}. The data

were visualised with dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP) to project into 2D space, and aligned canonical correlation vectors (1:30) were applied to identify nearest neighbour-based clusters. 17 clusters were identified through FindCluster Seurat graph-based clustering, at a resolution of 0.6. Cluster cell-types were assigned by identifying the most highly conserved genes of each cluster, and identifying known marker genes for each by comparison with published datasets^{75,76}.

2.7.3 Re-clustering and identification of ventricular conduction system cells

Cardiomyocyte- and fibroblast- associated clusters were subsequently selected and re-clustering was performed, with aligned canonical correlation vectors (1:40). I identified 16 sub-clusters with a resolution of 0.6. From this, the top 6 highly conserved markers for each cardiomyocyte-associated cluster were analysed for overlap with known VCS markers. Cluster 13 was identified as the putative VCS cluster based on 2 of the 6 most highly conserved markers being canonical VCS genes: *Slco3a1* (the CCS-LacZ locus) and *Etv1*. To confirm VCS identity, 7 VCS markers (*Cntn2*, *Gja5*, *Cpne5*, *Ntn1*, *Etv1*, *Sema3a*, *Slco3a1*) were used to assign a module enrichment score: the UCell Mann-Whitney algorithm was chosen, to be independent of dataset composition, and the result visualised via UMAP projection⁸³.

2.8 Electrophysiology

2.8.1 Micro-electrode array recordings

Cx40^{EGFP/+} litters from *Cx40*^{EGFP} homozygous males crossed with CD1 females were used for surgeries. P1 and P7 MI samples were harvested for micro-electrode array (MEA) recording. The VCS was dissected open in general accordance with **Chapter 2.4.4.1**, but all

buffers including PBS for the dissection stages were pre-warmed to 37°C. Dissection was performed as rapidly as possible, and the left ventricle was prized open without removal of either atrium. Immediately following pinning open of the left ventricle, the CX40-eGFP+ VCS was imaged and the heart was then transferred (exposed chamber facing downwards) to a 60-electrode MEA2100-System (Multichannel Systems, Harvard Bioscience) recording chamber filled with warmed CO₂-independent medium (1X) (Gibco, catalogue no.18045-054). The heart was pressed down from above onto the electrodes using a circular metal weight and re-adjusted as necessary to centre the ventricle on the electrode array. Recordings were collected and analysed using the Multichannel Systems Cardio2D software.

2.8.2 Electrocardiogram recordings

Litters from C57BL/6 males crossed with CD1 females were used for surgeries. Half of each litter underwent MI surgery and half underwent sham surgery to ensure littermate controls had minimal differences in genetic background. ECG recordings were collected from 6 P1 MI, 4 P1 sham, 6 P7 MI and 4 P7 sham animals, 21 days following surgery. General anaesthesia was induced in an anaesthetic chamber (4% isoflurane in oxygen) and maintained by delivering 1-2% isoflurane in oxygen via a facemask, whilst the animal was positioned on a 37°C heat mat. Both arms and the left leg of the animal were shaved to remove fur and the limbs were pinned down using sterile needles. Electrodes were attached to the upper right arm and the lower part of the left leg to achieve a lead II orientation, and to the upper left arm and lower left leg for a lead III orientation. Electrodes were connected to a Biopac ECG100C amplifier and ECG recordings collected using the Acqknowledge 4.0 software. The ECG experiment was performed in collaboration with Dr Neil Herring. We executed the experiment together; I then performed data analysis with supervisory input from Neil.

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 software. An unpaired, two-tailed T-test was used for comparing between two experimental groups. A one-way ANOVA with multiple comparisons was used for comparing more than two groups, and a Tukey-Kramer test applied to compare the means of every group. In all cases, a p-value of less than 0.05 was deemed significant. Significant p-values are reported as *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$). Non-significant p-values are provided to four significant figures. Individual points are plotted together with mean and error bars indicating ± 2 standard deviations.

2.10 Modelling and simulation

A multiscale computational framework for simulating human cardiac electrophysiology from ionic currents to electrocardiographic signal in myocardial infarction was developed to analyse the effects of reduced conductivity in patches of the Purkinje network post-MI, building on^{84,85}. The human biventricular model was calibrated and evaluated using extensive clinical and experimental data, both under healthy and MI conditions, and at multiple scales including cell (ion dynamics, action potential), tissue (conduction velocity, fibre anisotropy), and organ (activation sequence, electrocardiographic signal, electrophysiological heterogeneities) levels.

The post-MI patches with reduced VCS conductivity were designed to include Purkinje branches in areas matching the CX40 loss identified in my experimental imaging data. Baseline conductivity in the VCS (σ_{purkinje}) was set to 2.25 S/m, and baseline conductivity in the longitudinal myocardial fibre direction ($\sigma_{\text{ventricular}}$) was 0.26 S/m to yield a realistic activation sequence and QRS complex in simulations. Mild conduction delays were

modelled as ($\sigma_{\text{CX40-loss}} = (\sigma_{\text{ventricular}} + \sigma_{\text{purkinje}})/2$), severe conduction delays were modelled as ($\sigma_{\text{CX40-loss}} = \sigma_{\text{ventricular}}$) and extreme conduction delays were modelled as ($\sigma_{\text{CX40-loss}} = \sigma_{\text{ventricular}}/2$).

The modelling and simulation experiments were executed by Dr Hector Martinez-Navarro; I conceptualised the project and hypotheses to be tested, aided in the overall model design, and we together performed downstream data analysis of the resulting simulated ECG recordings.

2.11 Clinical Data

Written informed consent was obtained for the use of anonymised patient case reports at the John Radcliffe Hospital, Oxford, United Kingdom.

The anterior ST segment elevation myocardial infarction trace was obtained from a 70-year-old man with hypertension presenting with chest pain and out of hospital VF arrest, treated with immediate by-stander cardiopulmonary resuscitation and one successful automated external defibrillator (AED) shock. The patient was found to have anterior ST elevation on the ECG, which was treated with primary percutaneous coronary intervention (PCI) to the occluded proximal LAD with two drug eluting stents. Staged PCI was performed to severe mid vessel stenosis in the right coronary artery. The patient had an overall ejection fraction of 42%.

The left bundle branch block trace was obtained from a 69-year-old man with hypertension, type II diabetes mellitus and end stage renal failure (on haemodialysis via right arm AV fistula) presenting with heart failure symptoms. The patient had severely impaired LV systolic function (ejection fraction of 30%) and left bundle branch block (QRS duration 173 msec). The patient had a large anterior MI with no inducible ischaemia on his myocardial perfusion scan. Treated with optimal medical therapy and a primary prevention cardiac resynchronisation therapy defibrillator (CRTD).

The right bundle branch block trace was obtained from a 51-year-old man with type II diabetes mellitus presenting with heart failure symptoms 2 years following coronary artery by-pass grafting. He was found to have severely impaired LV systolic function on transthoracic echocardiography (previously normal), first-degree heart block (277 msec) and a right bundle branch block (QRS duration 161 msec) despite medical optimisation. A myocardial perfusion scan showed a fixed apical perfusion defect, further apical to mid inferior lateral defect, with minimal inducible ischaemia. The patient went on to have a primary prevention CRTD.

The interventricular conduction delay trace was obtained from a 63-year-old man with two previous MIs and subsequent coronary artery by-pass grafting, presenting with worsening heart failure symptoms. The patient had an ejection fraction of 15% and a primary prevention implantable cardioverter defibrillator (ICD). Broadening of the QRS duration (160msec) with interventricular conduction delay was treated with optimisation of medical therapy, and upgrade of the ICD to a primary prevention CRTD.

3 The three-dimensional anatomy of the murine ventricular conduction system through post-natal development

3.1 Background

3.1.1 Tissue clearing for whole-organ imaging

The opacity of organs has been a long-standing challenge in biological imaging. While tissues of interest are often located deep within an organ, due to light scattering only surface-level structures are visible using traditional light microscopy. Historically, this issue has been circumvented by dissecting or slicing organs so as to visualise internal structures. However, cutting samples in this way disrupts the 3D tissue architecture, which determines much of an organ's biology including: how structure relates to function; how cells interact with each other and with the extracellular matrix (ECM); how the microenvironment supports tissue architecture. An appreciation of tissue organisation in 3D space is of particular importance for understanding anatomical networks such as the blood and lymphatic vasculature, the nervous system, and in the context of the heart, the cardiac conduction system.

Tissue clearing approaches aim to alter tissue composition and reduce refractive index mismatch so that light can penetrate centimetres of tissue. In this way, whole organs can be imaged with good signal-to-noise at depth using light microscopy. Numerous tissue clearing methodologies have been developed and reviewed extensively elsewhere⁸⁶. In brief, three types of approaches exist: aqueous hydrophilic approaches; hydrophobic approaches requiring sample dehydration; and hydrogel-based approaches. Generally,

these all aim to replace light-scattering biological molecules including lipids and heme, and to homogenise the refractive index throughout the organ and surrounding solution.

The best-known aqueous methods are the clear, unobstructed brain/body imaging cocktails and computational analysis (CUBIC) approaches⁸⁷, but other methods including Scale, See Deep Brain (SeeDB) and ClearT have also been developed^{86,88-90}. CUBIC methods were designed through large-scale chemical screening to identify the best combination of reagents to depigment, delipidate and match refractive indices across a range of biological organs. While variants exist, optimised to particular applications, CUBIC protocols generally consist of two separate steps: first a delipidation step; and secondly immersion in a refractive index-matching reagent cocktail⁹¹⁻⁹³. A particular strength of CUBIC protocols is their high level of compatibility with immunostaining and with retaining endogenous fluorescent reporter proteins. Indeed, the initial delipidation step often improves subsequent antibody penetration into the tissue.

Hydrophobic tissue clearing methods generally involve sample dehydration through an organic gradient followed by immersion in organic solvents. A number of these protocols also involve a bleaching step after the initial dehydration to reduce autofluorescence. To be compatible with immunostaining, the samples must next be rehydrated and antibody steps undertaken in aqueous buffers, before dehydrating the sample a second time for refractive index matching. Several hydrophobic tissue-clearing protocols – especially three-dimensional imaging of solvent-cleared organs (DISCO) and related benzyl alcohol and benzyl benzoate (BABB) protocols – achieve a very high level of tissue transparency⁹⁴⁻⁹⁶. However, these protocols have a tendency noticeably to shrink the tissue, and a majority of the downstream refractive index matching clearing solutions are highly toxic and potentially very damaging to microscope systems. Recent efforts to overcome this limitation have, for example, involved ultimately rinsing organs and immersing them in the Ethyl Cinnamate (ECi) ester. ECi is a non-toxic reagent but has chemical properties and a refractive index compatible with deep tissue imaging⁹⁷.

The third category of tissue clearing involves embedding the sample in a hydrogel, before solvent immersion to improve tissue transparency. Initial cross-linking between the hydrogel scaffold and the embedded tissue is designed to prevent tissue damage or structural change to nucleic acids and proteins during the subsequent tissue clearing steps⁹⁸. Another difference seen in hydrogel-based methods is that many protocols including the cleared lipid-extracted acryl-hybridised rigid immunostaining/in situ-compatible tissue hydrogel (CLARITY) methods use electrophoresis to accelerate and improve clearing, often requiring a custom-built chamber with careful control of parameters such as temperature. In general, therefore, these methods are more involved than the aqueous or hydrophobic approaches.

The heart is especially challenging for whole-organ imaging. Most of the tissue clearing methods above were originally designed and optimised to image the brain. However, while the brain is relatively lipid-dense, the heart contrastingly is a myoglobin-rich muscular pump with an extensive and mature ECM. The heart is heme (iron)-dense, and markedly different to the brain in its molecular composition. As such, there is a bespoke need for tissue clearing methodologies optimised for cardiovascular imaging.

All three different tissue clearing approaches – hydrophilic, hydrophobic and hydrogel-based methods – have been applied to the heart, achieving varying degrees of tissue transparency. The most successful cardiovascular tissue clearing so far has relied on the iDISCO protocol. Using iDISCO, post-natal and adult murine hearts have been imaged compatibly with some antibody staining^{72,96}. However, a major limitation of this approach involves the high toxicity of the BABB-based clearing reagents, and the requirement to dehydrate the sample through an organic gradient which affects tissue composition and is particularly damaging to certain biological molecules including fluorescent proteins. ECI-based hydrophobic clearing provides an alternative approach, but with lower depth penetration and lower signal-to-noise than is achievable with iDISCO⁹⁹. Moreover, ECI-based clearing still requires dehydration through an organic gradient which bleaches

endogenous fluorescence. Many other protocols have been tested on, but not optimised for, cardiovascular imaging. Overall, imaging the heart through post-natal development with tissue clearing, using endogenous fluorescent reporters and/or immunofluorescence, remains an outstanding challenge that this Chapter will attempt to address.

3.1.2 Application of confocal and lightsheet microscopy to large tissue samples

Confocal microscopy has been a central workhorse of cellular imaging over the past seventy years. In contrast to widefield microscopy, confocal microscopes direct illuminating light through a pinhole aperture to remove out-of-plane light. This achieves high optical resolution and results in relatively good contrast and low signal-to-noise ratios as compared to widefield microscopy. While confocal microscopy was initially applied to imaging of cells or thin samples mounted on microscopy slides, more recently it has been applied (quite effectively) to whole tissue and organ imaging (as reviewed in ¹⁰⁰).

Despite frequent adoption, confocal microscopy has limited depth penetration, restricted working distance and requires very long scan acquisition times. These drawbacks can be overcome by instead using light sheet or multi-photon imaging systems. Light sheet microscopes excite the sample using a thin sheet of light perpendicular to the microscope objective. In this way, only the focal plane of interest is illuminated. The sample may, therefore, be imaged plane by plane, which greatly speeds up acquisition as compared to confocal line-scanning approaches. Due to the optical sectioning achieved by light sheet illumination, the sample is less prone to bleaching or laser damage since only the focal volume of the sample is illuminated at any one time, and so the sample is exposed to lower laser power for shorter periods of time. Light sheet microscopy has consequently revolutionised our ability to image large samples quickly and with a high signal-to-noise at depth. Multi-photon systems by contrast do not have the speed advantages of the light

sheet, but enable better depth penetration than confocal microscopy and are particularly useful for intravital imaging applications. The size of the murine post-natal heart makes it a perfect system in which to take advantage of the benefits of light sheet microscopy.

3.1.3 Imaging the cardiac conduction system

The CCS is an especially challenging tissue to image. In part, this is due to its location: the CCS lines the cardiac chambers and runs along the intraventricular septum in the centre of the heart. Since it is not a superficial structure, imaging the intact CCS requires light penetration through the full extent of the myocardial wall or careful dissection to reveal the deep tissue anatomy. Indeed, Tawara undertook a detailed morphological study of CCS components including the AVN, bundle of His and neighbouring working myocardium in 1906, which paved the way for decades of careful CCS observation¹⁰¹. A great majority of the early CCS studies, since Tawara's monograph, have relied on sheep, cow, dog and pig models because they have significantly larger, free-running Purkinje fibres. These can be dissected out of the heart and studied in isolation *in vitro*, and can be identified histologically by electron microscopy or with Haematoxylin and Eosin (H&E) section staining based on cellular morphology.

A second reason why the CCS is challenging to image is because it is difficult to identify which cells make up the CCS. While some Purkinje fibres are free-running, conduction system cells are also sub-endocardial. Although they share many sub-cellular structures, Purkinje cells differ from working myocardial cells: they have distinctive features including a lack of T-tubules, large glycogen granules and characteristic mitochondria¹⁰². Nevertheless, Purkinje cells are indistinguishable by eye from surrounding working myocardium. This has led to a need for VCS-specific markers to robustly identify and study Purkinje cells. However, relatively few VCS-specific molecular markers have so far been identified. While Cx40 is generally considered amongst the most specific and well-

characterised markers for VCS cells⁴, other VCS markers that have been identified include *Etv1*^{40,103}, *CCS-LacZ*¹⁰⁴, *Cntn2*¹⁰⁵ and *Irx3*¹⁰⁶. Murine reporter lines have been developed for these markers and represent an important tool for CCS research (**Table 3.1**). In this Chapter, I will employ the *Cx40-EGFP* reporter line to visualise the CCS. It should be noted that this reporter line does not uniquely mark VCS cells: Cx40 is also expressed in the cardiac atria and coronary arteries. Nevertheless, this represents the best validated reporter for the HB, BB and Purkinje fibres, which collectively are the focus of this thesis⁶⁸.

Mouse reporter line	Genetic manipulation	Developmental stages	Cardiac conduction system expression					Other cardiac expression
			SAN	AVN	HB	BB	PF	
minK–Cre ERT2 ^{107,108}	Transgenic	E9.5–adult	–	+	+	+	+	A, V, IVS
Cx40–CreERT2 ¹⁰⁹	Knockin	E9.5–adult	–	±	+	+	+	A, EC, CA
HCN4–Cre ERT2 ^{110–112}	Knockin	E6.5–adult	+	+	+	+	+	LV, EC
Hcn4–CreERT2 ¹¹³	Transgenic	E7.5–adult	+	+	+	+	+	LV, EC
Pitx2c–CreERT2 ¹¹⁴	Transgenic	Neonate–adult	+	+	+	+	+	CS, AV rings
Sema3a–CreERT2 ¹¹⁵	Knockin	E12.5–Adult	–	–	–	–	+	No?
Tbx2–Cre ^{116,117}	Knockin	E9.5–adult	–	+	–	–	–	AVC
cGATA6–Cre ¹¹⁸	Transgenic	E8.5–neonates	–	+	+	–	–	A, LV
Tbx3–Cre ¹²	Knockin	E11.5–adult	+	+	+	–	–	No EPI, EPDCs, SH
Tbx18–Cre ^{111,117}	Knockin	E10.5–adult	+	–	–	–	–	

Isl1– Cre ^{111,119}	Knockin	E8.5–adult	+	–	–	RB B	RPF	OFT, RV, A
Shox2– Cre ¹²⁰	Knockin	E9.0–adult	+	–	–	–	–	No
Shox2– LacZ ¹²⁰	Knockin	E9.5–12.5	+	–	–	–	–	IVS LV (~E12.5) , EC
HCN4– EGFP ^{111,119}	Knockin	E7.5–adult	+	+	+	+	+	LV (~E12.5) , EC
HCN4– nLacZ ¹¹¹	Knockin	E7.5–adult	+	+	+	+	+	
cGATA6– lacZ ¹¹⁸	Transgenic	E7.5–neonate	–	+	+	–	–	A, LV
Troponin I– lacZ ¹²¹	Transgenic	E9.5–adult	–	+	–	–	–	A, ring
Des1– nLacZ ¹²²	Transgenic	E8–18	–	–	–	+	+	No
HF-1b– LacZ ¹²³	Knockin	E8.5–adult	–	+	+	+	+	RA
CCS– lacZ ¹⁰⁴	Transgenic	All stages	±	+	+	+	+	RA, VV OFT, IVS, AS, VV
mink– lacZ ^{107,108}	Knockin	E8.5–adult	–	+	+	+	+	
HOP– lacZ ¹²⁴	Knockin	E16.5–adult	–	+	+	+	+	A, V
Cntn2– lacZ ¹⁰⁵	Knockin	Adult	+	+	+	+	+	No
Cntn2– GFP ¹⁰⁵	Transgenic	Adult	+	+	+	+	+	No
Etv1– EGFP ^{40,103}	Transgenic	E13.5–adult	–	–	+	+	+	A
Popdc1– LacZ ¹²⁵	Knockin	Adult	+	?	+	+	–	CM
Popdc2– LacZ ¹²⁵	Knockin	Adult	+	+	+	+	–	CM
Cx40– EGFP ¹⁷	Knockin	E9.5–adult	–	±	+	+	+	EC, A
Cx45–	Knockin	E9.5–adult	+	+	+	+	–	A, V

LacZ ^{126,127}									
Cx30– LacZ ¹²⁸	Knockin	Adult	+	–	–	–	–	–	No
Cx30.2– LacZ ^{126,129}	Knockin	E10.5–adult	+	+	+	+	+	+	No
Tbx3– GFP ¹³⁰	Transgenic	E9.5–adult	–	+	–	–	–	–	AVC EPI, EPDCs, SH
Tbx18– GFP ¹³¹	Knockin	E10.5–?	+	–	–	–	–	–	OFT, RV, A
ISL1– nLacZ ¹¹⁹	Knockin	E9.5–adult	+	–	–	–	–	–	OFT, RV, A
Irx3– tauLacZ ¹⁰⁶	Knockin	E10–adult	–	–	+	+	+	+	No
mlrx3– EGFP ¹⁰⁶	Transgenic	E14.5–P3	–	–	+	+	+	+	No
Id2– LacZ ¹³²	Transgenic	Transient E14.5	–	–	+	+	–	–	No

Table 3: Mouse reporter lines for the Cardiac Conduction System.

Abbreviations: A, atria; AS, atrial septum; AVC, atrioventricular canal; AVN, atrioventricular node; BB, bundle branch; CM, cardiomyocyte; EC, endothelial cell; CA, coronary artery; EPI, epicardium; EPDCs, epicardial-derived cells; His-bundle; IVS, interventricular septum; LV, left ventricle; OFT, outflow tract; PF, Purkinje fibre; RBB, right bundle branch; RPF, right Purkinje fibre; RA, right atria; RV, right ventricle; SAN, sinoatrial node; SH, sinus horn; V, ventricle; VV, venous valve. *Adapted and updated with permission from Liang et al., 2017, Trends in Cardiovascular Medicine, copyright of Elsevier.*

Classic studies of the CCS utilised histological sectioning of the heart combined with morphological, injected ink or dye-based identification of CCS structures. While informative, such sections rarely capture more than 10µm of tissue and, therefore, are of limited use for identifying cellular morphology and interactions. This is particularly problematic in the context of the His/Purkinje network, where three-dimensional anatomy and how cells are coupled across planes of the tissue is critical to CCS function. The overall 3D anatomy of the His/Purkinje network defines how the propagating electrical impulse organises synchrony of cardiac contraction such that blood passes unidirectionally through the heart with appropriate hemodynamic flow. As a result, understanding not just 2D but 3D spatial organisation of the His/Purkinje network has represented a significant and biologically-important challenge.

An alternative to histological sectioning for imaging the CCS involves exposing the His/Purkinje network by cutting open the ventricular chambers. By cutting down the middle of the left or right ventricle free wall, opening up each respective chamber, and pinning down the myocardium either side of the cut, the inner myocardial wall can be flattened and the His/Purkinje network lining the chamber inner wall can be visualised. While this approach disrupts some of the 3D spatial organisation of the network and does not allow all subendocardial VCS cells to be imaged, it provides important insight into how the network develops and its functional organisation.

The first intact 3D imaging of the CCS relied upon contrast-enhanced micro-computed tomography to resolve the rat and rabbit His/Purkinje network¹³³. More recently, an adapted iDISCO protocol was used to image the mature VCS in intact, tissue-cleared adult mouse hearts¹³⁴. This approach relied upon injection of an antibody, mCntr2-800, in order to visualise the CCS and is the only non-disruptive cardiac fluorescence imaging to date to capture full VCS structure. However, the method is not compatible with retaining endogenous fluorescence so cannot be combined with reporter lines (**Table 3.1**), and is only

compatible with limited antibodies. Moreover, the need for antibody injection limits the applicability of this approach for studying the CCS through developmental stages.

3.1.4 The *Nkx2-5*^{Cre/+} haploinsufficient mouse model

NKX2-5 is a key transcription factor during heart development and maturation. NKX2-5 was first described as essential for early murine heart development¹³⁵ and is also part of a core transcriptional network that regulates CCS phenotype and has a well-characterised role in conduction system development¹³⁶. NKX2-5 is required, together with TBX3, TBX5, ID2, ETV1, HOPX, SP4 and IRX3, for specification of the AVB and BBs of the VCS². Moreover, NKX2-5 has been implicated in the post-natal maturation of the VCS: *Nkx2.5*^{+/-} mice have a hypoplastic Purkinje network from birth, despite showing normal embryonic trabecular development^{137,138}. Recent work using a tamoxifen-inducible Cre mouse line to conditionally knockout *Nkx2-5* in the VCS revealed cell-autonomous functionality of NKX2-5 in maintaining proper conductivity within the mature VCS¹³⁹.

The *Nkx2-5*^{Cre/+} haploinsufficient mouse represents one of the best-characterised genetic models of an aberrant VCS. Mutations in *NKX2-5* are responsible for approximately 4% of congenital heart diseases. The mouse model mimics the phenotype seen in these congenital patients, in particular involving AV conduction disturbances associated with the hypoplastic His/Purkinje network^{138,140,141}. Based on clonal lineage analysis experiments in the mouse, it has been proposed that there is biphasic development of the VCS, and that the second wave of recruitment is disrupted and fails to occur when dosage of NKX2-5 is low¹⁴⁰. However, precisely how the *Nkx2-5*-dependent development of the VCS occurs at a cellular and mechanistic level through late embryonic and early post-natal development remains unclear.

3.2 Aims and Objectives

The aims of this chapter were to:

1. Develop a bespoke tissue clearing and imaging method for embryonic and post-natal mouse hearts
2. Characterise the ventricular conduction system through late embryonic and post-natal development
3. Model and quantify the His/Purkinje network architecture over murine post-natal development
4. Analyse the morphology of the hypomorphic His/Purkinje network in *Nkx2-5^{Cre/+}* mutant hearts

3.3 Results

3.3.1 A methodology to image the intact ventricular conduction system

Due to the cellular composition and opacity of the heart, and the location of the VCS lining the interventricular septum and within the sub-endocardial region, it has not previously been possible to image the intact VCS over post-natal development. I, therefore, aimed to design a novel methodology in order to image the VCS through post-natal development at near-cellular resolution. This required three stages of innovation. First, a tissue-clearing method to improve the optical transparency of the post-natal mouse heart, compatible with preserving endogenous fluorescence and with immunostaining using a wide spectrum of antibodies. Secondly, an optimised imaging set-up appropriate for each heart stage. Finally, a computational pipeline to segment and analyse the VCS network, quantifying network size and complexity through post-natal development.

I began by defining a shortlist of the most promising tissue-clearing methodologies applied in cardiovascular imaging to date, including: SUMIC, iDISCO, ECI (with and without bleaching in hydrogen peroxide), and CUBIC methods^{71,72,87,96,99,142}. I then trialled each of these methods on post-natal mouse hearts and compared their efficacy for clearing the heart to enable imaging at depth, while retaining endogenous fluorescence and compatibility with immunofluorescence (**Figure 3.1** and **Chapter 2.4.2**).

The SUMIC method applied to post-natal mouse hearts resulted in relatively good optical clarity of the sample. However, hearts cleared using this protocol showed a strong edge effect with significant (non-specific) fluorescence around the outside of the tissue, but very little specific signal in the central tissue regions (**Figure 3.1a**). This suggested that there was limited antibody penetration into the sample and/or too high an antibody concentration. In this case, it is more likely that the edge effect resulted from a penetration defect given that equivalent antibody concentrations did not produce such an edge effect across any of the other tissue-clearing methods tested. Moreover, endogenous GFP signal was almost entirely

lost from the CX40-eGFP SUMIC-cleared hearts despite repeating the protocol with addition of an antibody against GFP to help boost the signal. Given the almost-complete loss of endogenous fluorescence and poor antibody penetration, without a fluorescent read-out, it was difficult, therefore, to determine quality of tissue clearing at depth in these samples.

Use of iDISCO and ECI methods applied to post-natal mouse hearts resulted in good optical clarity, especially for hearts processed through the iDISCO protocol or hearts cleared in CUBIC and then bleached prior to ECI immersion. Furthermore, immunostaining was more successful using the iDISCO method than the SUMIC or CUBIC-ECI methods: antibody staining against HCN4 allowed wholemount identification of the nodal cells of the CCS with neither non-specific signal nor edge effects (**Figure 3.1b**). However, GFP signal was again almost entirely lost from the CX40-eGFP cleared hearts with these methods, despite trialling addition of an antibody against GFP or use of a GFP nano-boost probe to help increase signal-to-noise ratio (**Figure 3.1b-c**). This is probably due to quenching of the GFP and disruption to the structure of the GFP protein during the methanol dehydration gradient and subsequent steps. This issue may additionally have been compounded by only partial penetration of antibodies through the organ.

By contrast, use of the CUBIC method did not initially achieve optical clarity equalling the iDISCO method, but endogenous GFP signal was maintained, antibody penetration was good and early results in young post-natal hearts looked promising (**Figure 3.1d**). I consequently selected the CUBIC method to take forward for optimisation to enable fully cleared imaging of hearts throughout post-natal development.

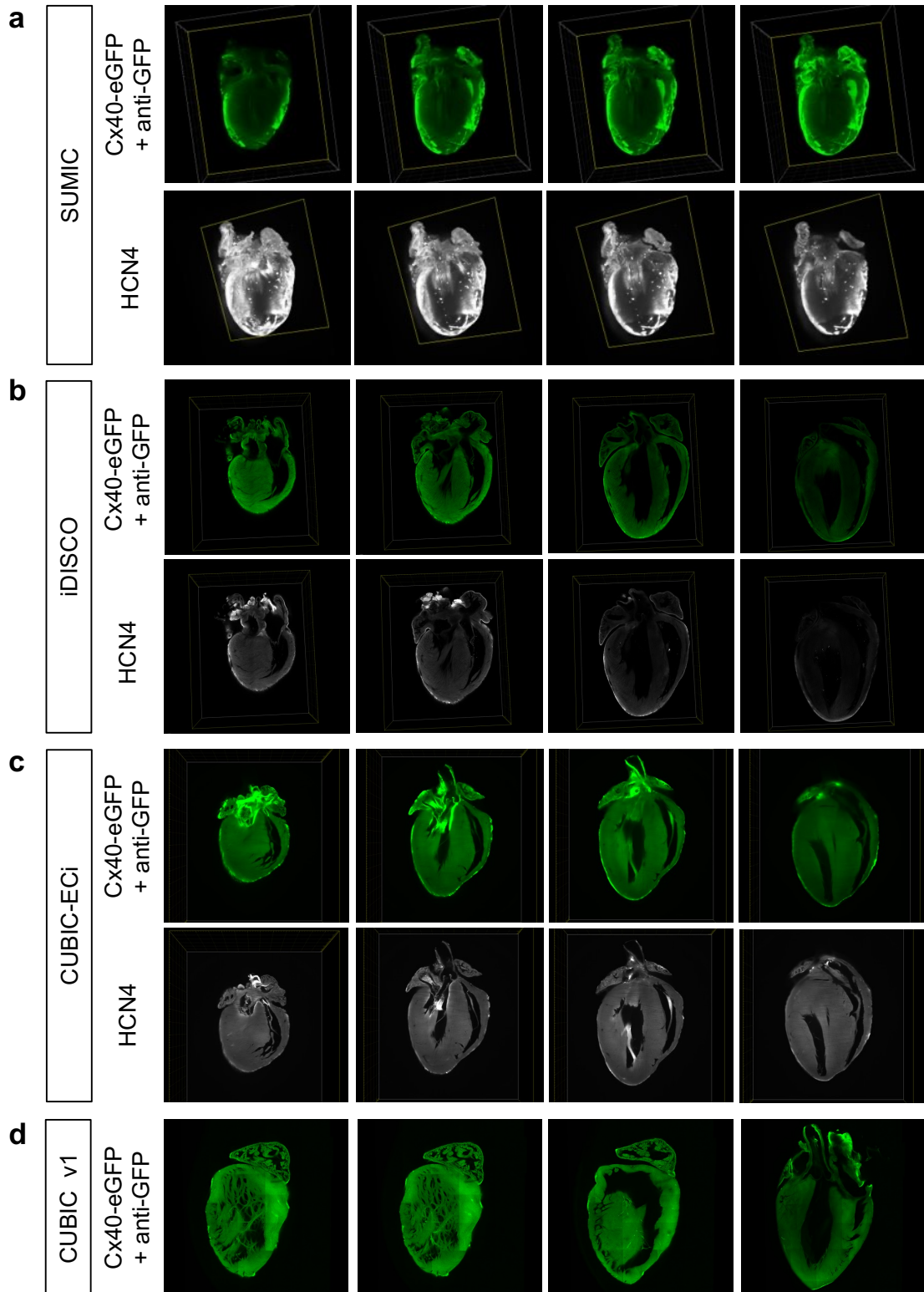


Figure 3.1: Comparison of tissue-clearing methods for the post-natal mouse heart

Post-natal day 8 hearts tissue-cleared using SUMIC (a), iDISCO (b), CUBIC-Eci (c) and un-modified CUBIC (d) methods. A z-slice series at increasing depth is presented in each case. Green: CX40eGFP and anti-GFP, which should label the developing ventricular conduction system (VCS), atria and coronary arteries; greys: HCN4, which should label nodal cells of the conduction system.

I next performed extensive optimisation rounds to establish a CUBIC-based tissue-clearing protocol bespoke to the post-natal heart. These optimisations included: significant extension of the initial delipidation step (adjusted for each post-natal stage), extending from 24 hours for embryonic hearts up to 2 weeks for P21 hearts; optimised temperatures for all steps; optimised incubation timings, conditions and solvent volumes; and optimally-adjusted antibody incubation conditions. To select the best-suited microscope for imaging samples following CUBIC clearing, I trialled imaging wholemount cleared hearts using Olympus and Zeiss inverted confocal microscopes (10X dry objective with 0.45 numerical aperture), the Zeiss Z1 light sheet microscope (5X air objective with 0.16 numerical aperture and 10X water objective with 0.5 numerical aperture) and the Miltenyi Ultramicroscope II (2X objective with 0.5 numerical aperture and a custom LaVision BioTec dipping cap). I determined that the Zeiss 980 inverted confocal microscope provided the optimal imaging strategy with highest resolution at depth for hearts through until P4. Beyond this time-point, length of acquisition and restricted working distance became limiting factors. While the Zeiss Z1 light sheet microscope did not cope well with large samples, I found that the Miltenyi Ultramicroscope II was optimal for imaging mouse hearts from P4 through to P21. Following these modifications, I successfully designed a protocol capable of clearing and imaging the murine heart throughout post-natal development, optimised for each stage from P1 through P21 (**Figure 3.2** and **Chapter 2.4.2.1**).

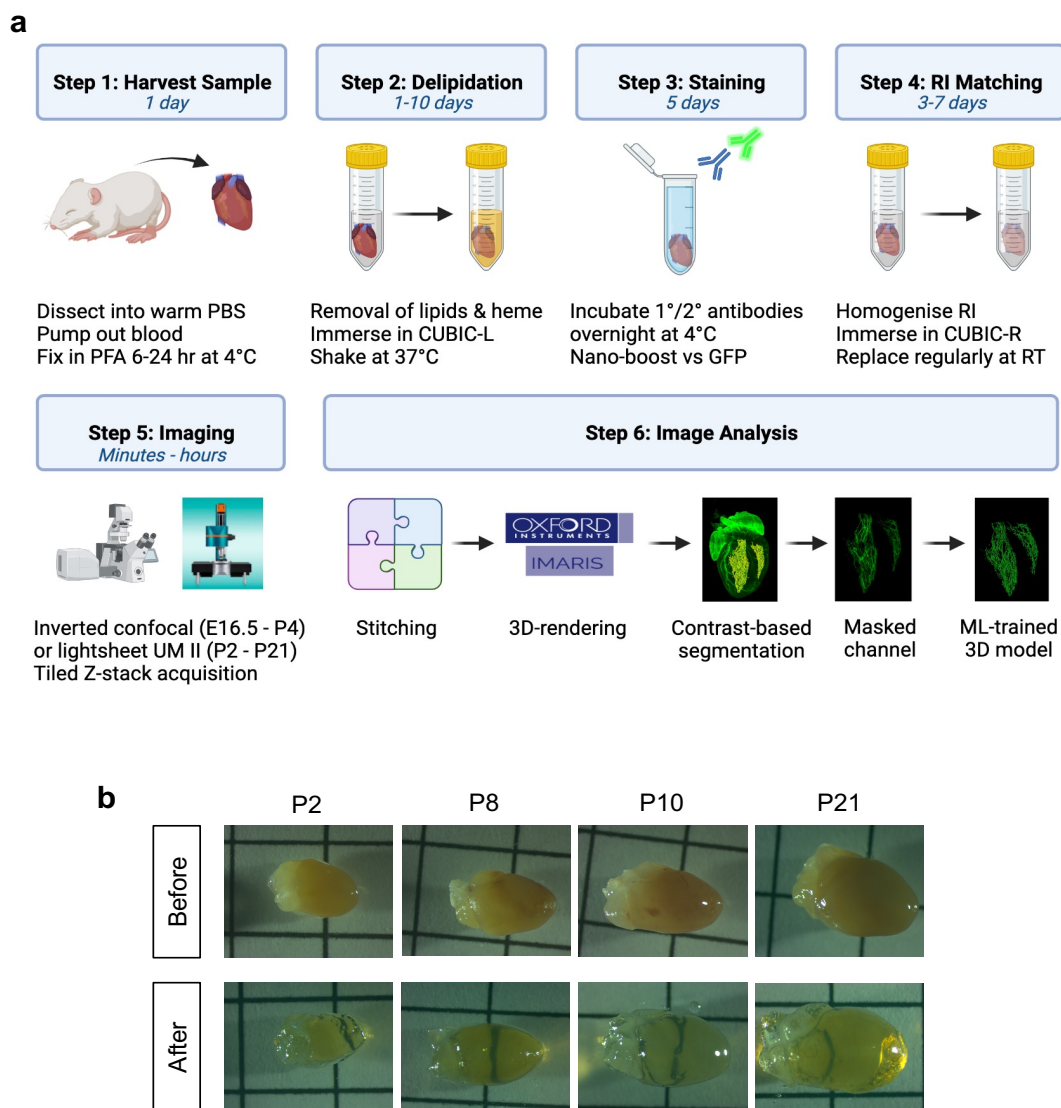


Figure 3.2: A pipeline for tissue clearing, imaging and analysis of the post-natal mouse heart

a. Schematic to illustrate the stages of the sample preparation, tissue clearing, immunostaining, imaging and analysis pipeline developed to enable wholemount imaging of the ventricular conduction system throughout post-natal mouse development. Abbreviations: PBS, phosphate-buffered saline; PFA, para-formaldehyde; RI, refractive index; RT, room temperature; UM II, Ultramicroscope II; ML, machine learning. *Schematic created using Biorender.com.* **b.** Time course of post-natal hearts imaged before and after processing through the tissue-clearing pipeline. Scale: imaged on 5mm graph paper.

3.3.2 The conduction system during late embryonic and post-natal development

3.3.2.1 The ventricular conduction system is refined from the trabecular myocardium during late-stage embryonic development

The VCS shares its developmental origins with working cardiomyocytes, but becomes specialised during embryogenesis through downregulation of the sarcomeric contractile apparatus and expression of specific ion channels and connexin isoforms^{4,5}. Lineage tracing studies have found progressive restriction of progenitors to VCS fate through until embryonic day E16.5, following which it is thought that limited cell division expands the VCS to its final form¹⁷. I, therefore, set out to characterise the late-stage formation and remodelling of the VCS from E16.5 through late embryonic development to birth. Using my optimised CUBIC and immunostaining protocol (**Figure 3.2**), I visualised the intact VCS through late embryonic development with the knock-in CX40-eGFP reporter line. I observed progressive restriction of CX40 expression, from broad expression across the trabecular myocardium at E16.5 through to restriction to the mature VCS structure by P1 (**Figure 3.3**).

3.3.2.2 The ventricular conduction system grows and expands during post-natal development

To confirm the mature VCS identity of GFP-positive cells in the CX40-eGFP heart by post-natal stages, I collected cryosections across a post-natal time-course from CX40-eGFP hearts and performed immunostaining against several well-known VCS markers: ETV1⁴⁰, CNTN2¹⁰⁵ and NaV1.5¹⁴³ (**Figure 3.4**). Overlap of all three markers with GFP indicated that by post-natal stages the GFP-positive cells represent the true VCS population, confirming that CX40-eGFP is a reliable read-out for the neonatal VCS.

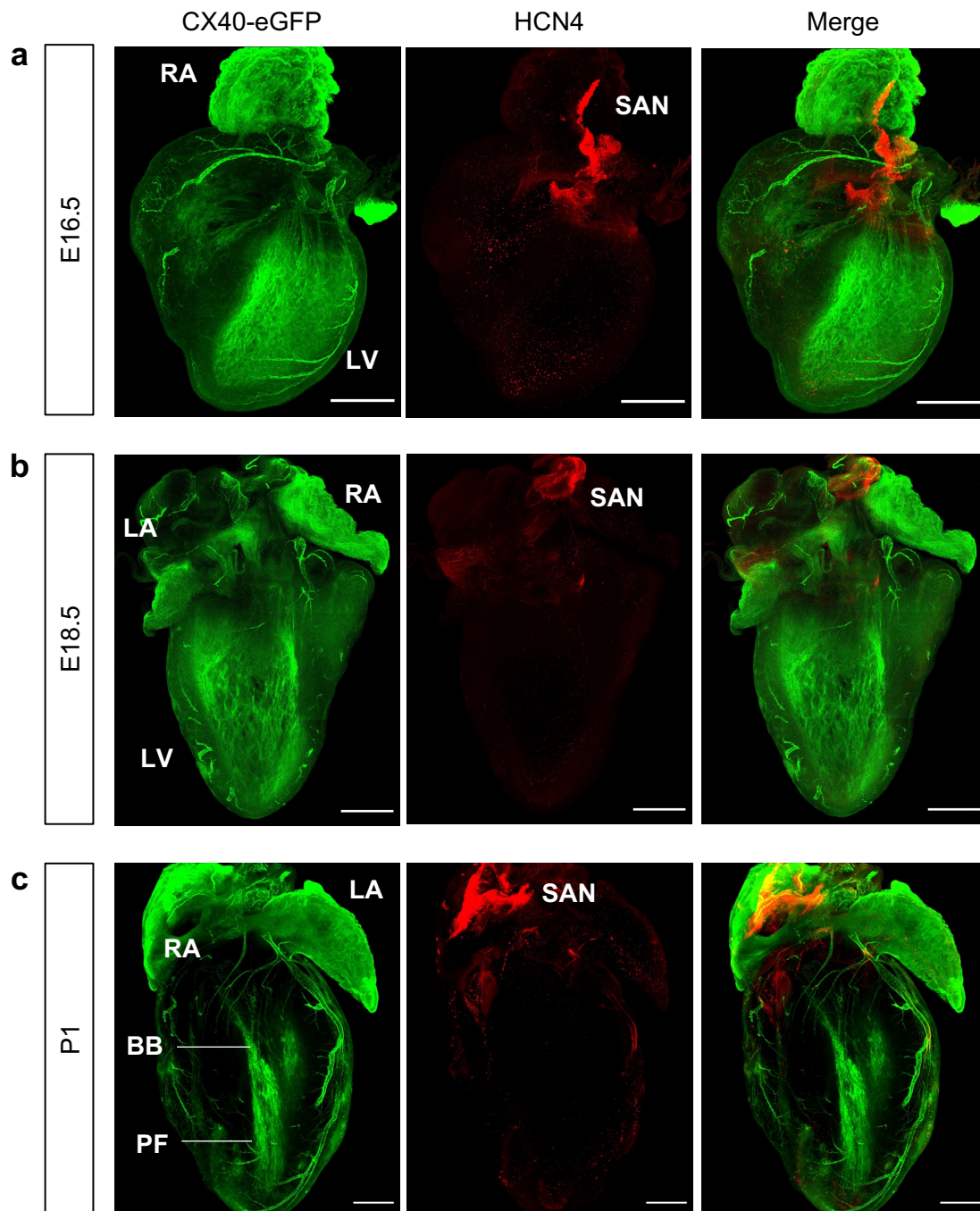


Figure 3.3: Myocardial connexin-40 expression is restricted to the His/Purkinje network by post-natal day 1

3D-rendered tissue-cleared Cx40-eGFP/+ embryonic day 16.5 (a), embryonic day 18.5 (b) and post-natal day 1 (c) hearts, revealing the refinement of trabecular CX40 expression to the developing His/Purkinje network. Green: CX40-eGFP and anti-GFP, labelling the developing ventricular conduction system (VCS), atria and coronary arteries; red: HCN4, labelling nodal cells of the conduction system. Scale bars: 500µm. Labels indicate left atrium (LA), right atrium (RA), left ventricle (LV), right ventricle (RV), bundle branches (BB), Purkinje fibres (PF), Sinoatrial Node (SAN).

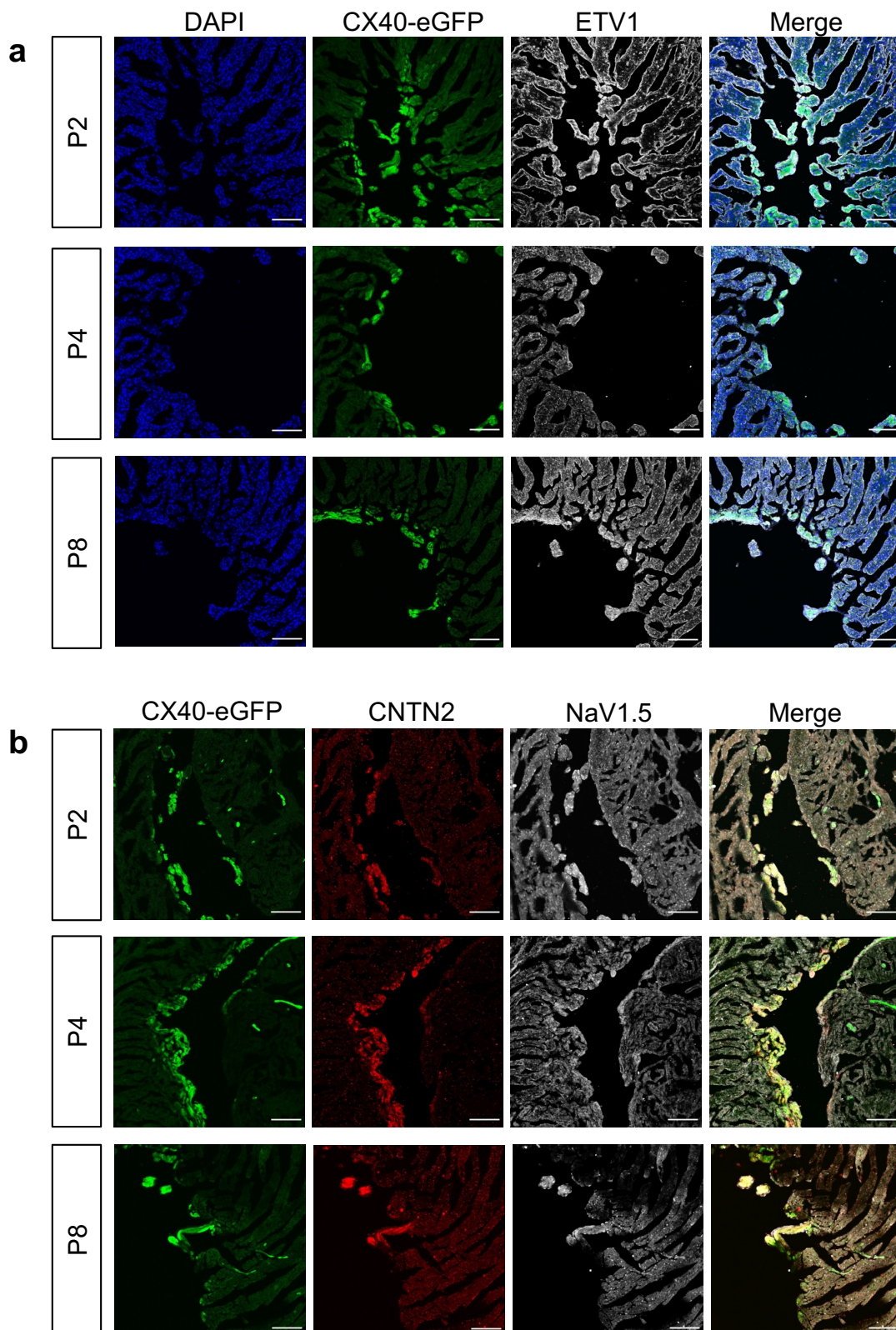


Figure 3.4: Expression of ventricular conduction system markers colocalise with CX40-eGFP in the post-natal mouse heart

ETV1 (a), CNTN2 and NaV1.5 (b) are enriched in the CX40-eGFP-positive cells in the post-natal ventricular conduction system. Histological section staining from post-natal day 2 (top), post-natal day 4 (middle) and post-natal day 8 (bottom) presented in each case. Scale bars: 100µm.

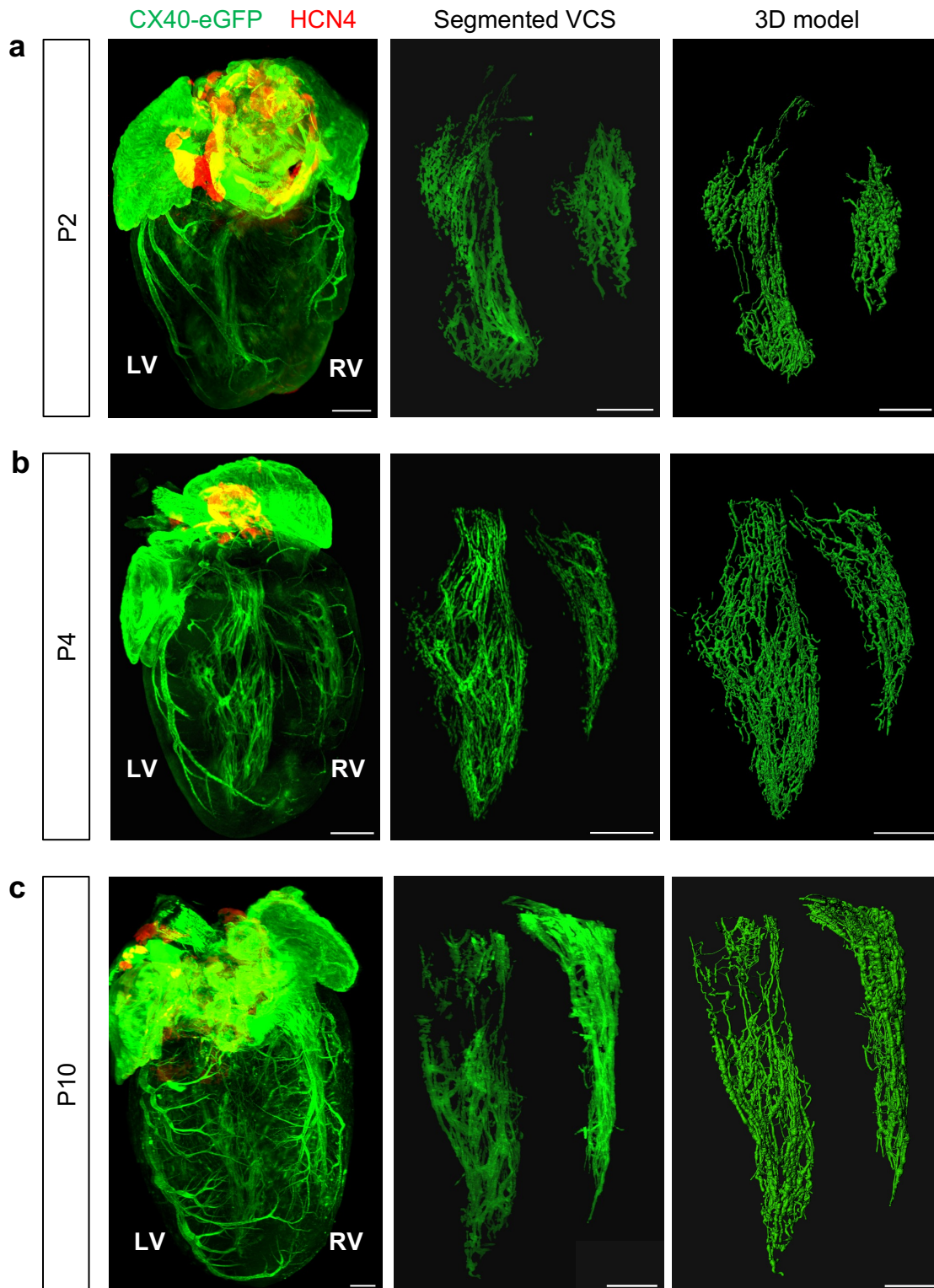


Figure 3.5: Post-natal growth and expansion of the cardiac conduction system
 Time series of tissue-cleared *Cx40-eGFP*^{+/+} hearts at post-natal day 2 (a), post-natal day 4 (b) and post-natal day 10 (c). Depicted from left to right: the 3D-rendered whole heart, the segmented VCS network, and its 3D filament model (built using Imaris 10.0 software). Scale bars: 500 μm. Green: CX40-eGFP and anti-GFP, labelling the developing ventricular conduction system (VCS), atria and coronary arteries; red: HCN4, labelling nodal cells of the conduction system. Labels indicate left ventricle (LV), right ventricle (RV).

Next, I used my optimised CUBIC and immunostaining protocol to visualise the intact neonatal CCS through post-natal development. Following birth, substantial growth and expansion of the VCS' branching network structure continued through post-natal stages (**Figure 3.5**), concurrent with overall growth of the organ. Segmentation of the VCS according to local contrast and subsequent construction of a 3D VCS model using the Imaris 10.0 Filament Tracer module depicted the network's increasing complexity over post-natal development. This represents the first description of the intact 3D architecture of the developing murine VCS.

3.3.2.3 Vascularisation of the ventricular conduction system during post-natal development

To date, the intact 3D anatomy of the vascular network supplying the VCS has not been extensively analysed. A previous study used histological analysis of serial sections in an attempt to define the arterial vascularisation of the mature human conduction system. In this way, the authors identified that blood supply to the AVB and left/right BBs of the VCS derives principally from the left coronary arteries¹⁴⁴. However, the nature and extent of the arterial vascularisation that supports the developing mammalian VCS has not yet been characterised.

Given the extensive growth and maturation of the His/Purkinje network over post-natal development, I hypothesised that the His/Purkinje network would be densely vascularised in order to receive a rich supply of arterial blood during these stages. Consequently, I characterised the relationship between the Purkinje network and its supporting vasculature over this time period. Since CX40 is expressed in the VCS and also in the coronary arteries, I designed and implemented a machine-learning guided segmentation pipeline in order to differentiate artery from VCS signal (**Chapter 2.4.5.1**). In this way, I was able to extract and visualise the arterial network supplying blood to the VCS

in 3D, and to track its development after birth (**Figure 3.6**). This analysis found extensive arterial vascularisation in close vicinity to the VCS through post-natal stages. Subsequent volume-rendering of both the VCS and the arterial vessels found arterial branches penetrating left and right BBs in the post-natal mouse heart at both P2 and P10 (**Figure 3.6a-b**). This is especially interesting given findings from the chick, where the coronary artery bed has been shown to play an instructive role in patterning the developing VCS¹⁴⁵.

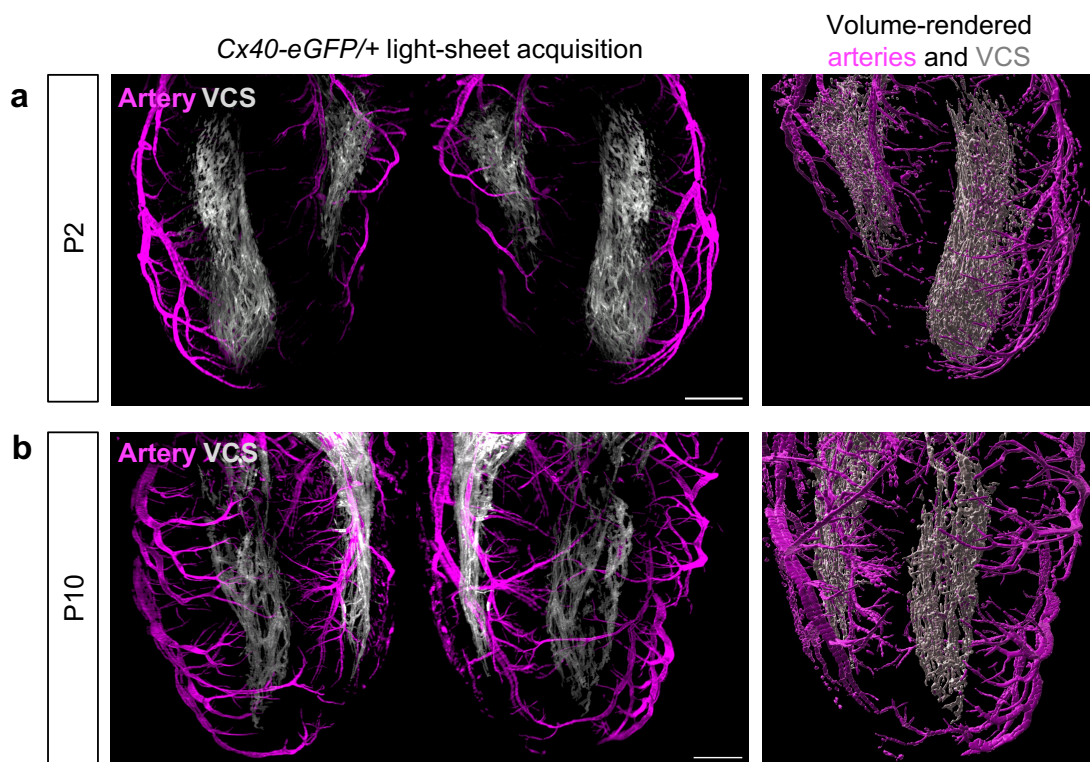


Figure 3.6: Vascularisation of the post-natal mouse ventricular conduction system

Post-natal day 2 (**a**) and day 10 (**b**) *Cx40-eGFP/+* hearts tissue-cleared using a modified CUBIC protocol. Depicted from left to right: 3D-rendered mirrored image of the ventricles following machine learning-guided segmentation to differentiate artery from ventricular conduction system (VCS) signal; volume-rendered segmented 3D surface (built using Imaris 10.0 software). Magenta: coronary arteries. Greys: His/Purkinje cells of the VCS. Scale bars: 500 μ m.

3.3.2.4 Macrophages wrap around junctions between Purkinje cells in the post-natal mouse heart

Following my analysis of the supportive vasculature of the VCS, I further wanted to establish whether other cell types might also have an instructive role in the developing post-natal conduction system by virtue of a close association with the VCS. In particular, tissue-resident macrophages have been shown to facilitate electrical conduction in the adult heart by coupling to working cardiomyocytes via Cx43, and have been found to be enriched in the nodes of the conduction system¹⁴⁶. I hypothesised, therefore, that tissue-resident macrophages might additionally interact closely with (and possibly couple electrically to) the conduction cells of the VCS. To assess whether macrophages interact with Purkinje cells in the post-natal heart, I performed immunofluorescence staining against CD68 (a well-established macrophage marker¹⁴⁷) in sections from post-natal *Cx40-eGFP/+* hearts. Image analysis revealed macrophages sitting in the junctions directly in between CX40-eGFP-positive Purkinje cells (**Figure 3.7a**).

Given the proximity of Purkinje cells and macrophages in the post-natal heart sections, I performed high-resolution imaging followed by local intensity contrast-based surface segmentation and 3D-rendering in Imaris (version 10.0), to assess extent of interaction between these cell types. Single-cell imaging coupled with 3D-rendering of the CD68+ macrophage cells showed cellular extensions wrapping around the Purkinje cells (**Figure 3.7b**), confirming that macrophages associate with the VCS in the post-natal mouse heart and as such may play a role in remodelling the conductive network analogous to their roles elsewhere in the coronary vasculature^{148,149}.

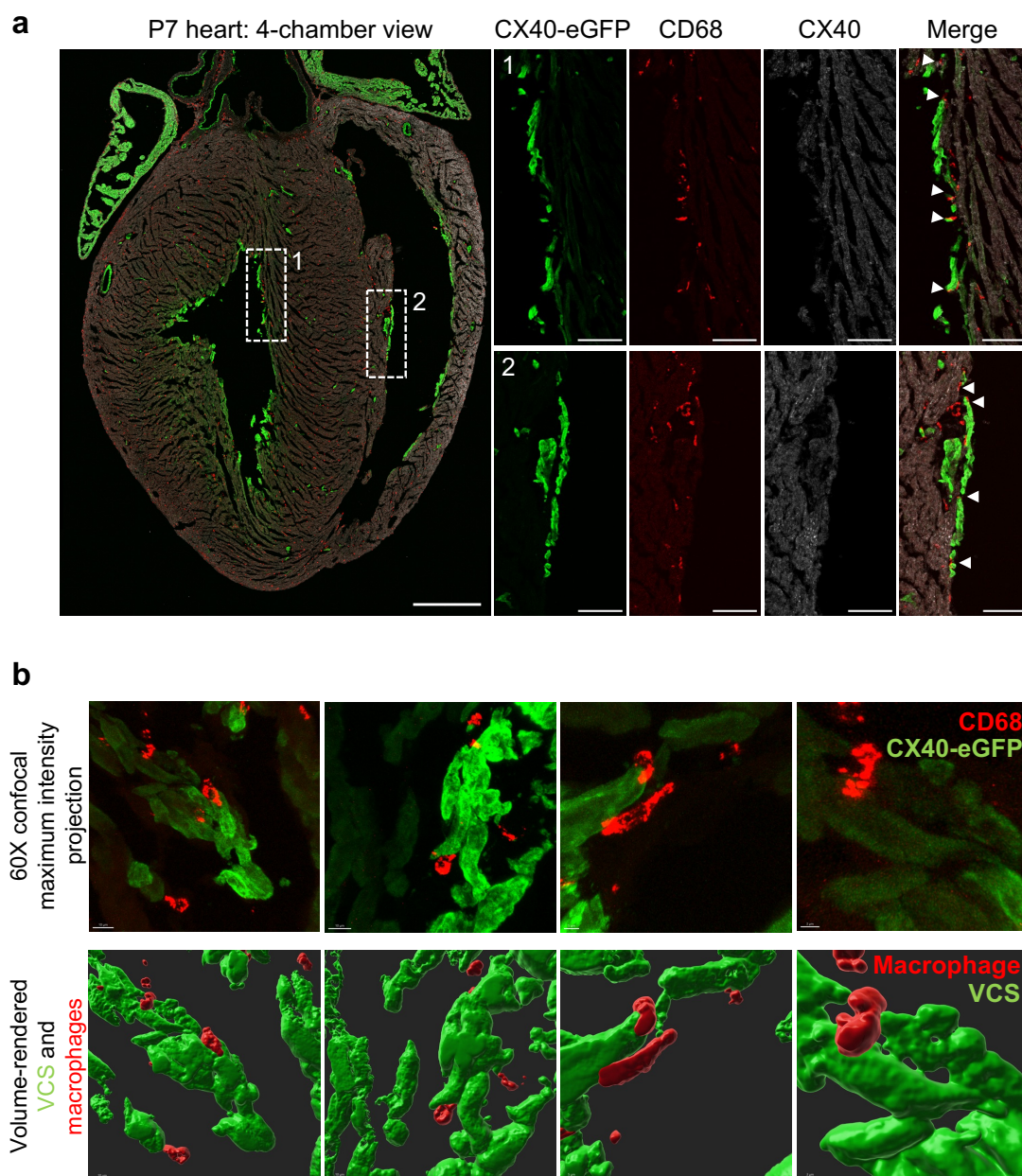


Figure 3.7: Macrophages wrap around junctions between Purkinje cells in the post-natal mouse heart

a. Cryo-section staining of a post-natal day 7 (P7) heart. Green: CX40-eGFP, labelling the developing ventricular conduction system (VCS), atria and coronary arteries; red: CD68, labelling macrophages; greys: CX40 antibody. Numbered panels on the right-hand side are zoomed inserts of the corresponding boxed regions. Arrows indicate CD68+ macrophages sitting at junctions between CX40-eGFP+ Purkinje fibres. Scale bars: 500µm (main image), 200µm (zoomed inserts). **b.** 60X high-resolution maximum intensity projection (above) and volume-rendered segmented 3D surface (below) built using Imaris 10.0 software, capturing CD68+ macrophage projections (red) contacting CX40-eGFP+ Purkinje cells (green). Scale bars: 100µm.

3.3.3 Modelling the ventricular conduction system network

3.3.3.1 *Quantitative analysis of His/Purkinje network growth and expansion during post-natal development*

Given the identified expansion of the murine VCS after birth, I set out to quantify VCS network growth over post-natal development from my imaging data of intact tissue-cleared *Cx40-eGFP/+* hearts. However, while CX40 is one of the best-known markers of the VCS, it is also expressed in the atria and coronary arteries. In order to perform quantitative modelling of the VCS network from my CX40-eGFP+ imaging datasets I designed an analysis pipeline to extract parameters systematically. This pipeline had to be capable of first subtracting the atrial and arterial signal, and second of building a network model from the VCS 3D fluorescence data in order to quantify parameters including VCS volume, network length, number of network junctions, and number of terminal points in the network.

Following trials of several software programmes (including Amira, Arivis and Imaris) and open-source packages (including Angiotool¹⁵⁰, AnalyzeSkeleton and Skeletonize3D¹⁵¹, and Labkit¹⁵²), and after numerous optimisation rounds, I designed an analysis pipeline using the Imaris 10.0 software (detailed in **Chapter 2.4.5.1**) capable of modelling and quantifying the VCS network. In brief, this first involved segmenting the VCS using machine learning to extract the VCS from artery and background signal. Next, using only the segmented VCS voxels, a 3D filament model of the VCS is built within the Imaris Filament Tracer module, and subsequently the network's growth is quantified from the filament-based model.

In this way, I identified significant growth in the VCS network through post-natal development. This included a 2-fold increase in network volume between P2 and P10 (unpaired t-test: $p = 0.0160$) (**Figure 3.8a**), accompanied by significant increase in filament length (unpaired t-test: $p = 0.0054$) and total filament area (unpaired t-test: $p = 0.0139$) (**Figure 3.8b-c**). Most surprisingly, despite expansion of the network during the post-natal

period, the complexity of the network was already established by P2: there was no significant change in extent of branching or number of network segments between P2 and P10 (**Figure 3.8d-e**). Moreover, while there was a trend towards an increase in the total number of branch points in the P10 network as compared to the P2 network (unpaired t-test: $p = 0.1653$), there was an accompanying trend towards a reduction in number of terminal points between these stages (unpaired t-test: $p = 0.0958$), meaning that fewer unconnected branch ends were identified in the P10 network (**Figure 3.8f**). This suggests, therefore, that the fibres become more connected as the network matures.

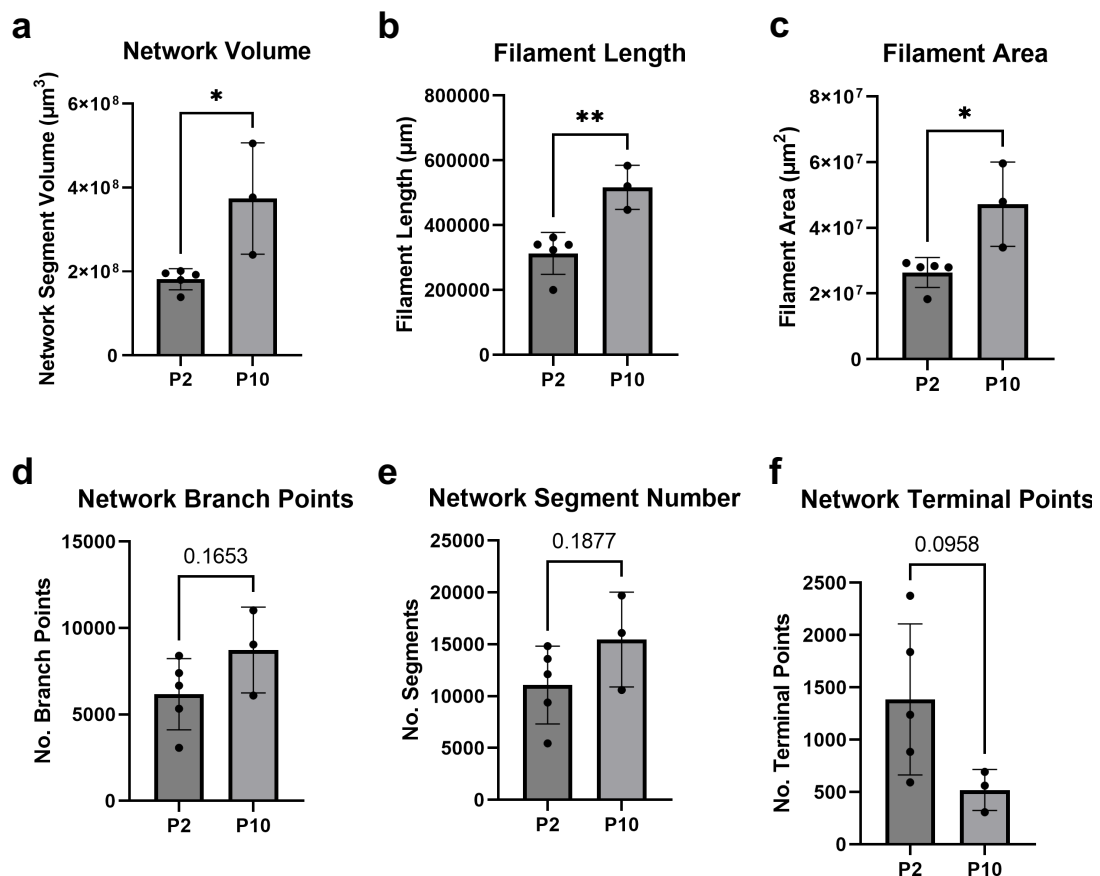


Figure 3.8: Quantitative analysis of the ventricular conduction system over post-natal development

Graphs quantifying network volume (**a**), filament length (**b**), filament area (**c**), number of branch points (**d**), number of segments (**e**), and number of terminal points (i.e., ends of branches) (**f**) across post-natal development between post-natal day 2 (P2) and post-natal day 10 (P10). Statistics: unpaired t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Non-significant p-values provided to 4 decimal places.

Interestingly, I found that expansion of the right ventricular network occurred later than the left network, and that overall, more growth was attributed to the right network relative to the left during post-natal development. Indeed, while there was a significant increase in network volume ($p = 0.0175$), filament area ($p = 0.0213$) and total filament length ($p = 0.0188$) in the right VCS network between P2 and P10, there was a modest but non-significant increase in the same parameters within the left VCS across the same time course (**Figure 3.9a**). This was accompanied by an overall trend towards increased complexity in the right network, which was absent in the left network (**Figure 3.9b**). This suggests that the left VCS has largely completed its developmental maturation by P2, whereas development of the right VCS network is delayed and involves substantial network maturation during the first week of life.

Overall, these findings reveal that the significant growth of the network during post-natal maturation results from extension and connection of existing Purkinje fibres, not from the genesis of new branch points, and that there is more extensive development in the right than the left His/Purkinje network during post-natal stages.

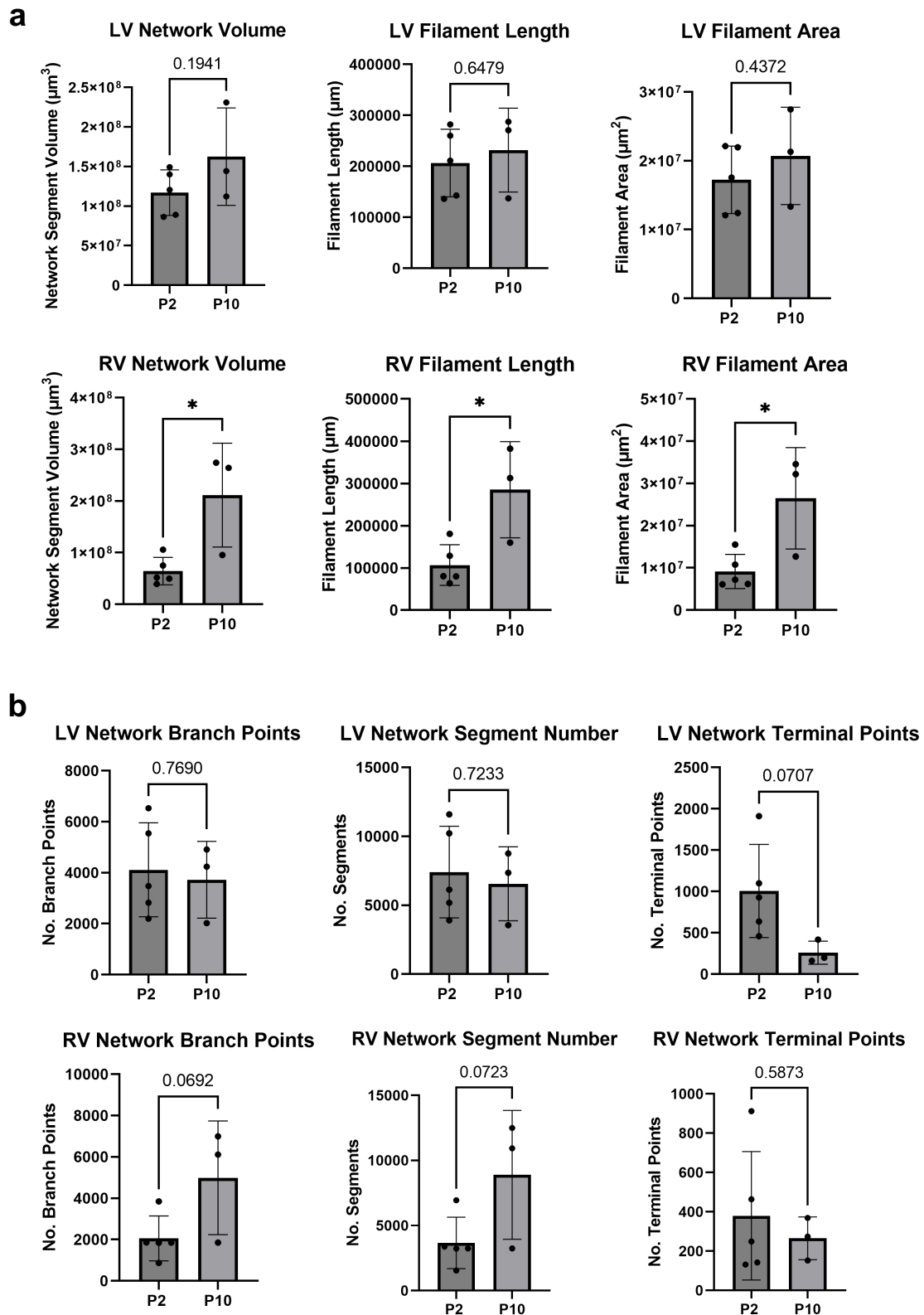


Figure 3.9: The right ventricular His/Purkinje network develops later than the left ventricular network

a. Quantification of the growth of left versus right His/Purkinje networks between post-natal day 2 (P2) and post-natal day 10 (P10). There is a significant increase in right ventricular network volume, total filament length and filament area, but a much more

modest increase in the left ventricular network. **b.** Quantification of the complexity of left versus right His/Purkinje networks between P2 and P10. There is no discernible difference in extent of network branching or number of segments in the left ventricular network across post-natal development, whereas there is a trend towards increased complexity in the right network over the equivalent time frame. The trend towards reduced number of terminal points is stronger in the left than the right ventricular network between P2 and P10. Statistics: unpaired t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Non-significant p-values provided to 4 decimal places.

3.3.3.2 Establishment of Purkinje hypoplasia in haploinsufficient *Nkx2-5*^{+/-} hearts over post-natal development

Finally, I applied the imaging toolkit developed in this Chapter to analyse the hypomorphic VCS network of the haploinsufficient *Nkx2-5*^{+/-} mutant. While it is known that *Nkx2-5*^{+/-} animals progress normally through early stages of embryonic VCS development, how hypoplasia is established in the network at later stages is less well understood. The predominant model in the literature suggests that VCS development is biphasic, and that the second wave of progenitor recruitment to the VCS network at late embryonic stages is *Nkx2-5*-dependent and, therefore, disrupted in the heterozygote mutant¹⁴⁰. More recently, it has additionally been proposed, based on genetic tracing of *Nkx2-5*-deleted VCS cells (analysed using section staining and dissected preparations) that Purkinje fibre hypoplasia may result from a cell dropout mechanism whereby cells lacking NKX2-5 fail to maintain a conductive phenotype¹³⁹. However, due to technical limitations it has not previously been possible to assess and quantify how the *Nkx2-5* hypomorphic network develops in 3D without dissecting the heart and disrupting tissue architecture. I, therefore, deployed my intact imaging and analysis pipeline to probe this defect and its underlying mechanism.

By tissue-clearing *Nkx2-5*^{+/-}; *Cx40-eGFP* hearts and visualising the VCS network, I first discovered that the VCS network is not strongly hypomorphic at P2. Strikingly, in young neonatal hearts harvested at P2 it was not possible in blinded analysis to determine with

confidence which hearts were wild-type versus haploinsufficient for *Nkx2-5* (**Figure 3.10**). However, on extending this analysis to P4 hearts (just 2 days later) the CX40-eGFP+ VCS network was clearly hypomorphic (**Figure 3.11**). I concluded from this qualitative assessment that the hypomorphic phenotype manifests in the P2-P4 window. This, therefore, suggested that a full dosage of NKX2-5 is not required to recruit cells to the VCS network in late embryonic development. Rather, that in the *Nkx2-5* heterozygous background development proceeds normally beyond birth, but that there is subsequent loss of Purkinje cells from the network after P2. This probably arises due to a failure of cells to maintain a conductive phenotype and/or due to Cx40 loss in a subset of VCS-like cells in conditions of low-level NKX2-5.

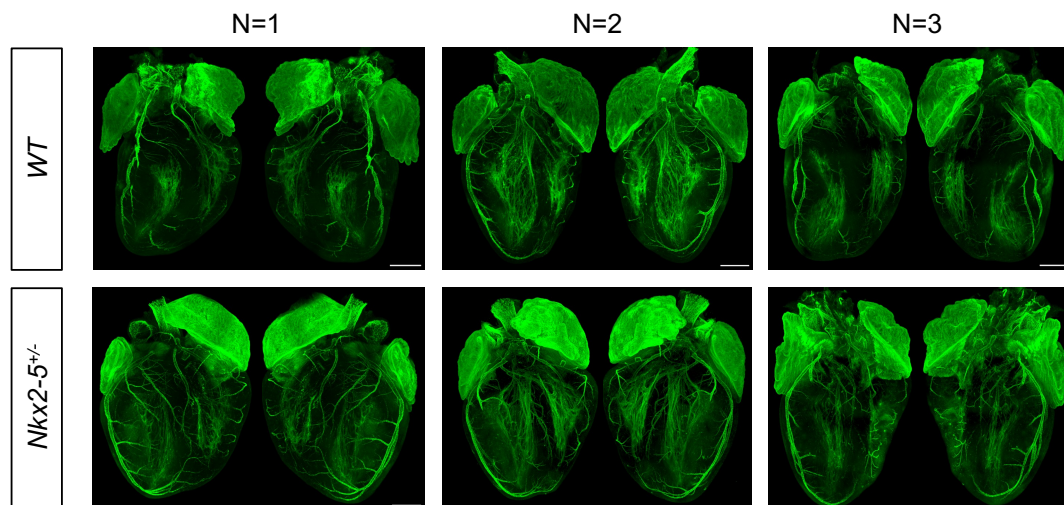


Figure 3.10: The *Nkx2-5*^{+/-} mouse conduction system is not substantially hypomorphic at post-natal day 2

3D rendering of tissue-cleared post-natal day 2 hearts. Top: *Cx40-eGFP*^{+/+}; *Nkx2-5*^{+/+} wild-type hearts, bottom: *Cx40-eGFP*^{+/+}; *Nkx2-5*^{+/-} haploinsufficient hearts. 3 different hearts are presented in each case. In blinded analysis it was not possible to determine which hearts were heterozygous for *Nkx2-5*. Scale bars: 500µm.

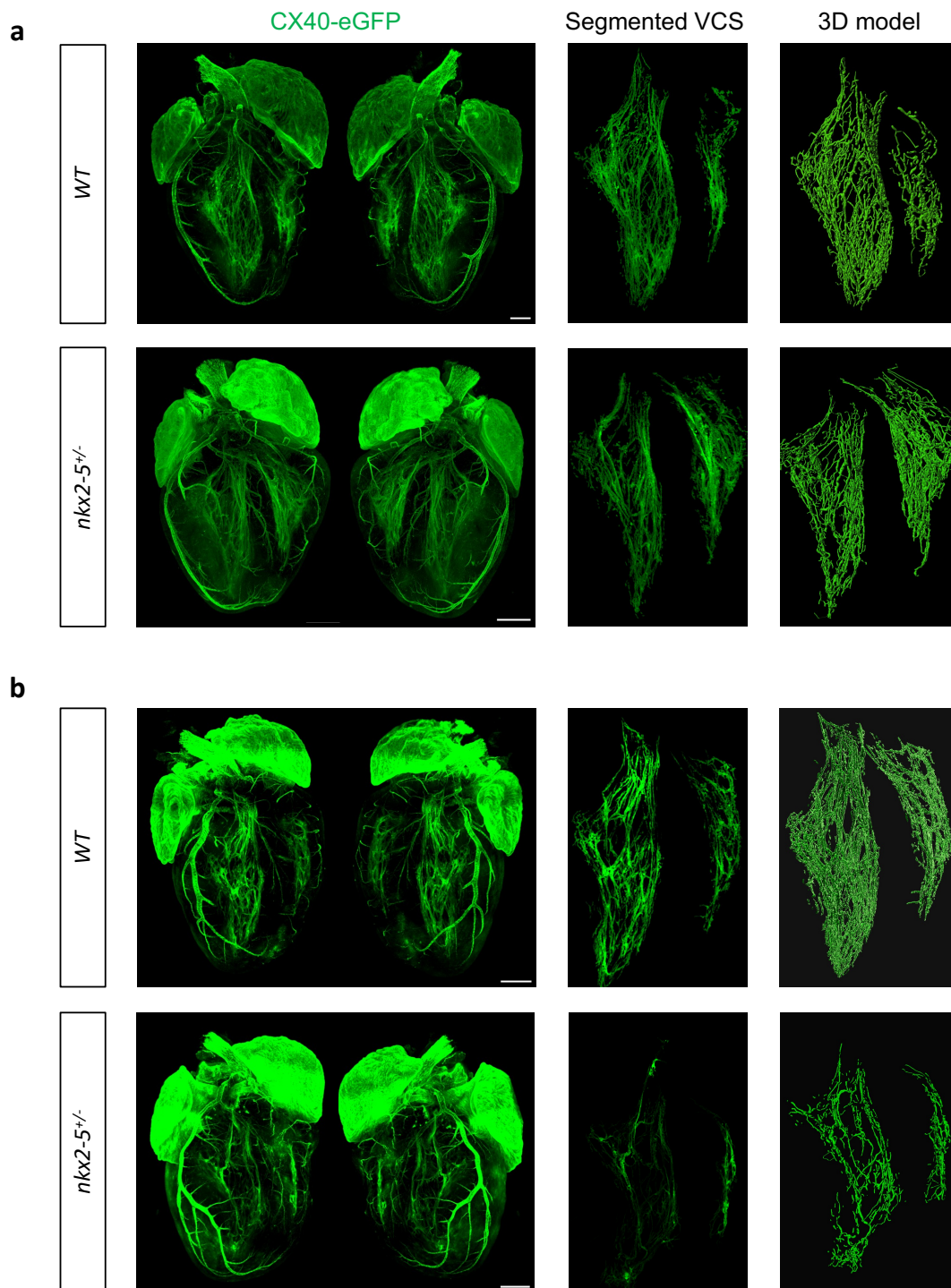


Figure 3.11: The *Nkx2-5^{+/-}* mouse develops a hypomorphic conduction system over early post-natal development

Time series of wild-type and *Nkx2-5^{+/-}* tissue-cleared hearts through post-natal development from post-natal day 2 (**a**) to post-natal day 4 (**b**). Depicted from left to right: mirrored 3D-rendering of the whole heart; the segmented VCS network; and its 3D filament model. While wild-type and mutant networks appear similar in structure, size and complexity at P2 (**a**), by P4 the network has become substantially hypomorphic (**b**). Green: CX40-eGFP and anti-GFP, labelling the developing ventricular conduction system (VCS), atria and coronary arteries. Scale bars: 500 μ m.

To quantify these findings and analyse the onset of Purkinje fibre hypoplasia in detail, I segmented the CX40-eGFP+ VCS network from the atrial and arterial signal and built 3D filament models of wild-type and *Nkx2-5^{+/-}* hearts at P2 and P4 stages. I quantified network volume, filament area and filament length in order to assess overall size of the network, and I quantified number of segments, number of branch points and number of terminal end points in order to determine the extent of complexity in the network. I detected no significant differences in any of these parameters between wild-type and *Nkx2-5^{+/-}* P2 hearts, confirming the qualitative finding of **Figure 3.10** that the *Nkx2-5^{+/-}* heart has an apparently normal VCS network at post-natal day 2 (**Figure 3.12**). Contrastingly, the size of the network was significantly reduced by P4 in *Nkx2-5^{+/-}* hearts, and moreover the complexity of the network had also decreased significantly by P4 between *Nkx2-5^{+/-}* versus wild-type hearts (**Figure 3.12**).

Finally, I assessed whether there were differences between onset and extent of hypoplasia in the left versus the right VCS network over early post-natal development. I found a significant decrease in network volume, filament area, filament length and segment number in the left VCS by P4, whereas although there was a clear trend towards a decrease in these parameters, none were significantly different in the right VCS between mutant and wild-type hearts (ANOVA with multiple comparisons: p-values of 0.0907, 0.0798, 0.0695, 0.1251 for P4 right VCS network volume, filament area, filament length and segment number, respectively) (**Figures 3.13** and **3.14**). This indicated that hypoplasia was more marked in the left than the right VCS of *Nkx2-5^{+/-}* hearts.

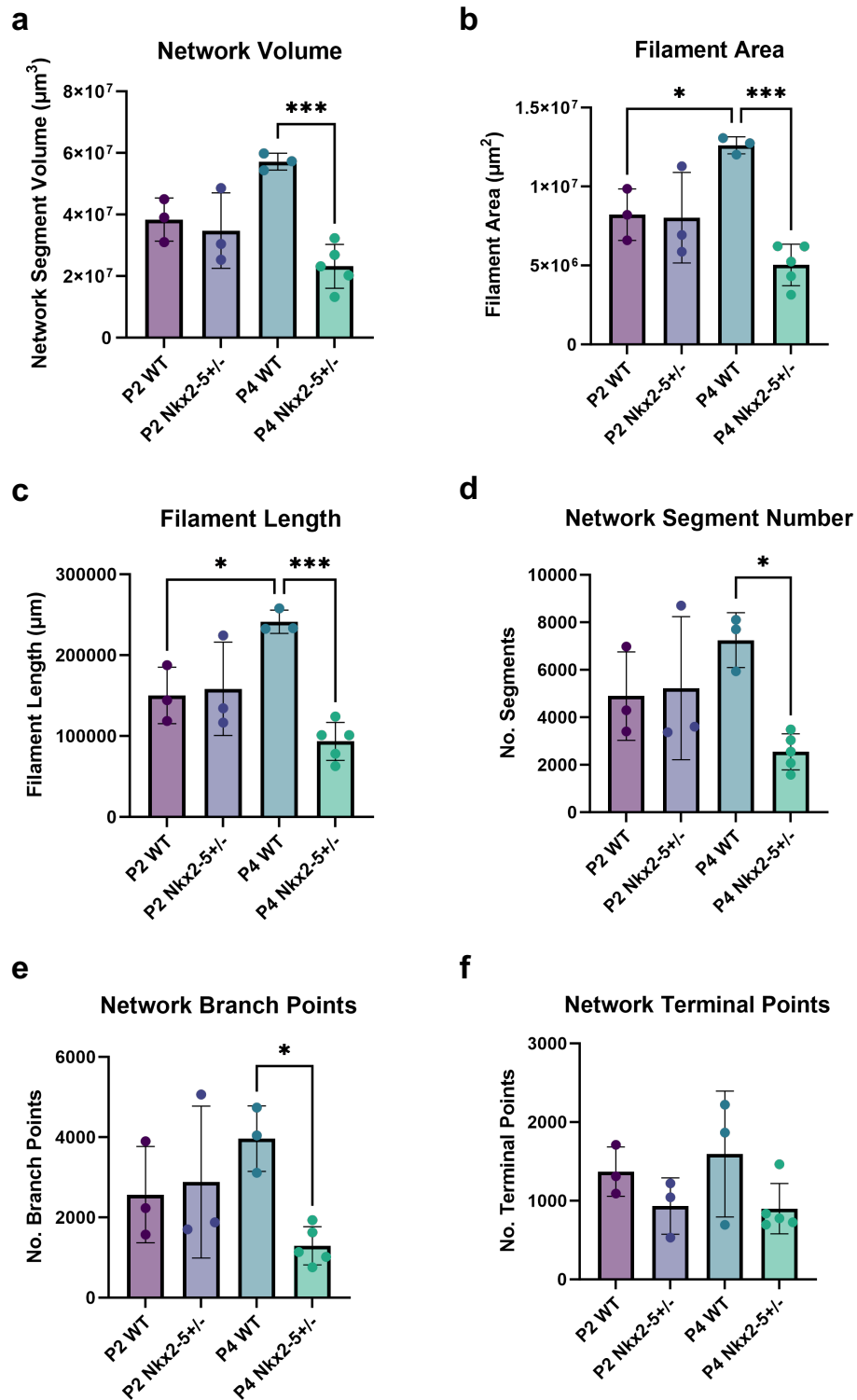


Figure 3.12: Quantitative analysis of the hypomorphic *Nkx2-5*^{+/-} ventricular conduction system

Graphs quantifying His/Purkinje network volume (a), filament length (b), filament area (c), number of branch points (d), number of segments (e), and number of terminal points (i.e., ends of branches) (f) across post-natal development between post-natal day 2 (P2) and post-natal day 4 (P4), wild-type and *Nkx2-5*-heterozygous hearts. Statistics: ANOVA with Tukey-Kramer test for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

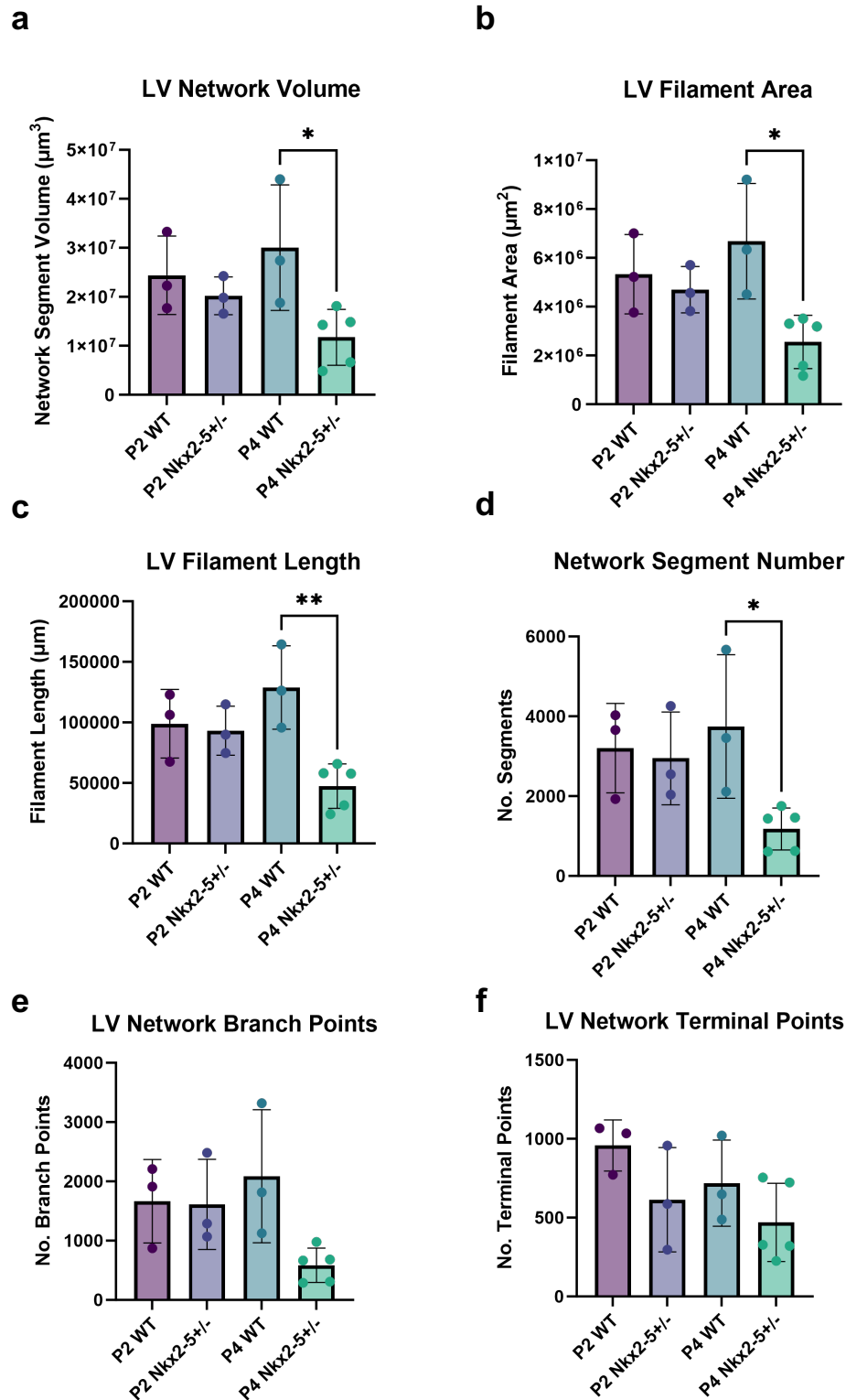


Figure 3.13: The left ventricular conduction system of the *Nkx2-5*^{+/-} heart is significantly hypomorphic by post-natal day 4

Graphs quantifying left His/Purkinje network volume (a), filament length (b), filament area (c), number of branch points (d), number of segments (e), and number of terminal points (i.e., ends of branches) (f) across post-natal development between post-natal day 2 (P2) and post-natal day 4 (P4), wild-type and *Nkx2-5*-heterozygous hearts. Statistics: ANOVA with Tukey-Kramer test for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

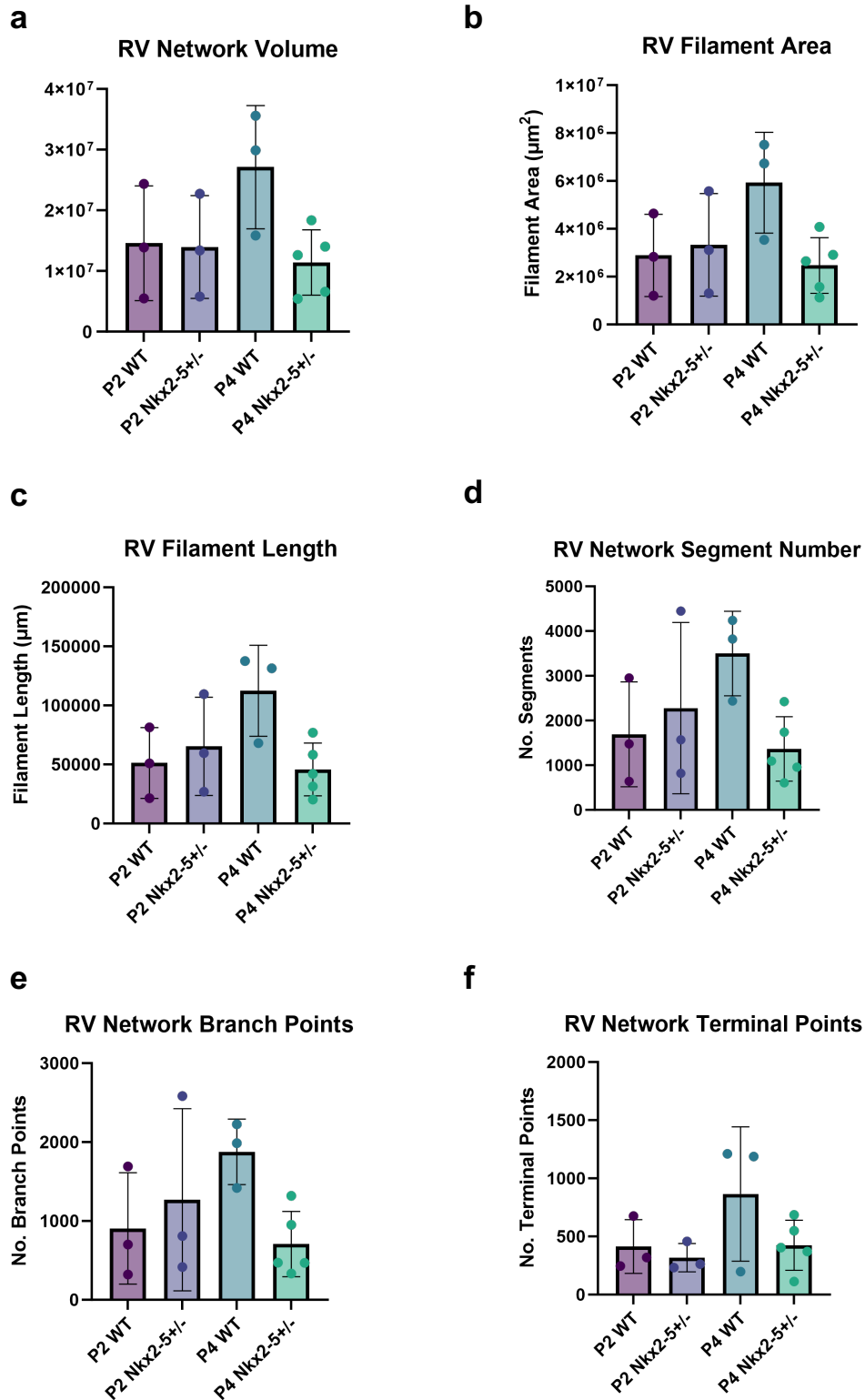


Figure 3.14: The right ventricular conduction system of the *Nkx2-5*^{+/-} heart is somewhat reduced but not significantly hypomorphic by post-natal day 4

Graphs quantifying right His/Purkinje network volume (a), filament length (b), filament area (c), number of branch points (d), number of segments (e), and number of terminal points (i.e., ends of branches) (f) across post-natal development between post-natal day 2 (P2) and post-natal day 4 (P4), wild-type and *Nkx2-5*-heterozygous hearts. Statistics: ANOVA with Tukey-Kramer test for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Overall, therefore, I was able to apply my imaging pipeline in order to perform a fully quantitative characterisation of the onset of hypoplasia in the murine *Nkx2-5*^{+/-} His/Purkinje network. I found, surprisingly, that there are no detectable differences between the mutant and wild-type VCS of hearts at P2, but that there is significant hypoplasia by P4, by which stage the mutant network volume is 3-fold smaller than the wild-type network. I further established that this hypoplasia extends both to reduced network size and reduced complexity of network branching at P4, and that this is most marked in the left VCS network. Altogether these findings highlight the power of the imaging/analysis pipeline, presented in this Chapter, and how it can be used to probe already-existing models in much greater detail.

3.4 Discussion

In this Chapter, I designed and optimised a CUBIC-based tissue clearing and whole-mount immunostaining protocol, imaging set-up, and analysis pipeline for whole-organ imaging of the mouse heart throughout post-natal development. I then leveraged this imaging toolkit to assess and quantify the maturation of the intact murine conduction system through post-natal development for the first time. My analysis identified that there is significant growth of the VCS network after birth, which occurs by extension of existing fibres, as opposed to genesis of new branch points. I further found that while the majority of left ventricular His/Purkinje network development is complete by P2, the right network's development is delayed with respect to the left and undergoes more extensive maturation between P2 and P10. Moreover, I characterised the vascularisation of the developing VCS and identified a previously unreported association between tissue-resident macrophages and Purkinje fibres in the post-natal mouse heart. Finally, I performed a quantitative analysis of the hypomorphic *Nkx2-5*^{+/-} VCS and determined that Purkinje fibre hypoplasia is established between P2 and P4 in these mutants, lending support to the cell dropout model

wherein low levels of NKX2-5 result in inability of VCS cells to maintain a conductive phenotype. Taken together, these insights significantly expand our understanding of conduction system biology and maturation after birth in neonatal mice.

3.4.1 Tissue-cleared whole-organ imaging as a methodology to study the biology of the cardiac conduction system

3D imaging of biological tissues is revolutionising our ability to interrogate the functional organisation of living systems. These approaches circumvent the need to dissect or section organs prior to imaging, enabling analysis of intact cellular and tissue architecture with ever-greater resolution. In the context of the CCS, this has proven to be particularly important. The 3D network architecture of the His/Purkinje system determines the propagation sequence of the electrical impulse responsible for organising synchronous cardiac contraction. Understanding its spatial morphology and architecture across cellular-, tissue-, and organ-level interactions provides the means to infer its functional organisation. Moreover, given the prevalence of congenital, inherited or idiopathic diseases affecting CCS function (e.g., atrial fibrillation, primary idiopathic VF, catecholaminergic polymorphic VT, long QT syndrome¹⁵³⁻¹⁵⁶), and the involvement of the CCS in acquired disease following injury or insult (e.g., viral myocarditis, myocardial infarction^{22,157}), these analyses provide a baseline understanding of healthy tissue architecture and enable analysis of how this is altered in the disease context. The ability to visualise and quantitatively analyse the CCS and surrounding cell types by immunofluorescence and/or endogenous fluorescent reporters in 3D as extended in this Chapter, will thus support future efforts to study CCS (patho)physiology. Moreover, the tissue-clearing methodology presented here could easily be adapted for imaging of intact adult mouse or embryonic human hearts.

3.4.2 Growth and maturation of the ventricular conduction system

The analysis presented in this Chapter supports and extends previous characterisation of VCS development. It has been shown that lineage restriction to Purkinje cell fate is largely complete by E16.5¹⁷. My wholemount 3D imaging of CX40 expression in the intact heart over late embryonic development shows that this lineage restriction precedes CX40 downregulation: CX40 expression persisted in the surrounding trabecular myocardium of E16.5-E18.5 hearts, only becoming restricted to mature VCS cells at birth (**Chapter 3.3.2.1**). It should however be noted that while the rate of change in connexin expression and protein turnover is very rapid, with most cardiac connexins having an approximate half-life of 1-2 hours¹⁵⁸, it is possible that there might be slightly longer perdurance of the eGFP in the cells of the trabecular myocardium. Since GFP expression is consequently an imperfect proxy for endogenous CX40 expression, real-time CX40 levels may be slightly lower over late embryonic stages than suggested by the images of Figure 3.3. This would temporarily align the downregulation of *Gja5* (encoding CX40) with the proposed lineage restriction more closely.

The results of the time series of the developing CCS over late embryonic development (**Figure 3.3**), together with the finding that other well-known mature VCS markers are already expressed by P2 (**Chapter 3.3.2.2**) indicate that the VCS' rapid conduction transcriptional programme is initiated and delimited to mature VCS cells just after birth. Going forwards, it will be important, therefore, to establish whether this time-course of VCS maturation correlates closely with that of other mammalian species, to allow further interpretation of previous work. Moreover, while VCS cells show a mature phenotype by this stage, the finding that network volume and 3D architecture continue to develop through the post-natal window is very significant. In order to understand the mechanism of this growth, future studies should also investigate the maturation of the connective tissue sheath that surrounds and insulates the proximal VCS. While major components of the sheath include collagen and elastin, future work should focus on identifying markers in order to distinguish

this sheath specifically. Given that this fibrous sheath fully encompasses the conduction cells of the His bundle and BBs and is thick in circumference when studied in histological sections, especially so in the proximal parts of the bundle branches¹⁵⁹, we can predict that recruitment of conduction cells to the network should be coordinated with deposition of the collagens, elastin fibres, and other constituent parts that make up the insulative sheath. However, how this is orchestrated at a cellular and mechanistic level remains unclear.

3.4.3 His/Purkinje network complexity

Prior studies have demonstrated extensive complexity in the His/Purkinje network. Indeed, it has previously been reported that there is bifurcation and merging of fibres at different levels of the VCS network, and consequently that the network's structure is not strictly hierarchical, but rather has fractal patterning within a semi-hierarchical tree^{160,161}. My imaging analyses of the His/Purkinje network in the mouse heart corroborate this finding (**Figure 3.5**). Furthermore, by performing quantitative studies through post-natal development, this Chapter presents novel insights into how the network complexity matures.

Notably, this analysis shows that much of the network's complexity has already been established in the left ventricle by as early as P2. Furthermore, this analysis provides the first intact imaging and quantitative assessment of the previously recognised structural asymmetry between left and right VCS networks in the post-natal mouse heart (**Chapter 3.3.3.1**)⁶⁸. In light of these findings, and given that there are known differences in trabeculation between the left versus right ventricle during development¹⁶², future work should focus on elucidating whether left-right VCS differences manifest at the cellular and molecular level as well as the differences in ontology, as identified here. This will help to establish whether development of the right VCS is simply delayed with respect to the left VCS, or whether cells destined for a conductive fate pass through a subtly different maturation trajectory depending on whether they are part of a left or right VCS lineage.

Going forwards, it will also be interesting to ascertain the extent to which these findings correlate with post-natal conduction system maturation in other animal hearts including those with relatively similar Purkinje networks to the mouse (rats and rabbits), but also in hearts which display differences in conduction cell size and ultrastructure (ungulates, cetaceans, dogs, cats and humans)¹⁶³.

3.4.4 Interactions between the conduction system and other tissue types during post-natal development

In this Chapter, I characterised extensive vascularisation of the growing VCS by the coronary arteries through post-natal development (**Chapter 3.3.2.3**). Given the known role that the vasculature plays in recruiting autonomic nerves during cardiac development^{164,165}, and the intertwined development of the VCS with its supportive vasculature identified here, it will be interesting to ascertain firstly to what extent there is signalling between the coronary arteries and the conduction cells in the post-natal heart, secondly whether nerves directly innervate the VCS as well as the arteries, and thirdly whether there is cross-talk between these three systems in development and in regeneration. Indeed, it will be important to establish whether the coronary vasculature actively patterns the VCS in an analogous way to how blood vessels pattern nerve growth in other systems, including in the developing hindbrain¹⁶⁶.

Moreover, the finding that macrophages wrap around the junctions between Purkinje cells warrants significant further investigation. Macrophages have previously been shown to interact with other developing networks including the coronary blood and lymphatic vasculature, raising the possibility that macrophages have similar functional interactions with the developing VCS network^{148,149}. A landmark study by Hulsman et al. established the unexpected involvement of macrophages in cardiac electrical conduction with comprehensive genetic and electrophysiological analysis¹⁴⁶. This study identified

macrophages in the SAN and AVN, demonstrated that working ventricular cardiomyocytes couple directly with macrophages via CX43, and found that genetic ablation of Cx43 in macrophages was sufficient to induce AV block. However, to the best of my knowledge, to date interactions between tissue-resident macrophages and the His/Purkinje system have not been investigated. Going forwards, it will be important to ascertain whether macrophages couple electrically to the Purkinje cells via CX40 and/or CX43, whether macrophages wrap around the Purkinje cells in mature adult hearts to the same extent as at post-natal stages, and whether they are playing other supportive roles in the VCS at steady state.

These preliminary findings further beg the question of whether there is a role for recruited macrophages in altering cardiac rhythm in inflammatory contexts including after MI. The recent finding that recruited macrophages elicit atrial fibrillation supports the hypothesis that in addition to interactions between the VCS and tissue-resident macrophages, recruited macrophages may interfere with electrical conduction through pathophysiological interaction with the conduction cells of the His/Purkinje network. Overall, therefore, future work should focus on elucidating the interactions between the VCS, vasculature, cardiac innervation and macrophages in healthy and diseased states (see **Chapter 7**).

3.4.5 The role of NKX2-5 in determining the conduction system lineage

It is well established that NKX2-5 plays a pivotal role in Purkinje fate determination. Indeed, NKX2-5 has a direct role in regulating CX40 levels: NKX2-5 regulates Cx40 expression in VCS cells both directly (through binding to the *Gja5* (CX40) genetic promoter) and indirectly (via upregulation of homeobox protein *Hop*)^{124,167}. Besides regulation of *Gja5*, NKX2-5 also sits at the top of the fast conduction programme and, therefore, controls expression of a broad suite of VCS genes (including *Cntn2*, *Scn5a*, *Hcn4*)².

NKX2-5 (at proper dosage) is, however, not the only transcription factor required for post-natal VCS maturation. Indeed, IRX3 and ETV1 (both of which also regulate *Gja5* (CX40) expression levels) are additionally required for post-natal VCS remodelling^{40,168}. Most interestingly, *Etv1* knock-out mice also display a hypomorphic left and right VCS. The *Etv1* KO network qualitatively appears similar to but slightly less hypomorphic than the *Nkx2-5* haploinsufficient mutant network investigated in this Chapter: the proximal parts of the *Etv1*^{-/-} network appear less reduced as compared to the *Nkx2-5*^{+/-} equivalent, whereas the hypoplasia of the terminal *Etv1*^{-/-} Purkinje fibres appear equally prominent. This suggests that there are specific spatial differences in the regulation of VCS lineage determination and maturation across the AVB, BBs and Purkinje fibres⁴⁰.

Contrastingly, the *Irx3*^{-/-} heart often seems to lack the right BB entirely, and to have a left VCS network that becomes progressively hypomorphic through post-natal maturation¹⁶⁸. This seems not to be due to apoptosis, but rather to lack of recruitment, maturation, and/or maintenance of cells with a conductive phenotype. Despite the precise architecture of the hypomorphic VCS networks differing between the three mutants, in all cases it appears that Purkinje cell number is depleted across the VCS in absence (IRX3/ETV1) or with reduced dose (NKX2-5) of these transcription factors. Furthermore, it is noteworthy that the extent to which the left versus right BB is affected differs in some of these mutants. This has also been seen in the *Ncam-1*^{-/-} mutant, in which the left VCS is dysmorphic but the right VCS appears to be unaffected¹⁶⁹. In light of these findings, differences between left versus right transcriptional regulation of cardiac conduction warrant further investigation.

While the onset of hypoplasia has not, to my knowledge, been assessed in the *Etv1* mutant background, it is noteworthy that in the *Irx3*^{-/-} left VCS there is no statistically significant hypoplasia in the P0 heart, but by P4 fibre density and thickness are depleted¹⁶⁸. This approximates to the timeline of hypoplasia onset that I have observed in the *Nkx2-5*^{+/-} mutant, in which hypoplasia is established between 2 and 4 days after birth (**Figures 3.11-3.12**). Collectively, these analyses indicate the requirement of the fast conductance transcription factors during the post-natal period for continued VCS lineage commitment.

Moreover, this suggests that in absence of sufficient levels of NKX2-5, IRX3 or ETV1, cells that would have been destined for a Purkinje cell fate fail to maintain expression of the fast-conductance VCS genetic programme. This suggests that the fast-conductance genetic programme is very tightly regulated in the murine heart.

Finally, it should be noted that the *Nkx2-5* mutant experiments in this Chapter were performed in a global *Nkx2-5*-heterozygous background. However, *Nkx2-5* has diverse roles in heart development, including in cardiomyocyte development and function^{170,171}. Going forwards, single and combinatorial deletion of *Nkx2-5*, as well as of *Etv1* and *Irx3* in a targeted way in VCS progenitors and in mature VCS cells, using a range of Cre drivers, could be combined with the imaging methodology laid out in this Chapter. This would help to determine the extent to which hypoplasia of the VCS results from a cell-autonomous role for these transcription factors in developing VCS cells that are already committed to a Purkinje fate, versus a role in progenitor cells prior to commitment and/or in cell recruitment to the network.

3.5 Conclusions

In this Chapter, I designed a tissue clearing, imaging and analysis pipeline for quantitative 3D examination of the intact murine cardiac conduction system. My results demonstrate significant growth and maturation of the His/Purkinje network during post-natal development, and elucidate the complex 3D architecture of the post-natal murine conduction system for the first time. Characterisation of interactions with other cell types identified extensive vascularisation of the His/Purkinje network from birth and close apposition of macrophages and Purkinje fibres in the post-natal mouse heart. Finally, a full quantitative assessment of haploinsufficient *Nkx2-5* heterozygous mutants demonstrated normal development through until P2, but formation of a hypomorphic conduction network by P4 due to loss of CX40+ conduction cells from the network, with significant differences between left versus right VCS development.

4 Morphological and network-level changes to the conduction system following myocardial infarction to regenerative and non-regenerative hearts

4.1 Background

4.1.1 The ventricular conduction system in infarcted hearts

One of the most common complications following MI involves cardiac rhythmic disturbance. This can arise due to damage to the cardiac conduction system, and/or due to changes to the electromechanical environment resulting from remodelling of cardiomyocytes and the laying down of fibrotic scar^{23,102}. The ensuing arrhythmias significantly increase mortality risk, worsen heart failure and lead to longer-term dyssynchrony of cardiac contraction.

Clinically, there are three main areas of focus regarding conduction disease following MI: AV block; left bundle branch block; and ectopic beats in and around the scar or border zone. Additionally, in rarer instances ectopic beats can derive directly from the Purkinje fibres themselves, necessitating catheter ablation of the relevant areas. Current treatment options for rhythmic disturbance following MI most commonly include pace-maker insertion or cardiac resynchronisation therapy if ejection fraction is severely reduced; however, therapeutic and drug treatment options remain limited. Indeed, despite the frequent occurrence of arrhythmias following MI, the cellular and molecular causes of conduction system pathophysiology in the infarcted heart are not well understood.

Evidence from the dog and pig has found that Purkinje cells can survive in infarcted regions where working myocardium is otherwise absent, suggesting that the Purkinje cells may be more resistant to ischaemic conditions than cardiomyocytes^{30,31}. However, it has been shown that overall, there is a decline in the Purkinje population of the adult pig heart following MI, and that those Purkinje cells that do survive undergo changes in subcellular morphology and extent of vacuolisation³¹. Furthermore, it is well established from classical canine studies that spontaneous ectopic ventricular beats can arise from surviving subendocardial Purkinje fibres underlying infarcted myocardial regions following acute MI^{29,172,173}. However, it remains unclear how Purkinje cells are affected following MI across different levels of the His bundle, BBs and distal Purkinje fibres. Moreover, to what extent Purkinje cells generally become damaged or undergo remodelling following MI, and whether the heart has any innate capacity to repair the VCS, are outstanding questions with significant consequence for cardiovascular patients.

4.1.2 Surgical LAD ligation in the neonatal mouse as a model for heart regeneration

Adult humans cannot regenerate their hearts following serious insult, for example due to viral infection (myocarditis) or following MI. However, neonatal mammals including mice, piglets and human infants can regenerate their hearts for a short window after birth^{47,52,53}. The neonatal mouse provides a tractable mammalian model in which to interrogate natural mechanisms of heart regeneration. Given that the ability to regenerate the heart is lost within the first few days of life, the long-term outlook for studies of the neonatal mouse is in translating knowledge of the natural regenerative processes lost soon after birth to improve heart repair after MI in adult patients.

In the following Chapters of this thesis, I use a surgical MI model for heart regeneration involving permanent ligation of the LAD coronary artery in the neonatal mouse,

which has been published and reported on extensively elsewhere^{70,174,175}. The model involves extrusion of the heart from the chest cavity and the tying of a suture knot around the LAD artery. Given the brief window during which neonates possess regenerative capacity, to power comparative analyses the surgeries undertaken in the following Chapters are performed on pups either at regenerative P1, defined as the first day following birth, or once the pups reach P7, by which point they no longer have cardiac regenerative ability⁴⁷. Given the invasive nature of the surgical procedure, throughout Chapters 4 and 5 I also include a sham surgical control in all experiments, in which the chest cavity is opened up, the heart extruded and a suture needle passed through the heart without the tying of a knot to occlude the LAD. Consequently, the sham control samples undergo the mechanical damage associated with performing the surgical procedure, as well as the stress and trauma of enduring the surgery, without suffering ischaemic damage to the heart.

I chose the permanent LAD ligation surgery in favour of apical resection⁴⁷ or cryoinjury¹⁷⁶, the other available neonatal cardiac injury models. The LAD MI is the only established murine ischaemic model for heart regeneration, is very well-characterised, and reliably produces a large infarct. In the context of assessing electrical damage, studying this model therefore provided the best conditions to identify conduction system pathophysiology and regenerative potential following MI. Moreover, given that the foundational heart regeneration studies that laid the groundwork for these experiments were performed on the LAD ligation model, including published datasets that I will go on to integrate with my own molecular analyses in Chapter 5, using this same approach provided powerful opportunities for comparative analysis.

It should be noted that the neonatal LAD ligation is an imperfect model of MI for three principal reasons. First, the permanent LAD ligation arguably mirrors patient morbidity less closely than ischaemia/reperfusion models since many individuals suffering heart attack undergo reperfusion by stent insertion, often within hours of the initial heart attack event. However, the LAD ligation provides valuable insight into the tissue response to prolonged ischaemia: permanent ligation is a better model than ischaemia/reperfusion for the

approximately 30% of patients who do not receive reperfusion clinically; as well as for patients who receive reperfusion but nevertheless progress to heart failure¹⁷⁴. Furthermore, ischaemia/reperfusion is not performed on neonates due to the small size of their hearts, and adult ischaemia/reperfusion models show much greater variability in outcome than LAD MI models so results from the former are arguably less reproducible¹⁷⁴.

Secondly, given the small size of the neonatal heart at P1, the ligation surgery causes additional damage to tissue surrounding the LAD artery. Consequently, the injury is not limited precisely to ischaemic damage from arterial blockage alone. The sham control mitigates this issue to some extent since passing the suture needle through the small neonatal heart incurs significant mechanical damage, but this does not exactly replicate the mechanical damage from the tying of a suture knot. Thirdly, the neonatal heart does not have the pre-conditioning and collateral damage that adult patients present with, so this models acute ischaemic damage in isolation from potential wider pathophysiologies of the diseased heart. Despite these limitations, the neonatal LAD MI model remains a very powerful system for heart regeneration studies thanks to the direct mammalian, intraspecies, and comparative approach that it supports between regenerative (P1) and non-regenerative (P7) stages. Indeed, studies of the neonatal mouse model have already led to numerous potential drug targets and promising avenues for heart repair in human patients.

A further consideration is the need to extricate developmental factors due to maturation over the first week of life, from regenerative processes that ultimately might be activated in an adult heart after MI. However, my quantitative assessment of VCS development in Chapter 3, and my neonatal electrophysiological ECG study and modelling of human arrhythmias from neonate post-MI phenotypes in Chapter 6, together provide support regarding the suitability of this model for studying the consequence of MI for the conduction system. The findings that CX40-eGFP expression is restricted to the mature VCS structure by birth (**Figure 3.3 and 3.4**), and that the left ventricular His/Purkinje network does not alter substantially over the post-natal period (**Figure 3.9**) indicated that many of the major developmental changes have largely taken place before the neonatal regenerative

window. This suggests, therefore, that any latent regenerative capacity in the conduction system would most likely not simply be a developmental response, increasing the potential for drawing transferrable insights with relevance to the adult heart.

Previous studies on neonatal heart regeneration have investigated: cardiomyocyte proliferation or trans-differentiation; neo-vascularisation, including via angiogenesis, collateral artery formation, and lymphangiogenesis; the role of innervation by cardiac sympathetic and parasympathetic nerves; diverse immune contributions to regeneration; and the mechanisms underpinning the fibrotic process^{3,64,66,67,177}. However, whether the CCS has the capacity to regenerate and how the heart maintains rhythm in the neonatal regenerative context has not yet been systematically investigated.

4.2 Aims and objectives

The aims of this chapter were to:

1. Determine whether the ventricular conduction system is disrupted following myocardial infarction in the neonatal mouse
2. Assess network-level damage to and subsequent restoration of the bundle branches and fibres of the His/Purkinje system following injury at regenerative versus non-regenerative stages
3. Determine whether there are changes in connexins and ion channels in the ventricular conduction system following myocardial infarction and how this differs at regenerative versus non-regenerative stages

4.3 Results

4.3.1 The His/Purkinje network is disrupted following myocardial infarction

I hypothesised that there is direct disruption to the His/Purkinje network following MI. I began, therefore, by investigating whether there are gross morphological and cellular changes to the His/Purkinje network following MI induced by LAD coronary artery ligation in the neonatal mouse. Given the LAD supplies the His bundle, left anterior fascicle and part of the left posterior fascicle¹⁷⁸, and given this vascularisation is already established by the earliest post-natal stages coinciding with neonatal regenerative capacity in the first week of life (**Figure 3.6** and **Chapter 3.3.2.3**), I anticipated disruption to these regions of the VCS following neonatal surgically-induced LAD ligation.

I first analysed network structure at 1- and 3- days following MI to *Cx40-eGFP/+* regenerative P1 and non-regenerative P7 neonates, in intact tissue-cleared hearts. I chose these time points in order to assess whether damage and cell death were apparent in the VCS in the days immediately after injury coinciding with the peak of the initial immune response (day 1) and with the onset and progression of the cardiomyocyte regenerative response (day 3) in the neonatal mouse (**Figure 4.1**)¹. CX40-eGFP signal and fibres within the network below the level of the suture site were discontinuous post-injury in MI but not in sham control hearts at both time points. This was most often in the left, but also in the right VCS network, indicating that VCS cells were either lost or molecularly altered post-MI (**Figure 4.1**). 3D reconstruction of MI hearts found that the surgical suture knot was located superficially and did not penetrate the myocardial layer to the sub-endocardial Purkinje fibres (**Figure 4.1, panels b' and d'**). This, therefore, suggests that loss of GFP expression proximally to the suture site resulted from ischaemic damage to the Purkinje cells.

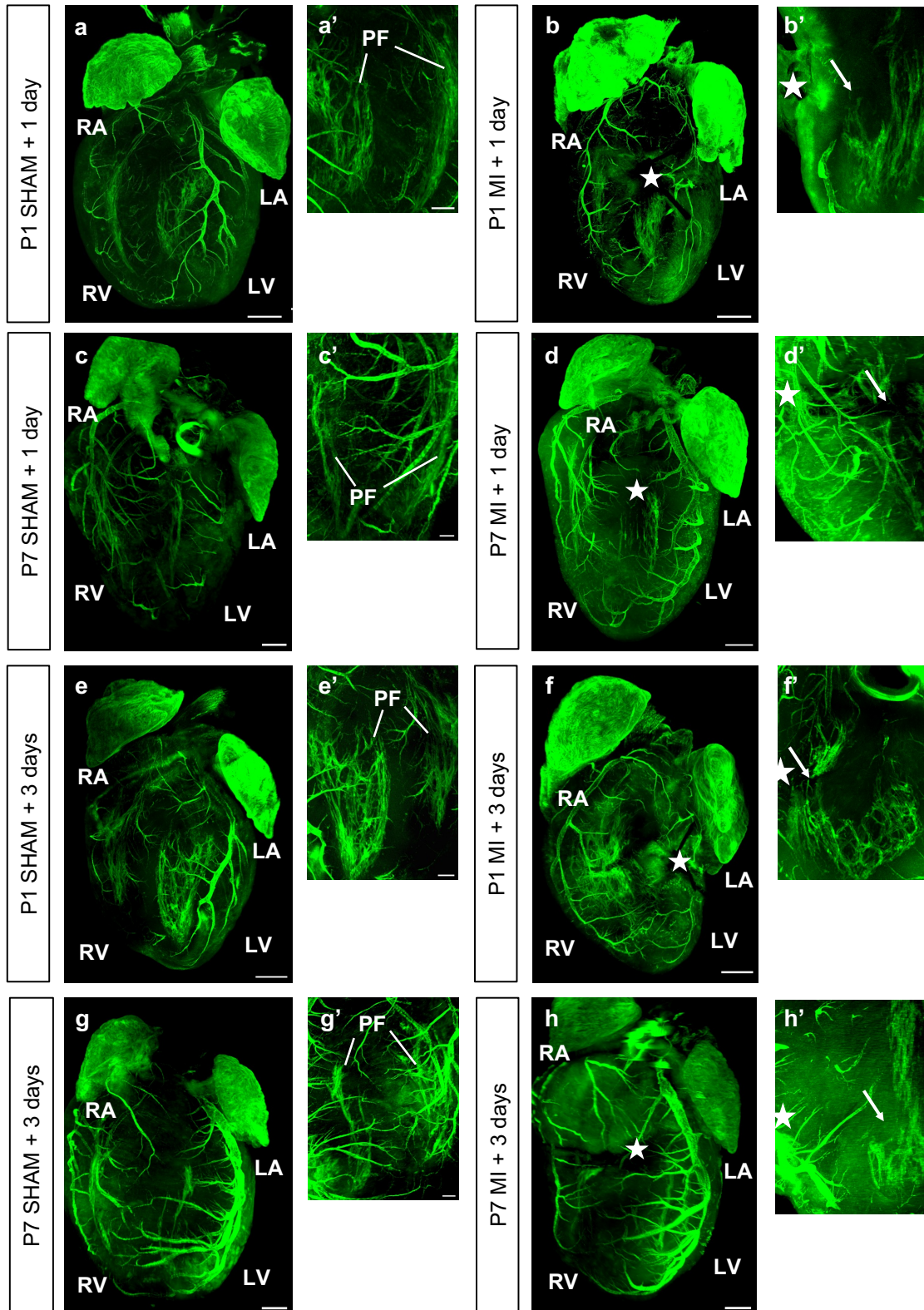


Figure 4.1: The His/Purkinje network is disrupted following myocardial infarction to both regenerative and non-regenerative hearts

3D-rendered tissue-cleared *Cx40eGFP/+* hearts following MI or sham surgery, showing disruption to the His/Purkinje network after MI. **a-b**: hearts harvested 1 day after P1 surgery. **c-d**: hearts harvested 1 day after P7 surgery.

e-f: hearts harvested 3 days after P1 surgery. **g-h:** hearts harvested 3 days after P7 surgery. Panel inserts show maximum intensity projections taken from the same heart, rotated through 90 degrees to look side-on through the ventricle. Stars indicate location of the LAD ligation. Arrows highlight regions of VCS disruption. Labels indicate left atrium (LA), right atrium (RA), left ventricle (LV), right ventricle (RV), Purkinje fibres (PF). Scale bars: 500µm for whole heart images, 200µm for magnified inserts.

4.3.2 The bundling of conduction fibres is altered by myocardial infarction with differences in regenerative versus non-regenerative hearts

To assess changes in bundling and fibre morphology, I dissected open the left ventricle of hearts following MI or sham surgery at P1 or P7, 3 days post-injury, cutting base to apex and pinning the myocardial wall flat to expose the His/Purkinje network (**Figure 4.2**). In both P1 and P7 MI hearts gaps were evident in the His/Purkinje network proximal to the LAD ligation site, and there were bright CX40-expressing bundles with altered morphology in the vicinity of the injury region which were particularly prominent in the P1 heart (**Figure 4.2, panels f' - j'**). However, MI-induced changes to VCS morphology differed between regenerative and non-regenerative hearts: following MI at P1 there was increased overall CX40-eGFP expression in the distal half of the network below the level of the suture site (**Figure 4.2f-j**), which was not seen following MI at P7 or following P1/P7 sham surgeries (**Figure 4.3**). In addition, MI at P7 resulted in altered bundling of fibres which were thinner, with more of the fibres running individually from base to apex, reduced bundle formation and a disorganised network (**Figure 4.2p-t, and panels p' - t'**). This is interesting since there is a close link between bundle anatomy and VCS function¹⁷⁹, whereby bundling of Purkinje cells within the fibrous sheath affects electrical insulation from the working myocardium to ensure fast electrical propagation through the network^{159,180}.

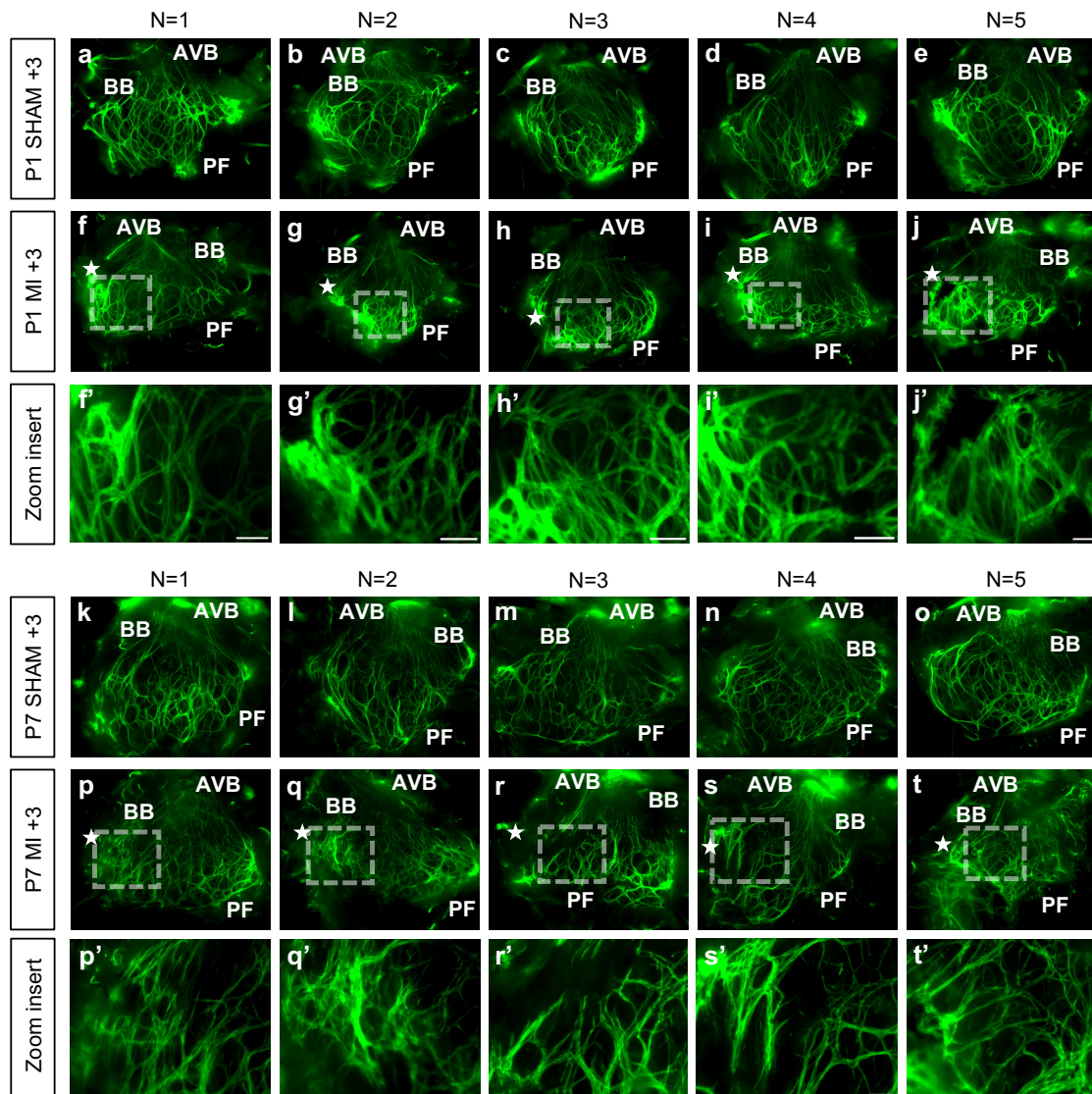


Figure 4.2: The bundling of conduction fibres is disrupted following myocardial infarction with differences between regenerative and non-regenerative hearts
 The dissected left ventricular His/Purkinje network of *Cx40eGFP/+* hearts 3 days following MI or sham surgery at regenerative P1 (a-j) and non-regenerative P7 (k-t) stages. Five representative hearts are shown in each case. Panels show magnified inserts from the boxed regions of the corresponding P1 and P7 MI images, highlighting the disruption in fibre bundle morphology after MI. Stars indicate level of the LAD ligation. Labels indicate atrioventricular bundle (AVB), bundle branches (BB), Purkinje fibres (PF). Scale bars: 100µm.

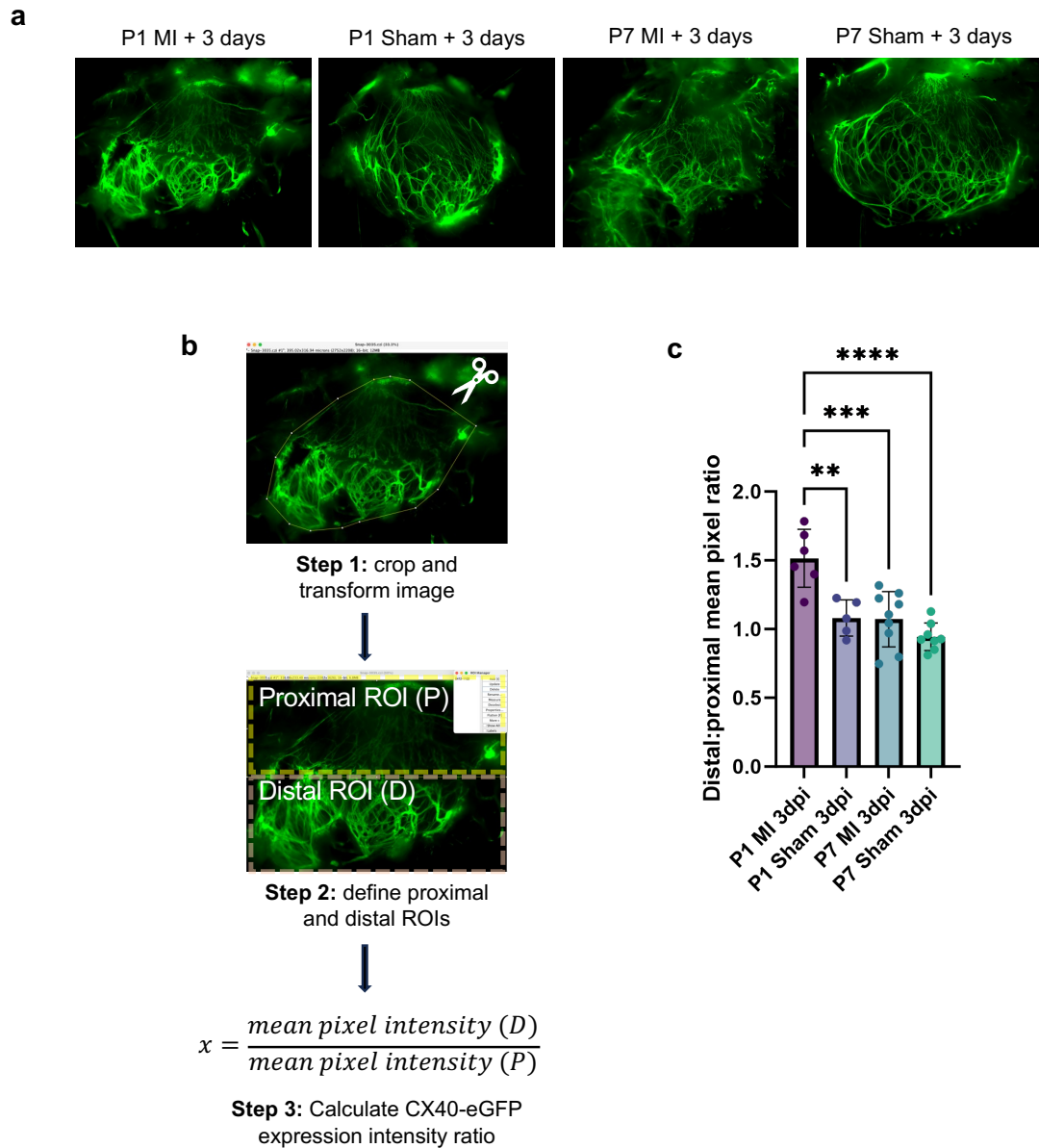


Figure 4.3: CX40-eGFP expression is increased in the distal Purkinje network of regenerative P1 hearts but not in non-regenerative P7 hearts following myocardial infarction

a. A comparison between P1 and P7 MI and sham hearts from *Cx40-eGFP/+* mice, 3 days following injury, identifies brighter CX40-eGFP signal in the distal half of the P1 MI Purkinje network towards the heart apex. **b.** Schematic to illustrate the pipeline used to quantify the distal: proximal mean pixel ratio. **c.** CX40-eGFP is significantly increased in the distal Purkinje network below the level of the LAD ligation in P1 MI hearts 3 days post-injury, but not in P7 MI or P1/P7 sham hearts at an equivalent timepoint. Statistics: ANOVA with Tukey-Kramer test for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

4.3.3 A full His/Purkinje network is reconstituted during neonatal heart regeneration

Next, I wanted to ascertain whether the disruption to His/Purkinje fibres and changes in bundle morphology identified in the immediate aftermath of the MI event result in longer-term damage after injury, or whether the regenerative P1 heart is able to reconstitute a full conductive network. To this end, following no surgery, MI surgery or sham surgery on P1 hearts, I allowed 21 days for recovery before I harvested the hearts and cut open the left ventricle to expose the His/Purkinje network (**Figure 4.4**).

In regenerative P1 hearts, there was strong CX40-eGFP expression in the part of the network closest to the injury site after 21 days (**Figure 4.4, panels k''-o''**). However, the VCS otherwise appeared morphologically similar to sham control and uninjured hearts, with apparent regeneration of full network architecture and proper bundling of fibres (**Figure 4.4k-o, and insert panels k'-o', k''-o''**). I concluded, therefore, that at a network level the VCS regenerates in the P1 neonatal heart following MI to reconstitute a network that has generally appropriate architecture, and does not show evidence of large gaps or major disruption.

4.3.4 A full connexin-40-positive His/Purkinje network is not restored following myocardial infarction in non-regenerative hearts

Given the general inability of the neonatal mouse heart to regenerate by P7⁴⁷, I hypothesised that following MI the P7 VCS would fail to regenerate to the same extent as the P1 VCS. To test this, I determined whether damage remains longer-term in the non-regenerative VCS. Following no surgery, MI surgery or sham surgery on P7 hearts, I therefore allowed 21 days for recovery before I again harvested the hearts and cut open the left ventricle to expose the His/Purkinje network (**Figure 4.5**).

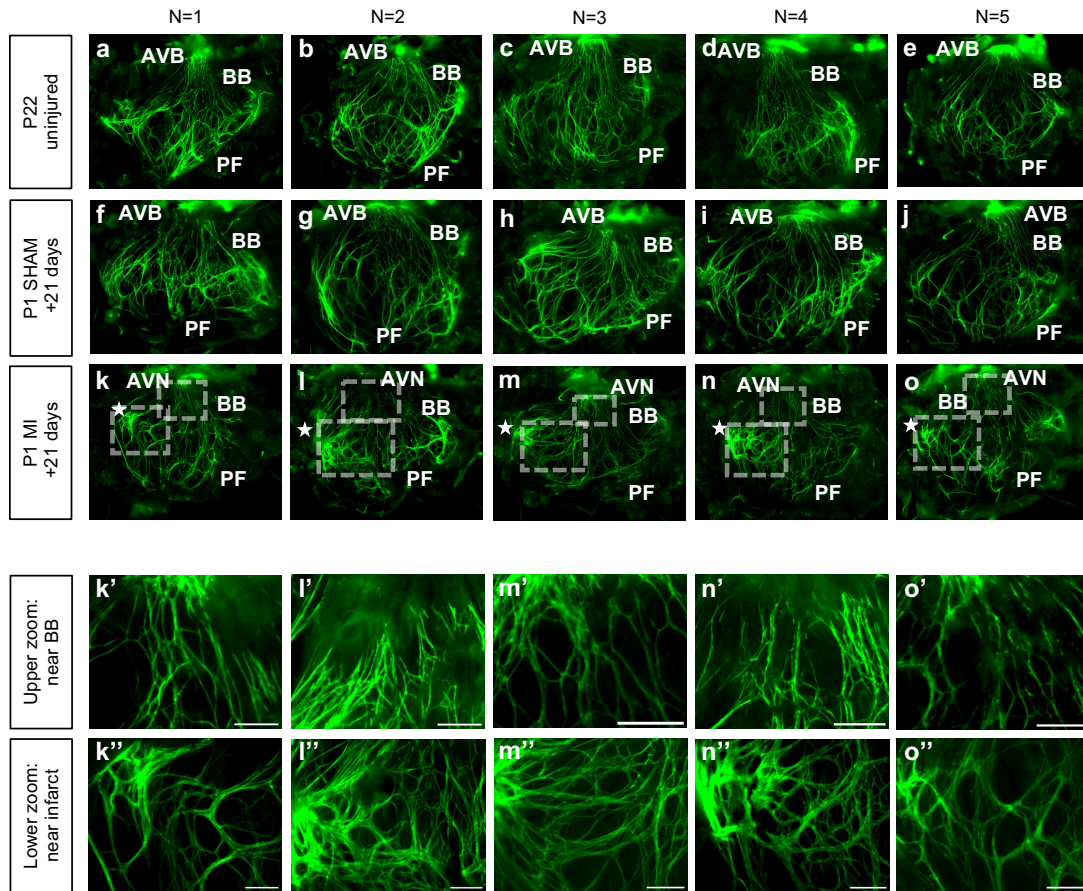


Figure 4.4: Reconstitution of a full CX40+ conduction network in the regenerative neonatal mouse heart

The dissected left ventricular His/Purkinje network of *Cx40eGFP/+* hearts that did not undergo surgery (**a-e**), hearts 21 days following P1 sham (**f-j**) surgery, or hearts 21 days following P1 MI surgery (**k-o**). Five representative hearts are shown in each case. Panels show magnified inserts from the boxed regions of the corresponding MI hearts. The P1 heart reconstitutes an apparently normal conduction network with bright CX40+ bundle branches (**k'-o'**) and full network coverage in the Purkinje fibre area underlying the infarct (**k''-o''**), but with especially bright CX40+ fibres still apparent nearest the LAD ligation region. Labels indicate atrioventricular bundle (AVB), bundle branches (BB), Purkinje fibres (PF). Scale bars: 100µm.

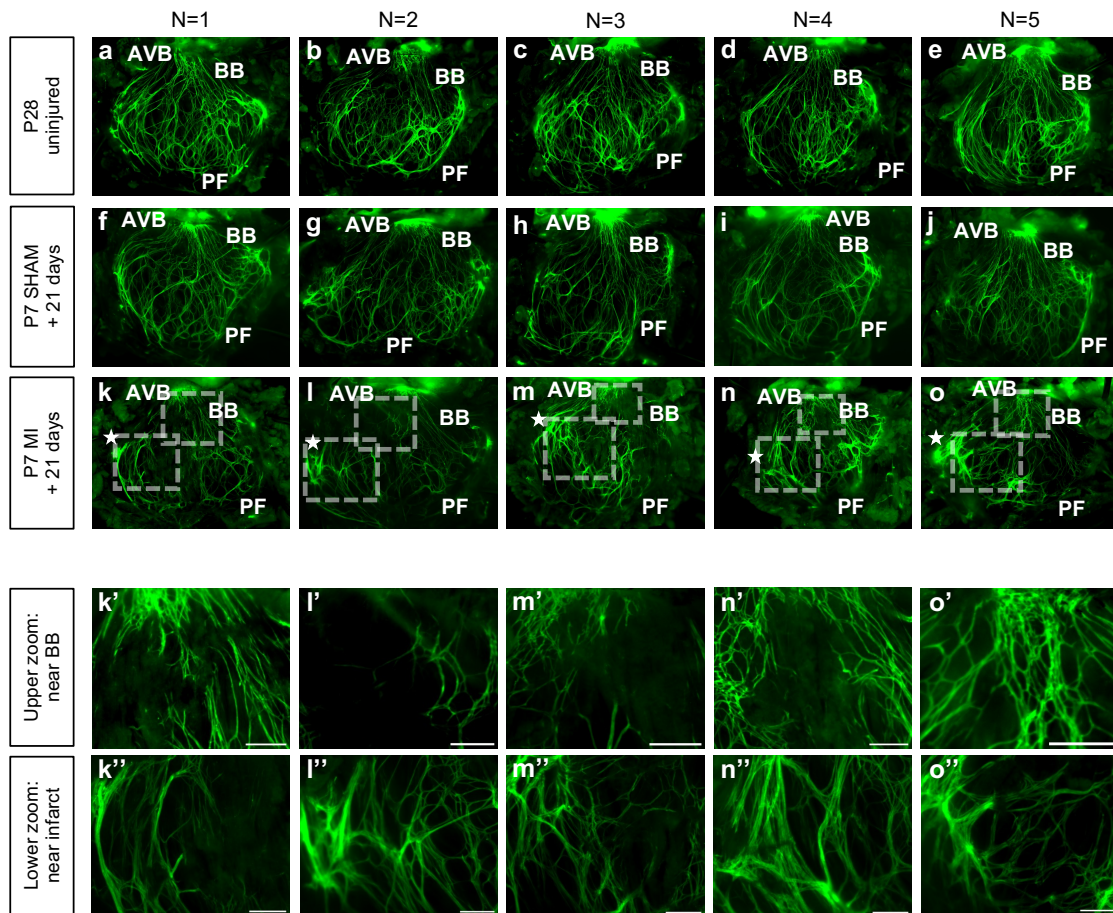


Figure 4.5: Large gaps in the CX40-eGFP+ bundle branches and Purkinje fibres of the conduction network are sustained in the non-regenerative infarcted mouse heart

The dissected left ventricular His/Purkinje network of Cx40eGFP/+ hearts that did not undergo surgery (**a-e**), hearts 21 days following P7 sham surgery (**f-j**), or hearts 21 days following P7 MI surgery (**k-o**). Five representative hearts are shown in each case. Panels show magnified inserts from the boxed regions of the corresponding MI hearts. The P7 heart fails to reconstitute a normal conduction network: there are large CX40-negative gaps in the bundle branches (**k'-o'**) and altered network bundling with gaps extending in some cases to the distal Purkinje region, including in the area underlying the infarct (**k''-o''**). Labels indicate atrioventricular bundle (AVB), bundle branches (BB), Purkinje fibres (PF). Scale bars: 100µm.

In the infarcted non-regenerative P7 hearts at 21 days there was increased CX40-eGFP expression nearest the injury site, as I had also seen in the P1 hearts 21 days after MI (**Figure 4.5, panels k''-o''**). Very strikingly, however, in the P7 infarcted network I identified broad gaps in CX40-eGFP expression which had not manifested in the infarcted P1 VCS and which were not present in sham control or uninjured hearts (**Figure 4.5k-o**). Surprisingly, this included loss of CX40-eGFP-positive signal in regions remote to the infarct area, incorporating the bundle branches (BBs) above the level of the regions of the network supplied by the ligated LAD artery (**Figure 4.5, panels k'-o'**).

Overall, these results revealed that the ability to reconstitute a full VCS is lost by the end of the neonatal regenerative window at P7. Moreover, the loss of CX40-eGFP signal across broad network regions including in BBs near the AVB indicated that damage to the electrical network following MI is not contained to the ischaemic region near the heart apex, but rather that disruption to the VCS includes long-term network alterations in the AVB and BB regions.

4.3.5 The His/Purkinje network remains intact but connexin-40 is downregulated regionally following myocardial infarction beyond the regenerative window

To determine whether this absence of signal in the VCS of P7 hearts at 21 days post-MI resulted from loss of Purkinje cells, or from downregulation of CX40 across the network, I fixed the infarcted hearts after dissection and performed wholemount immunostaining against contactin-2 (CNTN2), a well-established mature Purkinje cell marker¹⁰⁵. To this end, I designed an imaging set-up wherein the dissected surface of the heart's left ventricular inner-chamber would be pressed face-down onto an imaging chamber and weighted from above using an agarose block (**Chapter 2.4.4.2**). In this way, I was able to image the stained network in full, on an inverted confocal microscope. I could then cross-

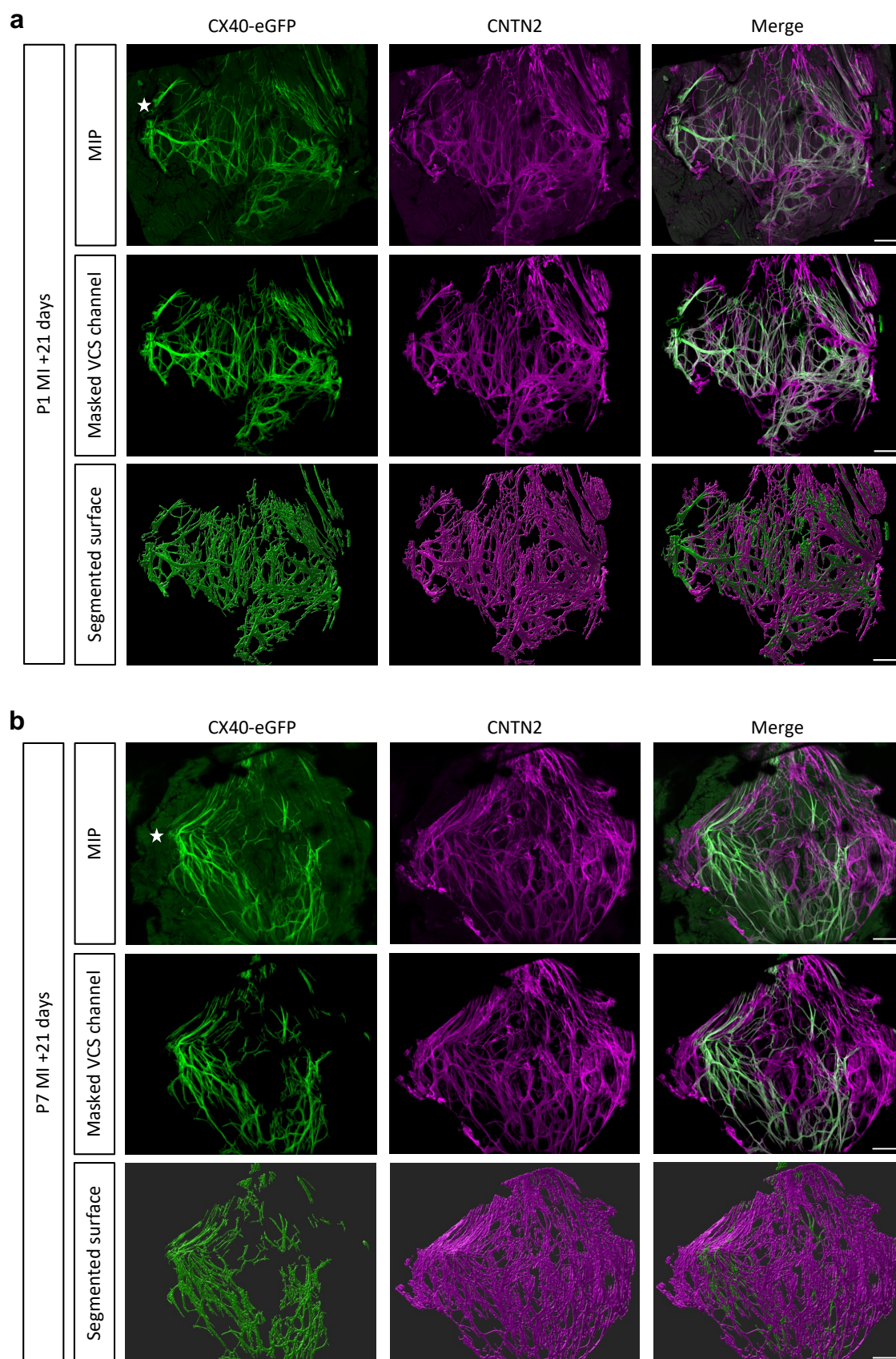


Figure 4.6: The fast-conductance gap junction channel Connexin-40 is downregulated regionally in the infarcted non-regenerative conduction network

The left ventricular His/Purkinje network of the *Cx40eGFP/+* heart 21 days following P1 MI surgery (a) or P7 MI surgery (b). Immunostaining against His/Purkinje marker contactin-2 (CNTN2) (magenta) revealed that the fibres of the network remained intact, but that CX40-eGFP was downregulated across patches remote from the LAD site in the non-regenerative P7 heart post-MI. Top to bottom: maximum intensity projection (MIP) of the immuno-stained network; His/Purkinje signal extracted from background fluorescence by segmentation to retain only the image voxels of the ventricular conduction system; 3D segmented surface model (built using Imaris 10.0 software). Stars indicate level of the LAD ligation. Scale bars: 500µm.

correlate these images with the original live image acquired pre-fixation to confirm that the protocol had not altered network morphology or CX40-eGFP expression.

This immunostaining analysis revealed that there was positive CNTN2 staining in fibres across all of the gaps in CX40-eGFP expression (**Figure 4.6**). This confirmed that VCS cells were not lost in the gaps identified in the P7 infarcted VCS network. Rather, taken together these results indicate that CX40 levels are greatly reduced in discrete regions of the network remote from the infarct area in the non-regenerative setting (**Figure 4.6b**) but are maintained in the regenerative setting (**Figure 4.6a**).

4.3.6 The pace-maker ion channel HCN4 is ectopically expressed in the infarct area after myocardial infarction and is dysregulated in non-regenerative hearts

I further wanted to ascertain whether differences in electrical remodelling between infarcted P1 versus infarcted P7 hearts extended beyond the VCS network to detectable changes in the surrounding myocardium. I, therefore, assessed the spatial expression of the pace-maker ion channel hyperpolarisation-activated cyclic nucleotide gated channel 4 (HCN4) in tissue-cleared regenerative P1 versus non-regenerative P7 infarcted hearts. HCN4 is normally expressed in the node, but has also been shown to be upregulated in left-ventricular working myocytes in rats post-MI^{181,182}.

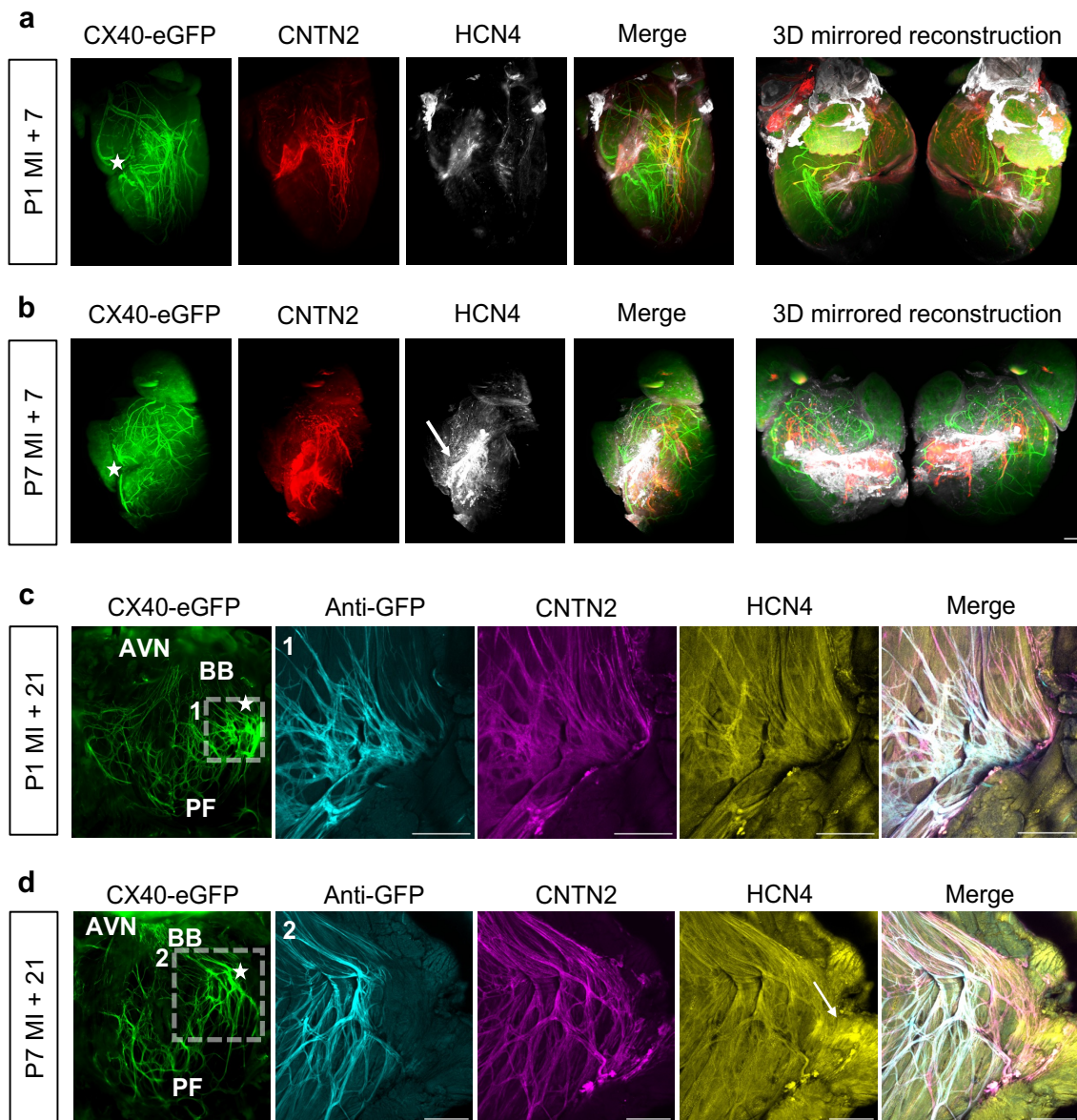


Figure 4.7: The pace-maker ion channel HCN4 is ectopically expressed in the infarct area after MI and is more dysregulated in non-regenerative hearts

Tissue clearing with immunostaining of wholemount *Cx40eGFP/+* hearts 7 days following MI or sham surgery at P1 (**a**) versus P7 (**b**) stages revealed upregulation of HCN4 in the infarct region which was particularly pronounced in the non-regenerative P7 heart. Hearts are shown in 4-chamber view (top) and in side-view (bottom). Stars indicate location of LAD ligation. **c-d**. Immunostaining on dissected left ventricular His/Purkinje network 21 days after MI injury. Antibody staining against GFP (cyan), VCS marker contactin-2 (CNTN2) (magenta) and pace-maker ion channel HCN4 (yellow) revealed upregulation of HCN4 in P7 (**d**) but not P1 (**c**) hearts adjacent to the VCS network nearest the site of LAD ligation. Stars indicate location of LAD ligation. Labels indicate atrioventricular node (AVN), bundle branches (BB), Purkinje fibres (PF). Arrows highlight regions of ectopic HCN4 expression. Scale bars: 500µm.

In P1 hearts 7 days post-MI, HCN4 signal was mainly restricted to the VCS network (**Figure 4.7a**), whereas in P7 MI hearts there was significant ectopic expression of HCN4 across the infarct area (**Figure 4.7b**). To ascertain the spatial relationship between distal Purkinje cells and HCN4 expression, I performed wholemount immunostaining on the dissected left ventricular His/Purkinje network. Ectopic HCN4 was evident in the infarcted myocardium adjacent to the Purkinje network nearest the LAD site in P7 but not P1 MI hearts 21 days after injury. Additionally, I identified CNTN2+ HCN4+ cells which were CX40-eGFP-negative, linking to the CX40-eGFP+ network (**Figure 4.7c-d**).

Overexpression of HCN4 and consequently of funny current (I_F) has previously been suggested to both destabilise resting potential in the ventricular myocardium of failing human hearts, and also to underlie ventricular arrhythmias in acute MI^{181,183}. My findings in Figure 4.7 reveal ectopic and significantly upregulated HCN4 in infarcted P7 hearts relative to the His/Purkinje network, consistent with an arrhythmogenic phenotype versus maintenance of lower level, discrete HCN4 expression in the P1 regenerative heart consistent with preserved normal sinus rhythm.

4.4 Discussion

In this Chapter, I assessed damage to the His/Purkinje system following MI in the neonatal mouse model of heart regeneration. Using the CX40-eGFP transgenic reporter mouse to visualise the VCS, I employed my tissue clearing and whole-organ imaging methodology (developed in Chapter 3) to image the VCS in infarcted neonatal hearts. In this way, I identified direct disruption to the His/Purkinje network at the level of the LAD ligation and discontinuous CX40 signal following MI in both regenerative and non-regenerative hearts. Through dissection of the left VCS, I further found that bundling of Purkinje fibres was disrupted in the immediate days following MI, with differences between P1 versus P7

hearts, including a loss of the characteristic thick conduction bundles in the non-regenerative P7 heart in place of a thinner, spindlier fibre arrangement.

By 3 weeks after injury, whereas the P1 heart reconstituted a full VCS network, in infarcted P7 hearts CX40 was downregulated in regions of the network including towards the top of the LBB, remote from the infarct. Moreover, sustained remodelling extended beyond the VCS: the HCN4 ion channel was greatly upregulated in the infarct area and near adjacent Purkinje fibres within non-regenerative infarcted hearts.

Collectively, therefore, the results of this Chapter show that proper His/Purkinje bundling is disrupted and the network is damaged following LAD MI. I found that the neonatal heart can broadly regenerate its electrical network to re-form a VCS with normal morphology. Contrastingly, the non-regenerative heart underwent pathological CX40 and ion channel remodelling. The molecular changes underpinning the different response of the electrical network in the regenerative P1 versus non-regenerative P7 heart will be the focus of Chapter 5. The implications of these changes for arrhythmogenesis will be addressed in Chapter 6.

4.4.1 Regeneration of a largely normal conduction system following myocardial infarction in neonatal mice

Since the finding that neonatal mammals are capable of full heart regeneration⁴⁷, significant progress has been made in elucidating the mechanisms by which cardiomyocytes proliferate and new vasculature forms during the regenerative window (recently reviewed in ^{184,185}). However, while there is associated recovery of ventricular function during neonatal heart regeneration¹⁸⁶, whether and how this translates to proper electrical and conductive maintenance or restoration had not previously been systematically investigated.

The findings presented in this Chapter identify reconstitution of an intact and generally normal His/Purkinje network by 21 days following MI in the regenerative P1 mouse heart, but

inability to regenerate a proper His/Purkinje system in the non-regenerative P7 mouse heart. The identification of a significant increase in CX40-eGFP expression across the electrical network below the level of the LAD ligation in P1 hearts in the days immediately following an MI event, but no equivalent broad CX40 upregulation in the non-regenerative P7 heart, is very interesting. This suggests that the regenerative response may be reinforcing the VCS rapid conduction transcriptional signature within the injured network, resulting in the different longer-term outcomes between P1 and P7 infarcted hearts by 21 days. It is also worth noting the observation that despite full network architecture appearing similar to sham and uninjured control hearts by 21 days following P1 surgery, a small area with particularly bright bundling remained evident in many hearts in the region of the network underlying the suture site. Whether or not this has any functional implications for the regenerating heart will be explored using an electrophysiological approach in Chapter 6.

A potential injury response previously reported in Purkinje cells after MI in the P1 mouse heart¹¹⁴, prompted the need to investigate VCS-specific pathophysiology with inclusion of morphological comparisons across both regenerative P1 versus non-regenerative P7 stages post-MI. This earlier study used a novel *Pitx2-Cre* allele to trace VCS cells and analyse these in histological sections; in this way, the authors suggested that there is a significant increase in Purkinje cell number following MI in the regenerative P1 mouse heart due to potential cardiomyocyte recruitment to Purkinje fate, however, they did not observe significant proliferation to explain such an increase in Purkinje cell number. While I found significant upregulation of CX40-eGFP within the P1 infarcted network (**Figure 4.3**), using wholemount analyses I did not find general hyperplasia in the P1 infarcted network. The previously identified increase in Purkinje cell number may perhaps relate to the particular Cre line used in this case; moreover, it is very challenging to quantify network architecture robustly in sections. Future lineage tracing studies, optimally combined with wholemount imaging will, therefore, be required to ascertain whether or not there is an overall increase in the number of mature Purkinje cells that form part of the conductive network following infarction in neonatal hearts.

Interestingly, in a separate study, better recovery of neonatal as compared to adult mice was found following genetic ablation of cells in the AVN, albeit in otherwise healthy animals. This finding suggested the possibility of latent neonatal CCS plasticity¹⁸⁷, although it is important to emphasise that there are differences both in ontogeny and physiology of the AVN versus the VCS (see **Chapter 1.1.2**). The morphological analyses of this Chapter have shown recovery of a full CX40+ VCS network following MI in neonatal mice, which provides the first evidence supporting the existence of latent neonatal His/Purkinje plasticity. This will be explored further through a full molecular characterisation of the neonatal VCS regenerative response in Chapter 5.

4.4.2 Normal and abnormal bundling of Purkinje fibres

Histological studies at the start of the 20th century initially established the existence of an insulative sheath surrounding CCS cells¹⁸⁸⁻¹⁹⁰. Indeed, these studies proposed that the presence of this fibrous sheath be used as a criterion for identifying conduction tissue¹⁹¹. Injection of Indian ink into the space between sheep Purkinje fibres and their connective tissue sheaths has since provided a powerful means to analyse bundle morphology and has confirmed the presence of the fibrous sheath around conductive bundles and Purkinje fibres throughout the CCS, through until the transitional cells linking to the working myocardium¹⁹². Unlike in ungulates where the connective sheath encapsulates 2-8 Purkinje cells, in human and rodent hearts thin fibrous tissue surrounds each individual Purkinje cell¹⁹³. Moreover, light and transmission electron microscopy studies have captured the organisation of the collagens and elastin fibres within the connective sheath at high resolution^{194,195}. While the main role of the sheath is in insulating the Purkinje fibres from adjacent myocardial cells, and so avoiding lateral spread of the electrical impulse, it has been suggested that the sheath may also provide mechanical protection from stress during the cardiac contraction cycles¹⁹².

The findings presented in this Chapter provide, to the best of my knowledge, the first evidence suggesting loss of normal VCS bundle architecture in the heart following MI. Given the importance of proper bundling within the fibrous sheath for insulated electrical impulse propagation, this loss is significant. The lack of organisation in the infarcted VCS network following MI at non-regenerative P7 stages that map onto the mature adult fibrotic response, suggests the replacement of thick conduction bundles with thinner spindly fibres may lead to slower and/or less synchronous conduction. Future studies could use MRI datasets from patients presenting following MI to assess whether this phenomenon of altered Purkinje fibre bundling also occurs in the human heart. Furthermore, electrophysiological follow-up to assess whether or not speed of conduction differs in the disorganised infarcted fibres as compared to the fibres of healthy hearts, will inform as to the clinical relevance of this observation.

Finally, it will be interesting to ascertain whether or not the altered bundling arrangement of the Purkinje fibres is influenced by changes in mechanical stress parameters in the infarcted heart. Following MI, changes to the contractile environment can lead to issues including reduced ejection fraction, and altered fluid shear stress and tissue stress. It is possible that the reason Purkinje cells have been found generally to be more resistant to MI damage than working myocardium^{31,196}, could in part be due to protection provided by the fibrous sheath. If so, disruption to the bundling arrangement could in turn affect longer-term Purkinje cell viability.

Overall, therefore, there are many outstanding unknowns regarding how Purkinje bundling is organised, and why it goes wrong in infarcted non-regenerative hearts. Given the importance of the connective tissue sheath for VCS function, the impact of changes to the VCS bundling architecture and how the neonatal heart restores proper fibre bundling, warrant further investigation.

4.4.3 Connexin remodelling in the conduction system of non-regenerative hearts

While it is well known that there is remodelling of the predominant connexin in cardiomyocytes (CX43) in the surviving myocardium of the border zone and that this has knock-on implications for arrhythmogenesis^{23,197}, previous studies have not investigated changes to the fast-conductance connexin (CX40) in the VCS following MI. In this Chapter, through analysis of the left ventricular conductive network in the *Cx40-eGFP/+* reporter line, I identified CX40 downregulation and VCS remodelling in broad regions of the His/Purkinje network of non-regenerative P7 hearts post-MI. Indeed, while CX40 expression appeared largely normal in the BBs of the P7 VCS in the days immediately following MI (**Figure 4.2**), surprisingly I found that by 21 days following P7 MI CX40 expression was almost entirely absent in large regions of the network (**Figure 4.5**). Most interestingly, this CX40 loss was often remote from the infarct area, in the BBs and proximal part of the Purkinje network above the level of the site of LAD ligation. Given the known importance of CX40 expression in order to ensure rapid conduction through the VCS, it seems likely that loss of CX40 across such large areas within the LBB of the acute chronic MI heart would result in delayed conduction of the electrical impulse through these regions, with probable consequences for the heart's ability to maintain synchronous cardiac contraction.

Findings by qPCR and western blot experiments of reduced total CX40 mRNA/protein levels in Purkinje fibres in the dog¹⁹⁸ and in left ventricular fibres of a rabbit congestive heart failure model¹⁹⁹ provide support for this being a conserved phenomenon in the non-regenerative stressed VCS. However, to the best of my knowledge the results of this Chapter provide the first indication that there is spatially dysregulated conductivity including down-regulation of CX40 in the His/Purkinje network distal from the infarct. This provides a potential molecular mechanism, which is distinct from scar-related re-entry, remodelling known to occur locally to the infarct/border zone or direct ischaemic damage to the BBs, to

explain BBB and the increased dyssynchrony of ventricular contraction that presents following MI.

Going forwards, it will be important to understand how remodelling of the VCS remote from the ischaemic zone takes place. It is possible that this is mediated by inflammation: increasing evidence indicates that inflammatory cytokines can down-regulate expression of CX40 and CX43, resulting in impaired cell-cell coupling, reduced conduction velocity and enhanced susceptibility to arrhythmia²⁰⁰. Moreover, it is known that healthy myocardial regions undergo electrical and structural remodelling in infarcted hearts when adapting to increased workload due to poorly contractile scar²⁰¹. It may be, therefore, that this CX40 remodelling in the VCS is a consequence of the heart adapting to a new electromechanical environment in which the heart must struggle to maintain sufficient ejection fraction to support bodily functions despite having a large anteroseptal area of ventricle that has lost its contractile ability.

It is interesting to note that CNTN2 expression remained uniform throughout the VCS network following MI despite extensive connexin remodelling. CNTN2 is not just a VCS marker, but also has well-established neuronal roles both at the Node of Ranvier (by localising voltage-gated potassium channels to the juxtaparanodal axonal region) and in modulating myelination by oligodendrocytes and rate of axonal conduction^{202,203}. Presumably, in the cardiac context CNTN2 may play important roles in controlling the rate of electrical impulse propagation through the VCS. It seems, therefore, that specific subsets of conduction-related factors (notably including CX40) are down-regulated, but that the affected cells remain intact and retain some mature Purkinje-like properties. It is interesting to note that *Nkx2-5* conditional deletion in the VCS results in loss of CX40, but in this context fibres also remain CNTN2-positive¹³⁹. In light of this, in the following Chapter I will explore global gene expression changes in the VCS of regenerative and non-regenerative hearts following MI. In this way, I will aim to elucidate the regulatory networks influencing which specific gene sets are pathologically remodelled in the non-regenerative heart.

The results of this Chapter demonstrate dramatic loss of CX40-eGFP expression in regions of the infarcted P7 VCS network. Going forwards, it will be important to further ascertain whether, in addition to the overall loss of CX40, there are changes across the network in sub-cellular distribution and/or the open versus closed state of CX40-containing channels. Indeed, it is known that ischaemia can cause CX43 channel state to change in cardiomyocytes near the infarct, and that CX43 remodels to the lateral side of the intercalated discs of border zone myocytes²⁰⁴. Whether channel opening is impacted outside of the ischaemic/border zone area has not previously been assessed, but warrants investigation. Super-resolution fluorescence or electron-microscopy imaging of the gap junctional organisation of VCS cells at different levels of the network (HB, BB and Purkinje fibres) would be informative in order to determine the precise molecular remodelling and channel localisation following MI in the non-regenerative context. Alongside this, it will be interesting to analyse whether expression of the other connexin sub-types changes. Given regional loss of CX40, it is possible that the slower-conductance CX43, the major cardiomyocyte connexin which is also expressed in VCS and especially in transitional cells linking Purkinje cells to working myocardium, becomes the predominant connexin in the affected BB and Purkinje areas. Overall, therefore, many outstanding questions remain regarding connexin distribution, activity state and sub-cellular localisation in the conduction system following MI.

4.5 Conclusions

The results of this Chapter demonstrate that the neonatal mouse heart can reconstitute a full CX40+ His/Purkinje network by 21 days following an MI event. Contrastingly, the P7 non-regenerative mouse heart failed to regenerate a normal VCS. In non-regenerative hearts, I identified down-regulation of the fast-conductance CX40 in regions of the VCS including in the BBs remote from the ischaemic area. Moreover, damage extended to global disruption to the fibrous bundling architecture of the BBs and Purkinje fibres in infarcted non-regenerative hearts. Finally, I found that the pace-maker ion channel HCN4 is ectopically expressed in the infarct area after MI in non-regenerative P7 hearts but maintained at lower levels in the regenerative P1 heart. Collectively, these findings are indicative of preserved normal sinus rhythm during heart regeneration, versus arrhythmogenesis beyond the regenerative window.

5 Elucidation of the molecular pathways associated with conduction system response to injury, (re-) growth and function

5.1 Background

5.1.1 Single nuclear and single cell RNA sequencing in the context of heart regeneration

Advances in next generation, high-throughput RNA sequencing (RNA-seq) technology have led to the development of bulk RNA-seq, and more recently single cell RNA-seq (scRNA-seq) and single nuclear RNA-seq (snRNA-seq) methods, that can delineate gene expression profiles across cell populations with increasing depth. The power of performing global gene expression analysis with single-cell resolution, is that this approach retains heterogeneity of cell states. By avoiding the averaging methods involved in bulk analysis, scRNA-seq and snRNA-seq thereby shed light on cellular sub-types and have allowed for granular analysis of cellular fate, heterogeneity and behaviour. This is of particular importance in the context of injury and regeneration, where factors including proximity to the injury site, extent of hypoxia and level of cell stress drive extensive variation in gene expression between neighbouring cells within a population.

Applying snRNA-seq and scRNA-seq to study cardiovascular biology has revolutionised our understanding of cardiac cell types in development and disease. However, the success of this approach has relied upon the design of appropriate workflows

to digest and dissociate hearts in order to retain viable cells. Furthermore, since a cell count over 10,000 viable cells is desirable for microfluidic scRNA-seq approaches, it has been more challenging to isolate and fully characterise rare cell types in the heart. To achieve successful cell isolation, careful consideration of parameters including size and morphology of the cell type(s) being isolated, and biological characteristics of the tissue being digested (e.g., extent of fibrous content) is required²⁰⁵. Certain cell types such as cardiomyocytes and adipocytes have proved particularly challenging to dissociate and capture using scRNA-seq methods; profiling by snRNA-seq is generally preferable where analysis of these cell types is desired²⁰⁶.

Recent applications of snRNA-seq and scRNA-seq to explore the gene networks underpinning heart regeneration have informed our understanding of the molecular determinants and gene programmes driving regeneration versus fibrotic repair. SnRNA-seq analysis on neonatal mouse hearts (enriched for cardiomyocytes) following MI or sham surgery at regenerative P1 versus non-regenerative P8 stages identified an immature population of cardiomyocytes in the P1 heart that enters the cell cycle after MI, but is lost in the P8 heart beyond the regenerative window¹. The authors further established the gene regulatory network supporting P1 cardiomyocyte regeneration, and found candidate protective (NFE2L1) and proliferative (NFY α) factors that are activated in response to injury. Moreover, scRNA-seq analysis on neonatal mouse hearts at P1 and P8 stages following MI or sham surgeries, elucidated the gene expression landscape of non-cardiomyocyte cell types including fibroblast, immune, endothelial, epicardial, glial, smooth muscle and pericyte cells⁷⁵.

Together, these studies have provided valuable datasets that capture most cardiac cell types during neonatal mouse heart regeneration. Complementary analyses have also been performed in the adult zebrafish, which can fully regenerate its heart following apical resection or cryoinjury⁴³. In particular, a study using the cryoinjury model to assess cardiomyocyte response during heart regeneration elucidated the transcriptomic signatures of the regenerating border zone cardiomyocytes, and implicated Erbb2 signalling in inducing

cardiomyocyte proliferation following injury²⁰⁷. Other studies have focused efforts on describing the involvement of epicardial-derived populations and activated fibroblast states in zebrafish heart regeneration²⁰⁸⁻²¹⁰. Overall, therefore, snRNA-seq and scRNA-seq studies have provided wide-ranging insight into the injury-responsive genetic networks of cardiomyocyte, epicardial, fibroblast and immune cells underpinning heart regeneration versus fibrotic repair.

5.1.2 Gene expression in the ventricular conduction system

Despite decades of extensive research on the CCS, our understanding of VCS gene expression remains relatively limited. However, over recent years mouse genetics coupled with microarray, bulk RNA-seq and scRNA-seq analyses, have begun to enhance knowledge of the molecular profile of the VCS (recently reviewed in ^{4,136,211}).

VCS gene expression analyses have relied heavily on transgenic reporter mice, including the Cx40-EGFP⁶⁸, Cntn2-EGFP¹⁰⁵ and CCS-LacZ²¹² lines, in order to enrich for VCS cells. While these reporter lines have proved invaluable, few are specific to the VCS alone (see **Chapter 3, Table 3.1**). Nevertheless, they have facilitated an increasingly refined understanding of the transcriptional regulatory network underpinning VCS development and function (**Figure 5.1**). Microarray and bulk RNA-seq analyses, including on micro-dissected¹⁰⁵ and FACS-sorted¹⁰³ Cntn2-EGFP+ Purkinje cells, and on laser capture micro-dissected Pitx2-CreERT2+ cells¹¹⁴, have identified many genes broadly upregulated in VCS versus working myocardial cells. The best-known genes upregulated in the VCS now include: genes for fast conduction (including *Gja5* (encoding CX40), *Scn5a*); genes encoding canonical ion channels (including *Hcn4*, *Kcnj3*); and genes with well-characterised roles in neuronal cell types (including *Cntn2*, *Pcp4*, *Sema3a*).

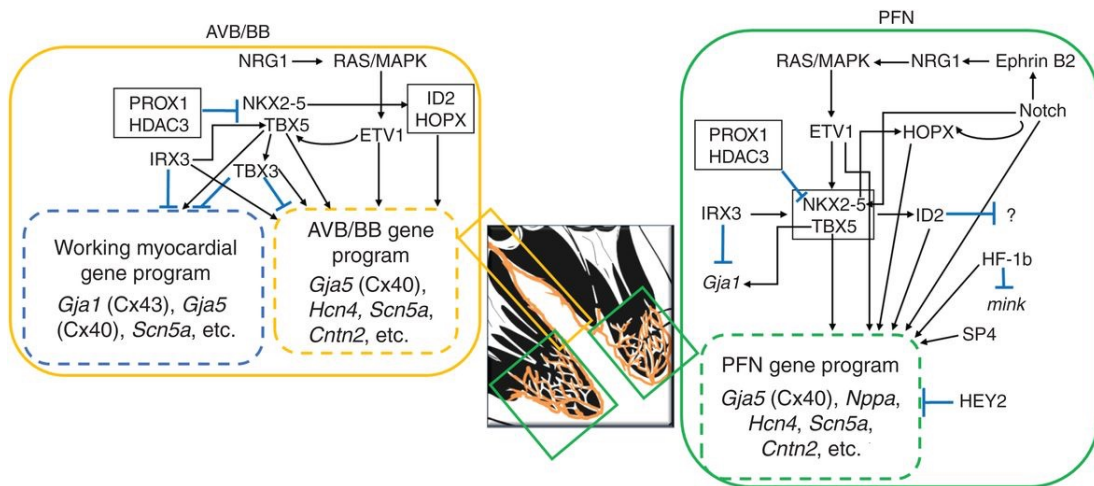


Figure 5.1: The transcription factor and gene networks underpinning ventricular conduction system development

Orange box: the signalling networks for atrioventricular bundle/bundle branch (AVB/BB) specification. Green box: the signalling networks for Purkinje fibre network (PFN) specification. Blue dotted box: the gene programme that shuts down in the AVB/BB. Orange dotted box: the gene programme that is activated for maturation of the AVB/BB. Green dotted box: the gene programme that is activated for maturation of the PFN. Black arrows indicate gene activation, blue lines with blunt ends indicate transcription factor repression of gene expression. *Figure reproduced with permission from Bhattacharyya and Munshi, 2020², copyright of Cold Spring Harbour Laboratory Press.*

Although a number of studies have profiled VCS gene expression through bulk approaches, to date few analyses have interrogated VCS gene expression at single-cell resolution. Notably, in the first scRNA-seq analysis to isolate and characterise cells of the CCS, Goodyer et al. identified a host of novel markers unique to CCS sub-populations by profiling of the micro-dissected E16.5 mouse conduction system⁷⁶. This study identified previously overlooked heterogeneity in the developing CCS both between and within CCS structures, including within the His/Purkinje compartment. Furthermore, a recent spatial multiomic profiling of the human CCS, which relied on “targeted” dissection and histological landmarks for CCS identification, provided the first spatially-resolved single-cell insights into human CCS gene expression²¹³. Collectively, these studies hint at divergence between cells

in the VCS, and consequently the need for future studies resolving VCS gene expression at the level of the single cell. However, to date no enriched scRNA-seq analysis has been performed on either post-natal or adult VCS sub-populations in the mouse heart.

Our limited knowledge of VCS cellular heterogeneity, and the relative paucity of scRNA-seq studies, results primarily from inherent difficulties in isolating and studying these cell types. The low cell number, difficulty in distinguishing and separating VCS cells from surrounding working myocardium, extent of cellular heterogeneity, and unusual cellular morphology, all present substantial hurdles to VCS molecular profiling. In this Chapter, I therefore aimed to advance our ability to isolate and analyse cells of the post-natal murine VCS using scRNA-seq approaches. Given the morphological disruption that I identified in the VCS following MI in Chapter 4 with differences between P1 and P7 infarcted neonatal mouse hearts, our limited understanding of how the CCS responds to injury, and my particular interest in determining the extent to which the CCS can regenerate, my ultimate goal was then to apply these tools to analyse VCS molecular heterogeneity across the regenerating and non-regenerating conduction system following MI.

5.2 Aims and Objectives

The aims of this chapter were to:

1. Establish the single-cell gene expression profile and extent of heterogeneity in the mouse ventricular conduction system across post-natal stages
2. Identify novel markers for the ventricular conduction system
3. Define the transcriptional signature of the regenerating and non-regenerating ventricular conduction system following myocardial infarction
4. Establish whether there are significant changes in conduction cell subtype composition and/or gene expression between the regenerating versus non-regenerating neonatal mouse heart after myocardial infarction
5. Determine whether there are sex-linked differences in the response of the ventricular conduction system to myocardial infarction

5.3 Results

5.3.1 Transcriptomic identification of a ventricular conduction system cluster in a cardiomyocyte-enriched single-nuclear RNA-sequencing dataset

The cardiac conduction system, and especially the VCS, have historically been challenging to analyse at a molecular level. This is due to low CCS cell number, extensive heterogeneity across cell types within the CCS, and difficulties in dissociating hearts so as to extract sufficient numbers of viable conduction cells for RNA sequencing analysis. I, therefore, determined firstly to characterise the post-natal VCS at single-cell resolution. Secondly, given the morphological disruption identified in Chapter 4 in the His/Purkinje network following MI, and the differences in response between P1 versus P7, I wanted to determine whether there is a loss of specific subsets of conduction cells within the network and/or changes in the VCS transcriptional signature across the regenerative to non-regenerative transition.

Since the VCS shares its developmental origins with working cardiomyocytes, I hypothesised that some VCS cells would have been captured alongside cardiomyocytes in cardiomyocyte-enriched RNA-seq datasets. Consequently, I began by analysing the previously published cardiomyocyte-enriched snRNA-seq dataset from P1 and P8 neonatal mouse hearts 1- and 3- days after MI or sham surgery¹, to extract Purkinje cells computationally from within the dataset (**Figure 5.2**).

Following high-resolution re-clustering of the cardiomyocyte nuclei in the dataset using Seurat (version 4.3.0), I found that a single cluster, cluster 13 contained known VCS markers amongst its 5 most highly-conserved genes, including *Etv1* (ETS transcription factor)⁴⁰ and *Slco3a1* (the CCS-lacZ locus)²¹². The other main VCS marker genes from the published (E16.5) VCS-enriched scRNA-seq dataset⁷⁶ were also all upregulated in cluster 13 (**Figure 5.2b**). Furthermore, assigning a VCS gene module score based on expression of 7 canonical VCS markers using the UCell package found considerably higher expression in

cluster 13 than in other cardiomyocyte clusters, indicating that this is indeed a VCS population (**Figure 5.2c**).

By performing Seurat differential expression analysis between cluster 13 and the rest of the cardiomyocytes, I identified both known and novel genes specific to the VCS (**Supplementary Table 1**). Known and novel markers identified included: *Etv1*, *Gja5* (CX40), *Kcnj3* (inwardly rectifying potassium channel member) (**Figure 5.2d**, known); *Grip1* (neuronal scaffold protein), *Meg3* (long non-coding RNA), *Vcan* (Versican, extracellular matrix component) (**Figure 5.2e**, novel). Of the five most differentially expressed genes, three have previously been implicated in the VCS (*Cpne5*, *Etv1*, *Cacna2d2*), but the remaining two genes, *Robo1* and *Slit3*, although well-known for their roles in axon-guidance, and in early cardiac development^{214,215}, were not known to be expressed in the CCS prior to this analysis. To validate my snRNA-seq analysis, therefore, I performed immunostaining against ROBO1 and SLIT3 on sections from P2, P4 and P8 *Cx40-eGFP/+* hearts. ROBO1 and SLIT3 expression was highest in the CX40-eGFP-positive conduction cells (**Figure 5.3**). Consequently, this analysis confirmed that both *Robo1* and *Slit3* are novel markers of the VCS.

Next, I separated the datasets by sample type and performed Seurat differential expression analysis. I did this within cluster 13, between regenerative P1 versus non-regenerative P8 cells, and between injured versus sham conditions in each respective group. The top differentially expressed genes were analysed. However, I found that genes differentially expressed within cluster 13 between condition types were either also differentially expressed across the wider cardiomyocyte cluster (i.e., involved in a general cardiomyocyte as opposed to a VCS-specific response), or were not injury- or stage- specific on cross-comparison between conditions.

The lack of a distinctive VCS injury or regenerative (P1 v P8) signature in this dataset is most likely to be a function of the low number of cells recovered in cluster 13: there were only between 11 and 37 cells per condition (**Supplementary Table 2**). Overall, therefore, while re-analysis of the snRNA-seq dataset identified a suite of novel VCS markers (**Figure**

5.2e and **Supplementary Table 1**), the low number of VCS cells captured in this dataset provided limited power for analysing Purkinje-specific cellular responses to MI.

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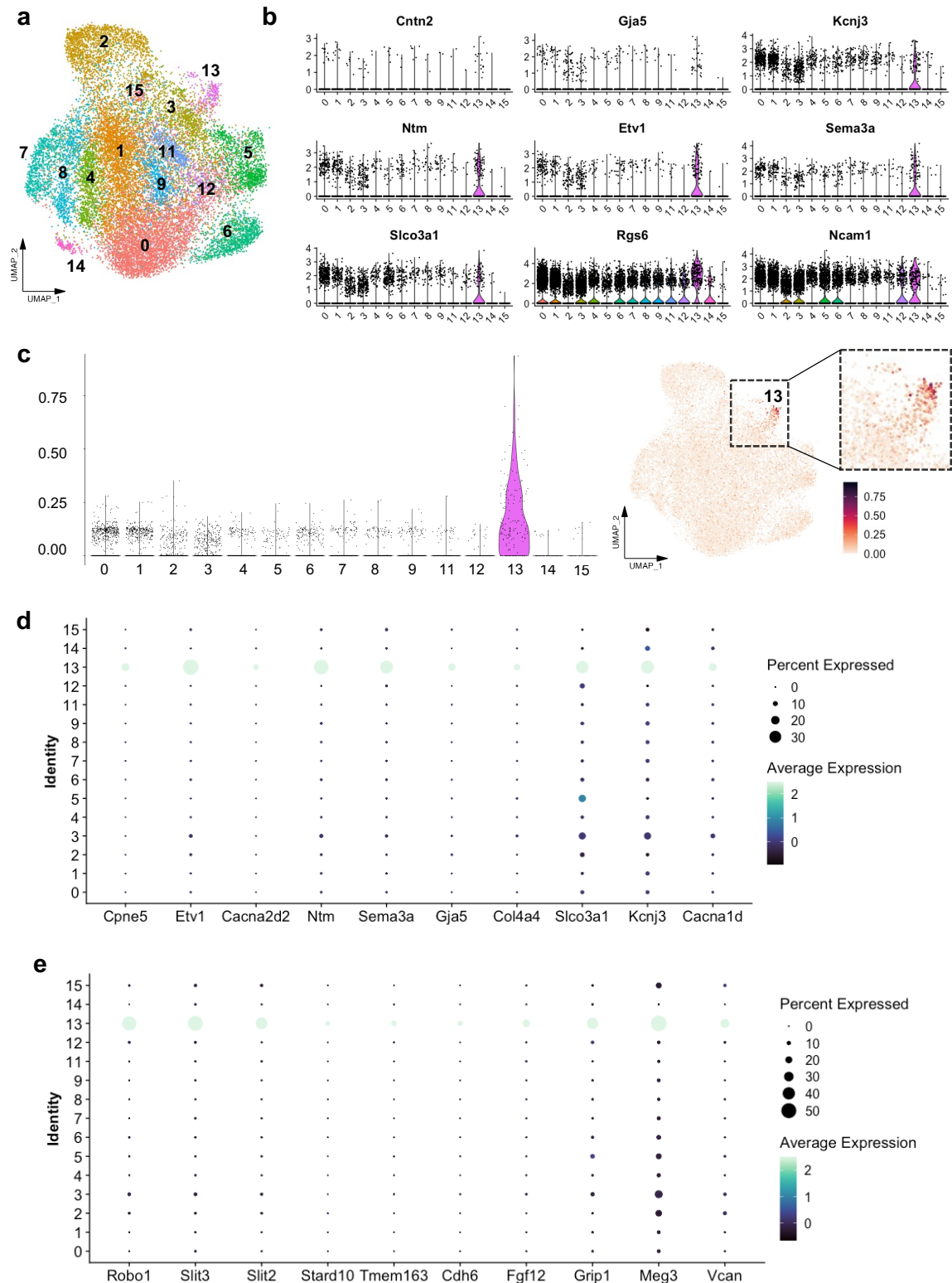


Figure 5.2: Identification and analysis of a Purkinje population in cardiomyocyte-enriched single-nuclear RNA sequencing

a. Uniform Manifold Approximation and Projection (UMAP) visualisation of cardiomyocyte nuclei following re-clustering. **b.** Violin plots show that the ventricular conduction system (VCS) marker expression is upregulated in cluster 13 of the re-clustered dataset. **c.** Violin and UMAP plots of UCell VCS gene set module expression shows upregulation in cluster 13. **d-e.** Dot-plots of gene expression find cluster 13-specific upregulation of known (**d**) and novel (**e**) VCS markers.

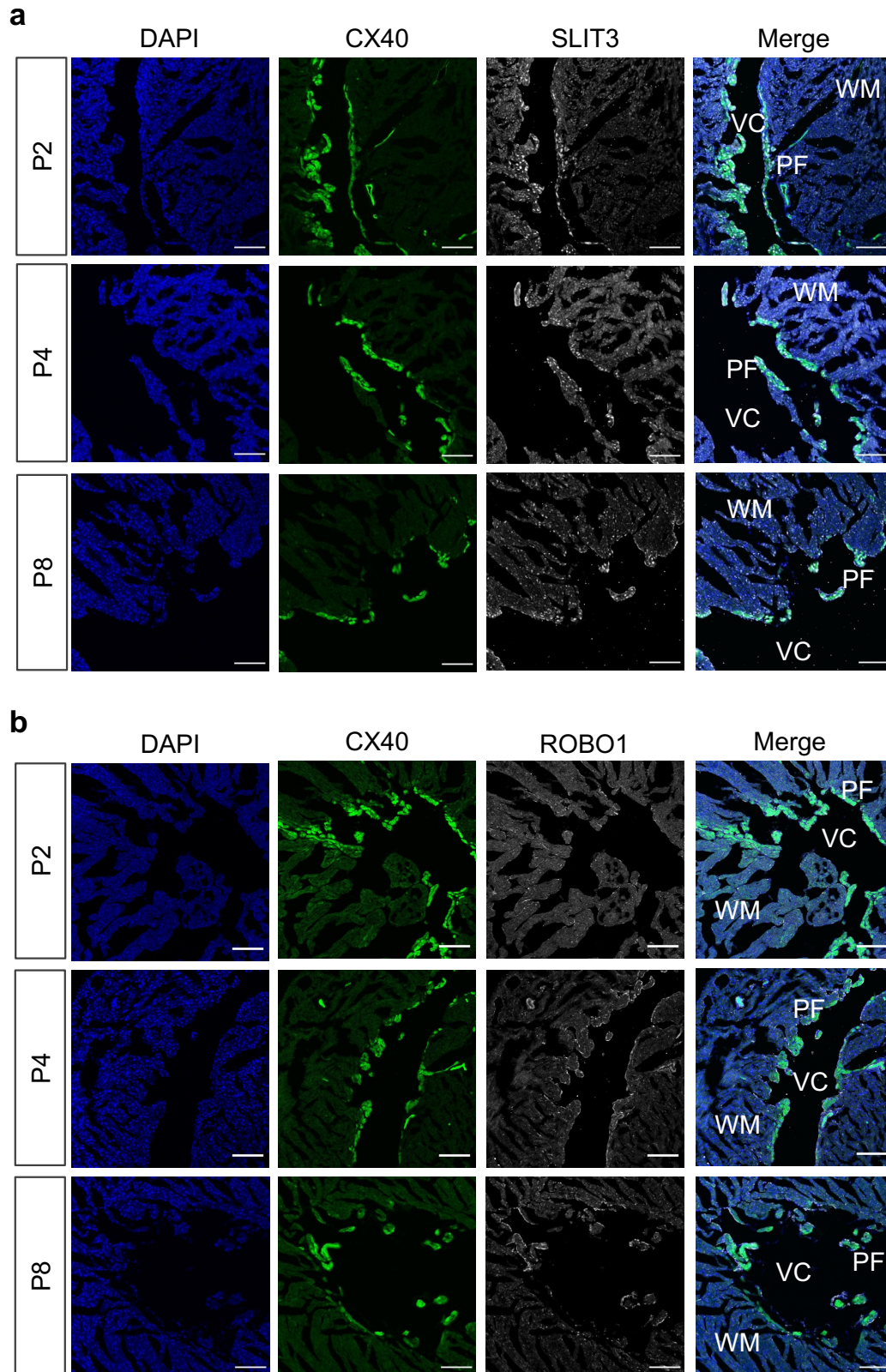


Figure 5.3: SLIT3 and ROBO1 are expressed in the post-natal ventricular conduction system Cryosection immunostaining across post-natal day 2 (P2), post-natal day 4 (P4) and post-natal day 8 (P8) Cx40eGFP/+ hearts. Expression of both SLIT3 (a) and ROBO1 (b) overlap with the CX40-eGFP-positive Purkinje cells. Scale bars: 100µm. Labels indicate Purkinje fibres (PF), ventricular chamber (VC), working myocardium (WM).

5.3.2 Enriched single-cell RNA-sequencing of the neonatal ventricular conduction system following myocardial infarction

5.3.2.1 *Experimental design to isolate and transcriptionally analyse cells of the post-natal ventricular conduction system*

Given the morphological disruption identified in the His/Purkinje network following MI and the differences in response between regenerative P1 versus non-regenerative P7 hearts (**Chapter 4**), I set out to determine whether certain groups of conduction cells within the network are lost following injury and/or whether the molecular response to myocardial infarction differs between P1 and P7 hearts. Given the low number of VCS cells captured in the published cardiomyocyte-enriched snRNA-seq dataset (**Supplementary Table 1**), however, I designed my own RNA-seq experiment in order to do this.

Cardiomyocytes are a notoriously difficult cell type to extract, keep alive and analyse using either FACS or microfluidic-based scRNA-seq approaches due to their size, morphology and metabolic profile. Since the VCS cells share their origins with the working cardiomyocytes, they too represent a challenging population to characterise molecularly. Indeed, to date the only scRNA-seq study to characterise mouse VCS cells focused on E16.5, and so there is no published protocol for viable mouse VCS cell dissociation appropriate for RNA-seq; there have been no previous single-cell studies on the post-natal VCS. Moreover, the VCS cells are a relatively rare population in the heart. I planned, therefore, to enrich for VCS cells through fluorescence-activated cell sorting (FACS) for CX40-eGFP-positive cells, and then to perform RNA-seq analysis.

Importantly, while I identified regional down-regulation of CX40-eGFP across significant areas of the infarcted P7 VCS network at 21 days post-surgery (**Chapter 4, Figure 4.5**), at the earlier time-point of 3 days after surgery CX40-eGFP expression was still present across the full extent of the VCS network (**Chapter 4, Figure 4.2**). Furthermore, the time-point of 3 days after MI captures relatively early molecular changes, concomitant with a

full cardiomyocyte regenerative response in P1 compared to P7 hearts, and that might define longer term VCS restoration or remodelling. Enrichment for CX40-eGFP-positive cells at this early stage following injury, therefore, represented a promising means by which to capture regenerating and non-regenerating VCS cells from across the HB, BBs and Purkinje fibres. However, while prior studies (including the datasets re-analysed above) have often relied upon snRNA-seq of cardiomyocytes to circumvent the aforementioned challenges associated with cardiomyocyte RNA-seq, when I analysed cryosections from *Cx40-eGFP/+* post-natal hearts using high resolution 60X microscopy, I found that GFP expression was cytoplasmic but not nuclear. In this case, therefore, I needed to use FACS to sort for GFP+ cells, not extracted nuclei. I was also keen to proceed using scRNA-seq rather than snRNA-seq thanks to the richness of the data that results from retaining cytoplasmic mRNAs in scRNA-seq approaches. Indeed, in a direct comparison between snRNA-seq and scRNA-seq using cortical tissue, it was found that the number of gene transcripts detected was elevated from approximately 7,000 per nucleus to 11,000 per cell, and that the diversity of retained transcripts was higher with single cells than with single nuclei²¹⁶. I decided, therefore, to proceed with optimisation of sample preparation for CX40-eGFP-enriched scRNA-seq.

It was first necessary to design a method to dissociate post-natal hearts into viable single cell suspensions and to sort for CX40-eGFP+ VCS cells. I trialled two approaches for neonatal heart dissociation to accomplish this. First, I minced the heart manually using a scalpel blade before processing the heart through enzymatic digestion with Type II collagenase in a 37°C orbital shaker, as had previously been done in house in the Riley group. Secondly, I used the Miltenyi GentleMACS Dissociator with heaters to perform controlled mechanical and enzymatic digestion according to manufacturer's instructions for neonatal hearts. The second approach resulted in much higher GFP+ cell viability both using a cell Countess to visualise proportion of live cells versus debris, and in FACS analysis using a Zombie live/dead stain. As a result, I proceeded to optimise this second approach over multiple dissociation and FACS experiments. In this way, I designed a dissociation, staining

and FACS protocol capable of isolating c. 5,000 GFP+ viable cells from each P4 heart (equivalent to P1 surgery + 3 days), and c. 2,500 GFP+ viable cells from each P10 heart (equivalent to P7 surgery + 3 days) (**Chapter 2.5**). A primary reason for the lower number of P10 cells extracted in this way was lower cell viability following dissociation: viability dropped from over 90% for cells collected from P4 hearts, to between 40-60% for cells collected from P10 hearts, even following optimisation rounds. This was probably on account of the increased fibrous content of the P10 tissue, and due to the larger size and elongated morphology of the more mature VCS cells by P10.

Finally, I also optimised a lipid-labelling cell multiplexing strategy to enable tagging of individual hearts on a cell-by-cell basis (**Chapter 2.6.1**). This was done for several reasons. Firstly, this strategy allowed samples to be pooled prior to processing through 10X GEM bead capture, cDNA amplification, library preparation and sequencing, thereby removing batch effects between lanes on the 10X chip. Secondly, this meant that each cell in the dataset was barcoded according to its heart of origin and so could be grouped with other cells deriving from the same individual heart. Thirdly, this approach empowered statistical analysis of the resulting datasets. Finally, in the context of using the neonatal LAD surgical model this provided a powerful means by which to ascertain extent of variability between injury samples and ensure any anomalous individuals could be excluded in post-processing prior to analysis. The choice to use the 10X microfluidic droplet method over plate-based techniques reflected a desire to sequence a larger number of cells. Using 10X with cell multiplexing, I was able to sequence tens of thousands of cells, whereas plate-based methods only capture between 100 and 1,000 cells per experiment²¹⁷.

Following optimisations as above, I performed scRNA-seq on injured P1 and P7 hearts, enriching through FACS for CX40-eGFP-positive VCS cells, to determine the transcriptional signature of the regenerating VCS. Given that CX40 is also expressed in the atria and coronary arteries, I dissected off the atria and excluded endothelial cells using negative selection for CD31 (**Figure 5.4**). Hearts were harvested for sequencing 3 days following MI or sham surgery, to coincide with the time-point at which I identified clear

morphological changes in the VCS (**Chapter 4.3.1-4.3.2, Figures 4.1-4.3**), and the stage known to coincide with the peak of the proliferative cardiomyocyte regenerative response¹.

I first completed a pilot scRNA-seq experiment, in which I dissociated 4 P1 and 3 P7 *CX40-eGFP/+* hearts, 3 days after MI surgery. Following dissociation, staining, lipid cell multiplexing oligo (CMO) labelling and pooled FACS to separate GFP+ CD31- live cells, I isolated 47,322 cells for 10X scRNA-seq. However, during the pilot 10X chromium run, the 10X machine encountered an electronic fault due to broken sensors and terminated the run early. The machine ran the samples to completion upon re-starting the run with the faulty sensors still in place. Following sequencing of the resulting libraries, I was able to determine that VCS cells had successfully been collected and to perform preliminary clustering and gene expression analyses, validating the enrichment strategy. The machine error resulted in very high background levels in the cell multiplexing libraries, however, such that I was unable to de-multiplex the samples to determine which were from P1 versus P7 hearts, or indeed which individual each cell derived from. As a result, I determined that the dataset was unreliable for more extensive analysis and so did not use these results to draw further conclusions.

With my optimised protocol for neonatal heart dissociation, staining, cell multiplexing and VCS cell sorting in-hand (**Chapters 2.5 and 2.6.1**), I proceeded to perform the main scRNA-seq experiment to transcriptionally characterise the regenerating and non-regenerating VCS (**Figure 5.4**). I dissociated 8 P1 MI, 6 P1 sham, 5 P7 MI and 5 P7 sham hearts, 3 days following surgery. At both P1 and P7 timepoints, littermate controls from *Cx40-eGFP* homozygous males (BL6 background) crossed with CD1 females were used for the MI and sham surgeries, to minimise differences in genetic background between treatment groups. By performing lipid labelling of the single cell suspensions from individual hearts and subsequent demultiplexing, following quality control I determined that there were no major differences in cell type composition across different hearts and extent of injury within the same timepoint (**Figures 5.5 and 5.6**). I next removed any cells that could not be assigned to a single sample with confidence during demultiplexing steps. I then retained all

correctly assigned cells for downstream analysis. Following quality control, I analysed 8,778 cells from P1 MI hearts, 12,431 cells from P1 sham hearts, 7,646 cells from P7 MI hearts and 3,922 cells from P7 sham hearts.

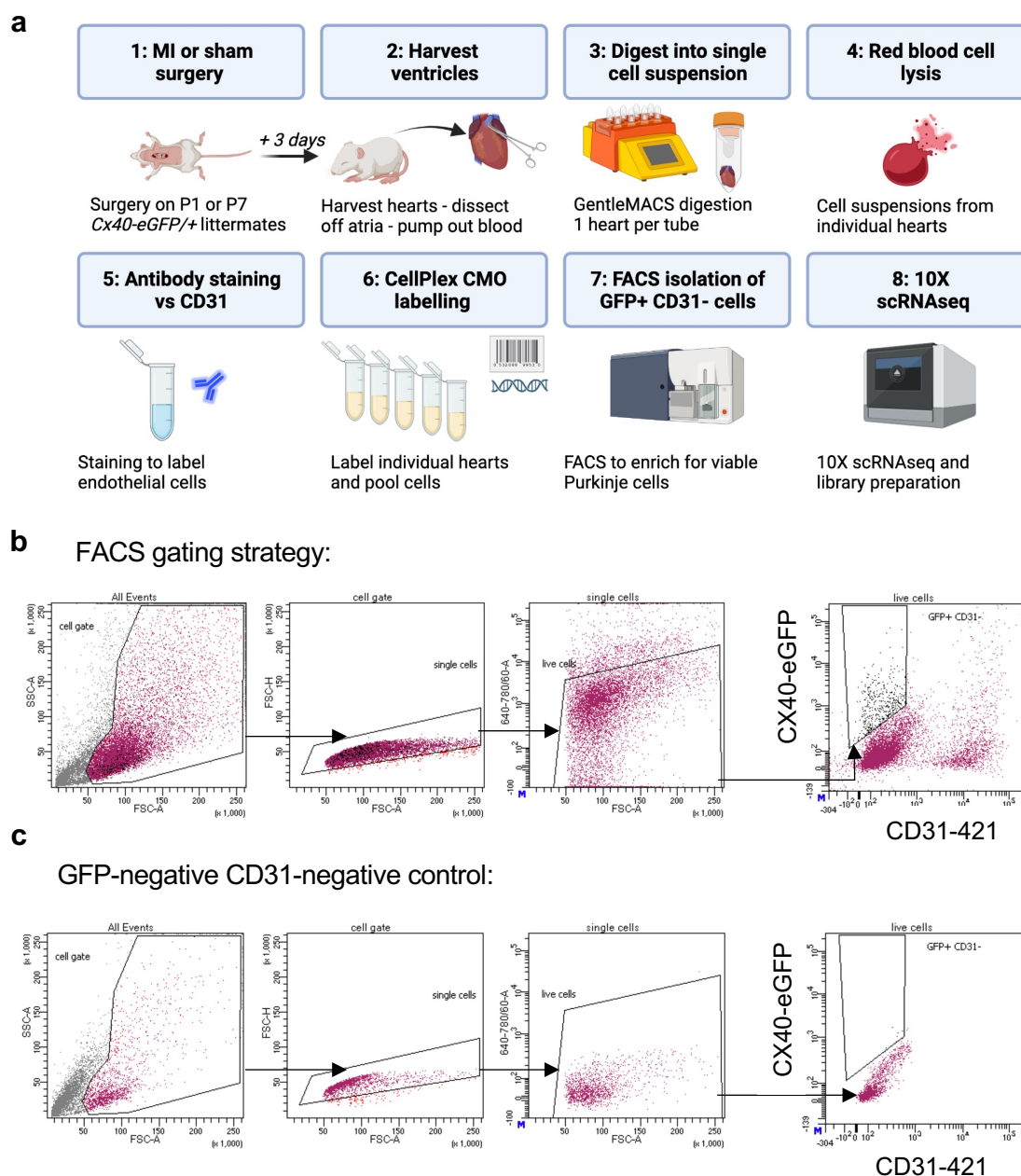


Figure 5.4: A single-cell RNA sequencing strategy to enrich for cells of the ventricular conduction system

a. Schematic to illustrate the stages of sample harvesting, digestion, labelling, ventricular conduction system (VCS) cell enrichment and 10X single-cell RNA sequencing. *Schematic created using Biorender.com.* **b-c.** Fluorescence-activated cell sorting plots showing the gating strategy employed to enrich for viable CX40+ CD31- VCS cells.

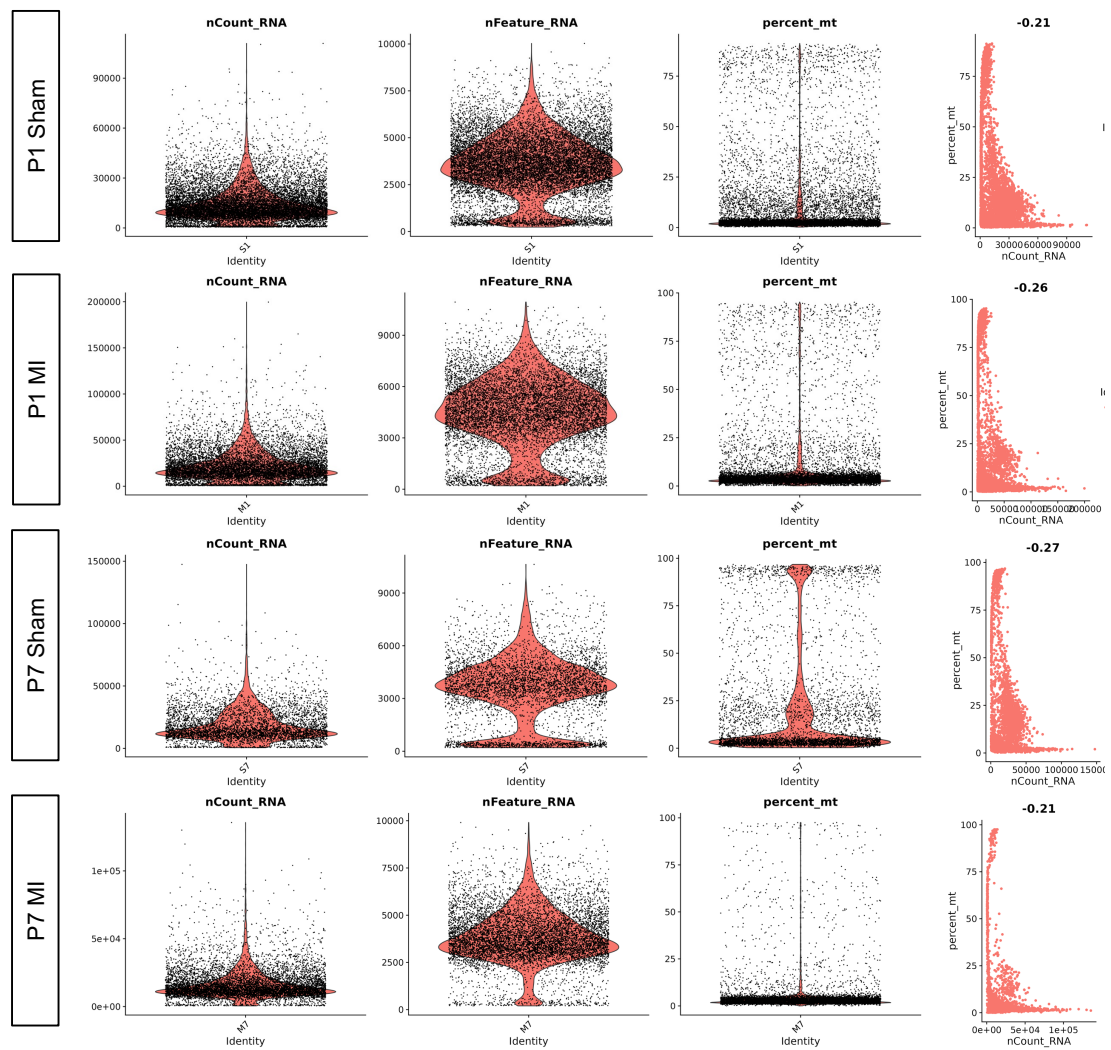


Figure 5.5: Single-cell RNA sequencing quality control

Violin plots showing from left to right: number of unique molecular identifiers; number of genes; percent of mitochondrial reads.

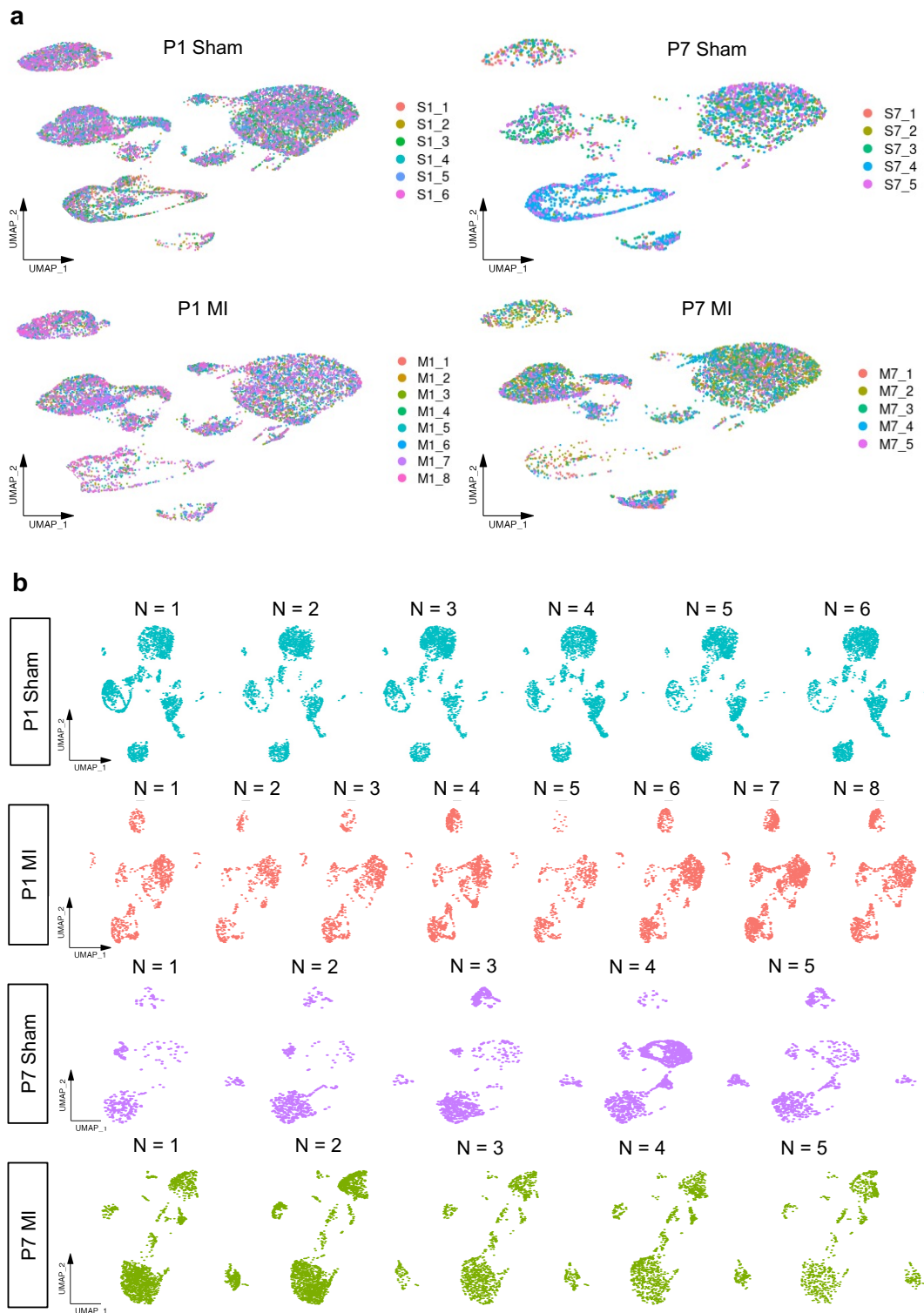


Figure 5.6: Demultiplexing found no major differences in cell type composition between the Purkinje populations of individual hearts following MI or sham surgeries

a. Uniform Manifold Approximation and Projection (UMAP) visualisations of P1 sham, P7 sham, P1 MI and P7 MI hearts after integration across conditions. Cells are coloured according to individual heart of origin within each UMAP plot. **b.** Uniform Manifold Approximation and Projection (UMAP) visualisations of P1 sham, P1 MI, P7 sham and P7 MI hearts before integration across conditions, split by individual heart of origin. No major differences are apparent in cell composition between individual hearts within each experimental group.

5.3.2.2 *Single-cell RNA sequencing identifies a distinct transcriptional signature between regenerative and non-regenerative conduction system cells*

Having performed quality control and filtering, I began by analysing the range of cell types captured within the scRNA-seq dataset (**Figure 5.7**). Clustering revealed striking heterogeneity across the VCS with two major, distinct VCS clusters, showing either more cardiomyocyte-like or fibroblast-like expression patterns. Additionally, I identified three further populations. First, an epicardial-like population, indicating either isolation of a conduction population with epicardial origin or epicardial cell bleed-through during the FACS enrichment. Secondly, a small immune population, indicating either CX40 expression in an immune subset, interesting in light of the finding in Chapter 3.3.2.4 that tissue-resident macrophages associate closely with VCS cells, or further non-specific sorting during the FACS enrichment. Thirdly, a smooth muscle cell (SMC) population resulting from CX40 expression in the SMCs of the coronary arteries (**Figure 5.7b**). While known VCS markers and those I identified through snRNA-seq re-analysis (**Supplementary Table 1**) showed varied patterns of expression across the dataset (**Figure 5.8b-c**), I identified a core cluster, CmVCS.3, with particularly conserved expression of the canonical Purkinje genes (**Figure 5.7c** and **5.8a**). To gain deeper insight into the defining features of each of these clusters, I analysed the most highly conserved genes of every individual cluster (**Figure 5.7d** and **Supplementary Table 3**). Interestingly, this analysis revealed many markers of VCS clusters which had either: previously identified connections to conductivity or to heart rhythm disorders; roles in cardiomyocyte metabolism; known roles in neurons, glia and/or at neuronal synapses; and roles in cell-cell adhesion, ECM production (including collagens) or ECM modification.

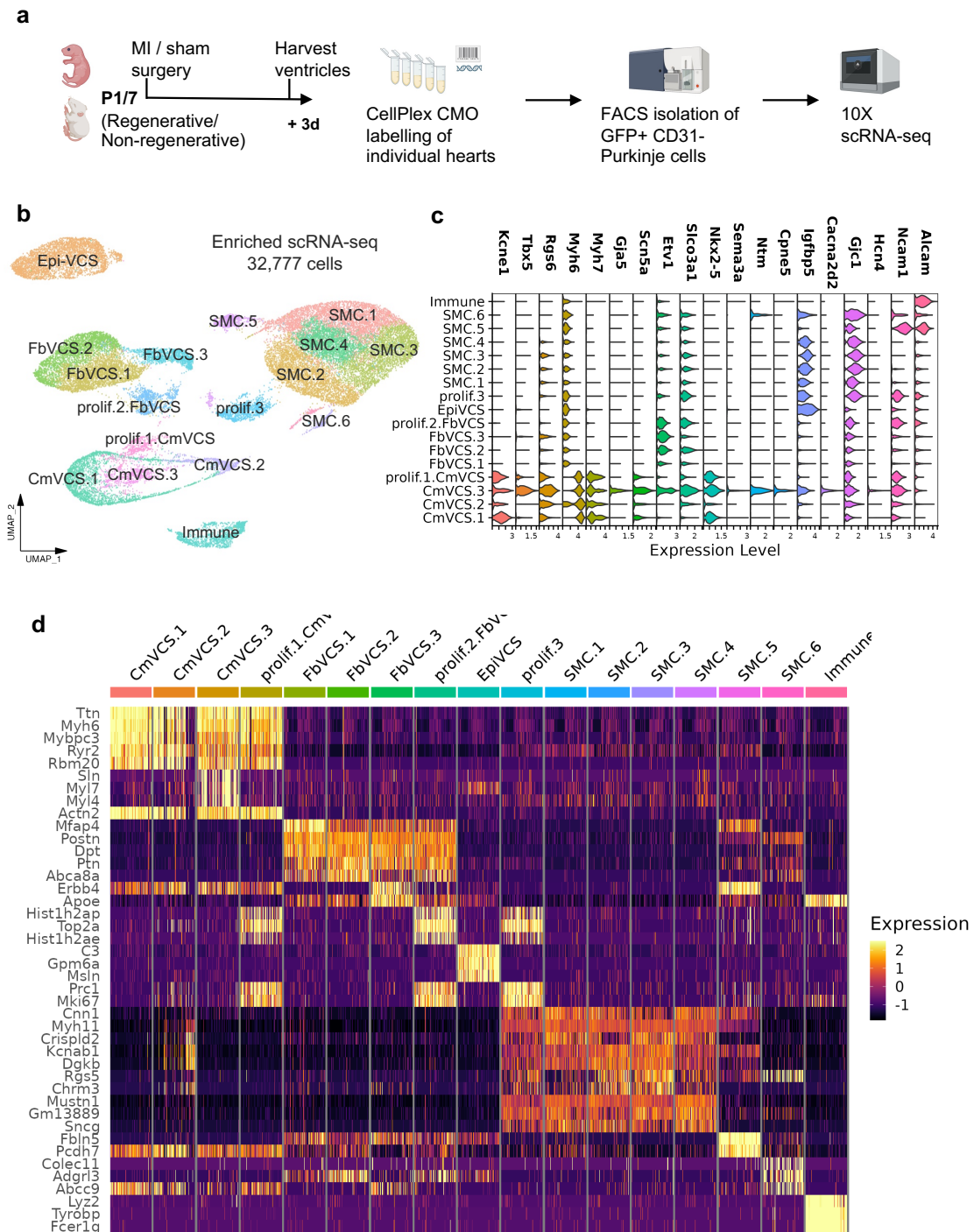


Figure 5.7: Single-cell RNA sequencing identified a heterogeneous transcriptomic signature across the ventricular conduction system of injured and regenerating murine hearts

a. Schematic to illustrate experimental design, including neonatal mouse surgeries, 3 days post-injury harvest timepoint, CellPlex multiplexing strategy, FACS enrichment and 10X single-cell RNA-sequencing. *Schematic created using Biorender.com.* **b.** Uniform Manifold Approximation and Projection (UMAP) visualisation of cells integrated across all conditions reveals significant heterogeneity across the dataset. **c.** Violin plot showing expression of known conduction system markers across clusters in the dataset. **d.** Heatmap showing the top three most highly conserved genes of each cluster in the integrated CX40+ CD31- single-cell RNA-sequencing dataset.

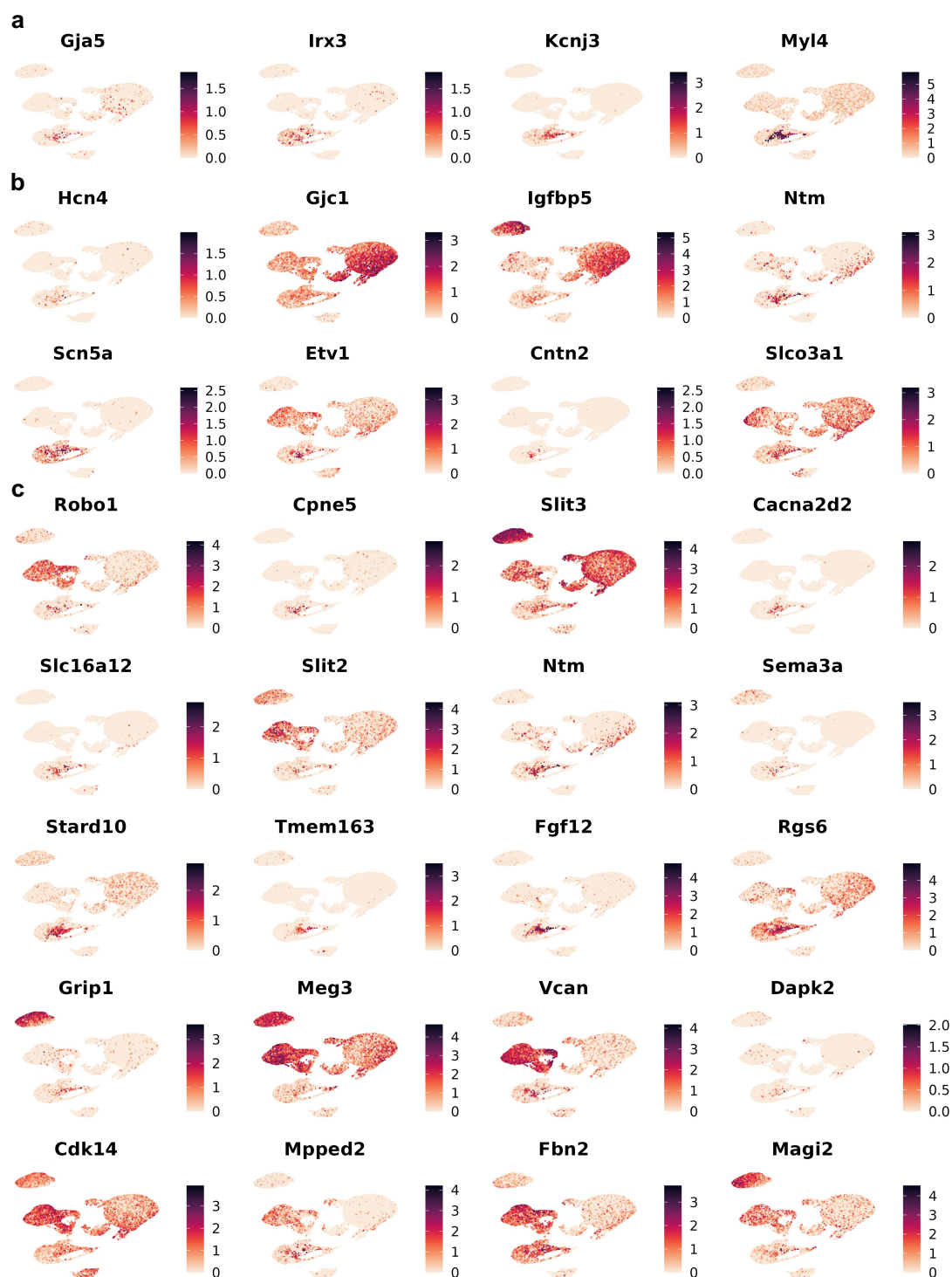


Figure 5.8: Expression of known ventricular conduction system markers show heterogeneous expression across the enriched CX40+ CD31- single-cell RNA-sequencing dataset

Featureplots of Gja5, Irx3, Kcnj3 and Myl4 identify a core Purkinje cluster with most highly conserved expression of ventricular conduction system (VCS) markers. **b.** Featureplots of other well-known VCS markers show varied expression patterns across the CX40+ CD31- single-cell RNA-sequencing dataset. **c.** Featureplots of the most differentially expressed genes of the conductive VCS cluster 13 isolated from the cardiomyocyte-enriched single-nuclear RNA-sequencing dataset¹ also show varied expression patterns across the CX40+ CD31- single-cell RNA-sequencing dataset.

To interrogate the differences between the wider VCS clusters, I integrated my scRNA-seq data with a stage-matched whole-heart (minus cardiomyocytes) scRNA-seq reference dataset from hearts 3-days following MI or sham injury⁷⁵ and found that after anchor selection, while the cardiomyocyte-like VCS cells clustered separately from the reference, the epicardial-like and fibroblast-like populations integrated with epicardial and fibroblast clusters, respectively (**Figure 5.9**). I then performed re-clustering after removing the epicardial-like, immune and smooth muscle cell clusters to elucidate the specific differences between the major cardiomyocyte-like versus fibroblast-like Purkinje populations, which showed the most interesting expression of known VCS markers (**Figure 5.10 and Supplementary Table 3**). In this way, I found enrichment of gene ontology (GO) terms associated with neuronal differentiation and Wnt/smoothened signalling pathways in the fibroblast-like VCS cluster grouping 1; versus control of calcium ion release and contraction-associated gene sets in the cardiomyocyte-like VCS grouping 2 (**Figure 5.11**).

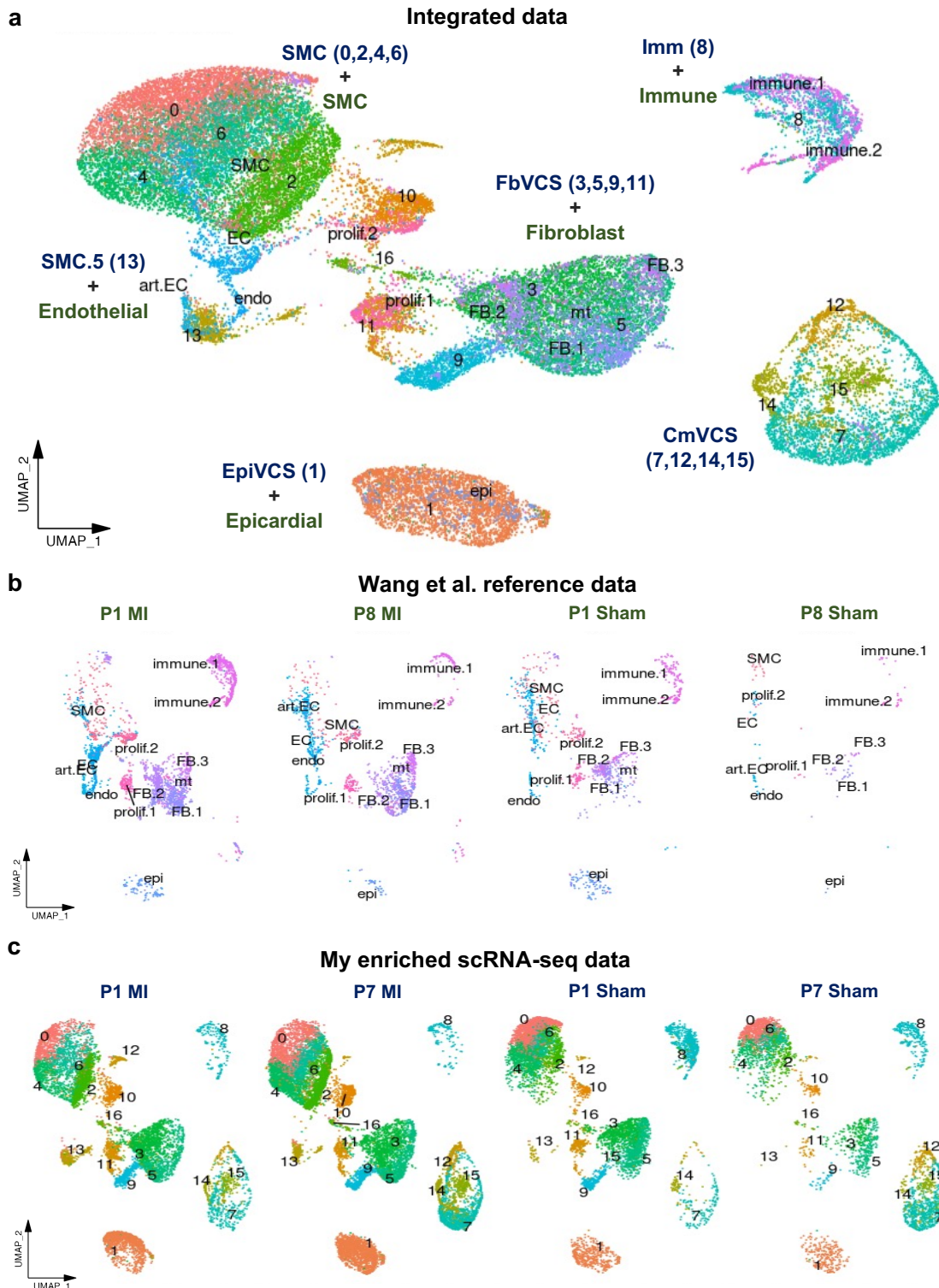


Figure 5.9: Integration of a reference post-natal murine cardiac single-cell RNA-sequencing dataset with the enriched ventricular conduction system dataset

a. Uniform Manifold Approximation and Projection (UMAP) visualisation following integration of my enriched CX40+ CD31- single-cell RNA-sequencing data with a reference whole heart post-natal murine dataset. Black text labels identify cell types in the reference dataset, black numbers identify clusters of cells from my enriched dataset, green text labels identify wider clusters of reference dataset, navy text identifies wider

clusters from my enriched dataset. The cardiomyocyte-like (CmVCS) populations clustered separately from the reference, the epicardial-like cells (EpiVCS) clustered with the epicardial cells of the reference, and the fibroblast-like (FbVCS) conduction cells clustered with the fibroblast cells of the reference. **b.** UMAP visualisation of cells deriving from the reference dataset only, split by age (P1 v P8) and by treatment (MI versus sham).

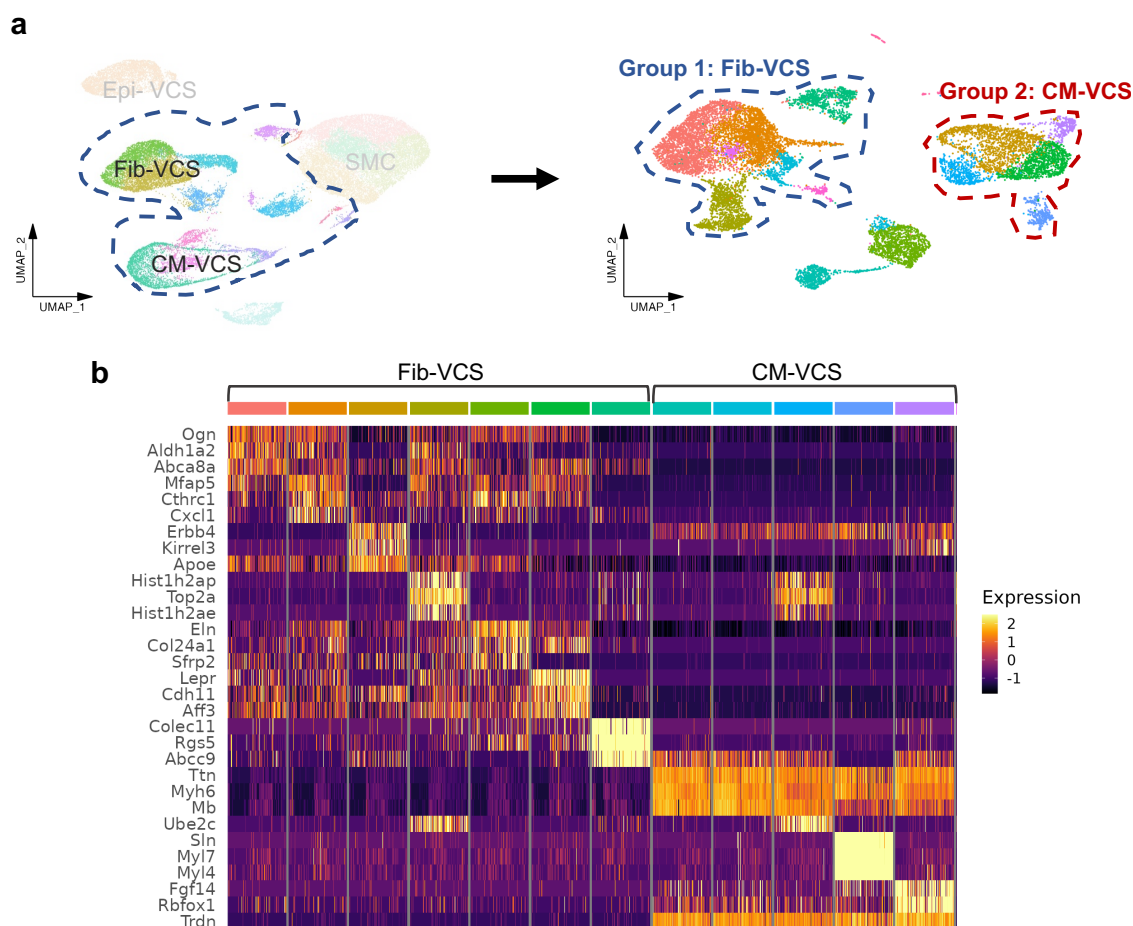


Figure 5.10: Gene expression across the two major sub-populations of conduction system cells in the CX40+ CD31- enriched dataset following re-clustering

a. Re-clustering following removal of smooth muscle, immune and epicardial-like cell populations. The re-clustered Uniform Manifold Approximation and Projection (UMAP) visualisation identified two major groups of fibroblast-like (Group 1: Fib-VCS) and cardiomyocyte-like (Group 2: CM-VCS) conduction cells. **b.** Heatmap showing the top three most highly conserved genes of each cluster in the dataset following re-clustering to remove smooth muscle, epicardial-like and immune cell populations.

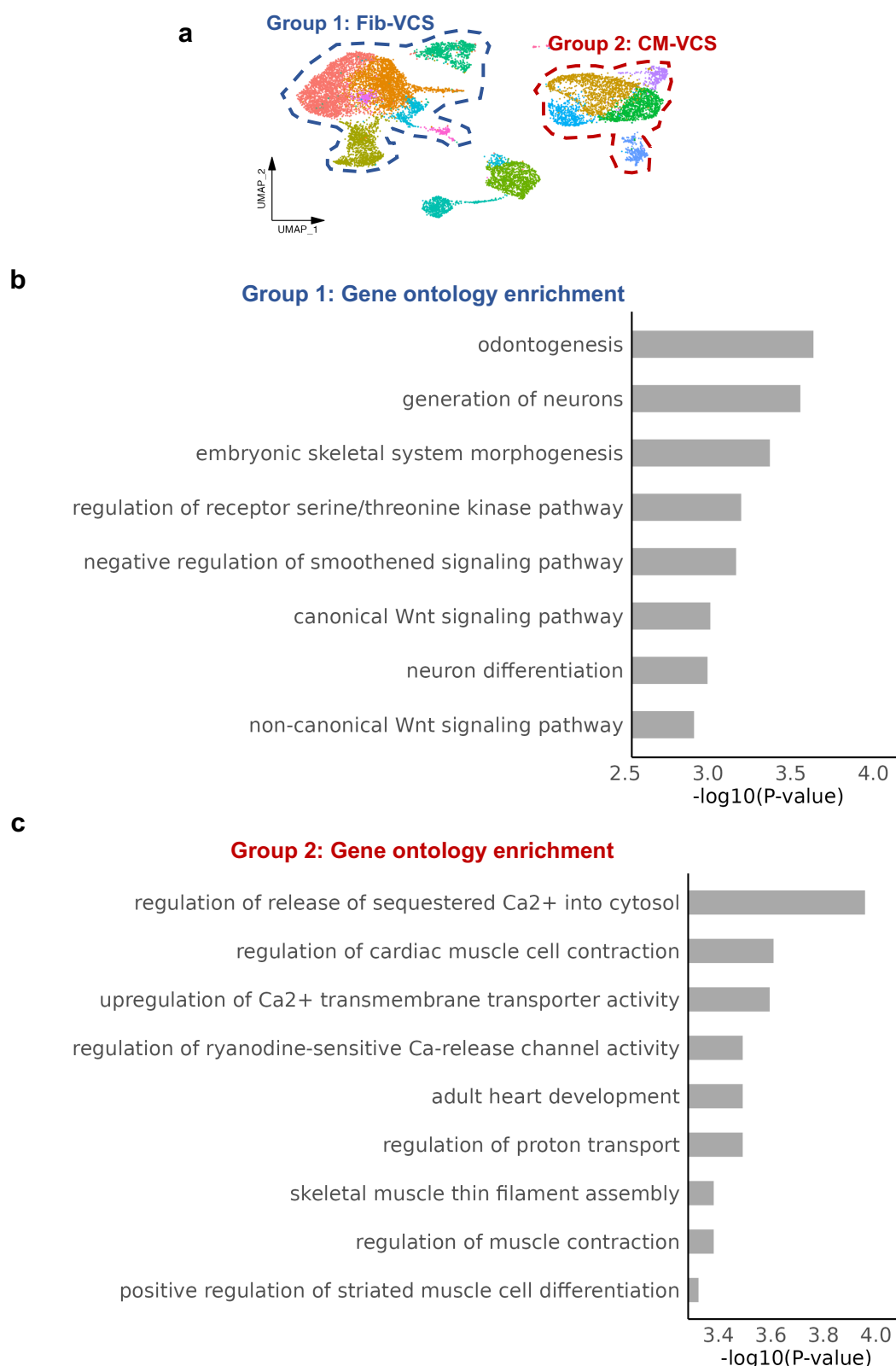


Figure 5.11: Gene ontology enrichment analysis of conduction system subtypes in the regenerating and non-regenerating heart

a. Uniform Manifold Approximation and Projection (UMAP) visualisation of the re-clustered CX40+ CD31- single-cell RNA-sequencing dataset following removal of smooth muscle, immune and epicardial-like cell populations. **b.** Top gene ontology enrichment terms for genes upregulated in the fibroblast-like ventricular conduction system (VCS) cells. **c.** Top gene ontology enrichment terms for genes upregulated in the cardiomyocyte-like VCS cells.

5.3.2.3 Cells of the His/Purkinje network lose electrical identity and undergo ionic remodelling in the non-regenerative heart after injury

Next, I interrogated differences in VCS cell type composition between MI versus sham and regenerative versus non-regenerative conditions (**Figure 5.12**). While cell clusters were more equally represented across P1 MI and sham datasets, there was a significant shift in the P7 MI hearts, where the cardiomyocyte-like VCS cluster grouping was proportionally under-represented as compared to P1 MI and P1/7 sham hearts (**Figure 5.12a/c**). This was even more apparent after re-clustering of the VCS cells only, where a major shift in cellular representation is seen from more cardiomyocyte-like (group 2) VCS cells in P7 sham, to more fibroblast-like (group 1) VCS cells in P7 MI hearts (**Figure 5.12b/d**). Indeed, by assigning cells to neighbourhoods on a k-nearest neighbour graph using Milo differential abundance testing⁸⁰, I found that almost all the local neighbourhoods containing group 2 cardiomyocyte-like VCS cells were significantly reduced in P7 MI versus sham hearts (**Figure 5.12e**). When comparing cell proportions in P1 versus P7 MI hearts, local neighbourhoods containing the group 2 cardiomyocyte-like VCS cells were significantly reduced in infarcted hearts at P7, including those containing the core Purkinje cells with most highly conserved VCS marker expression (**Figure 5.12f**). Strikingly, within these reduced local neighbourhoods, cell cycle analysis identified a cycling VCS group 2 population which was lost in P7 MI hearts as compared to sham or P1 MI hearts (**Figure 5.12g**).

I further analysed differential gene expression in the VCS between P1 versus P7 hearts. Focusing on the core Purkinje cluster with most highly conserved expression of VCS marker genes (**Figure 5.13a**), and on the proliferating VCS cluster significantly reduced in P7 MI hearts (**Figure 5.13b**), I identified a cohort of genes upregulated in the VCS of infarcted P1 hearts which were down-regulated in infarcted P7 hearts. These genes generally fall into at least one of three categories: regulators of gene transcription (e.g., *Nkx2-5*, *Cdk8*, *Hmgn2*, *Prdm16*); implicated in arrhythmia in genome-wide association or

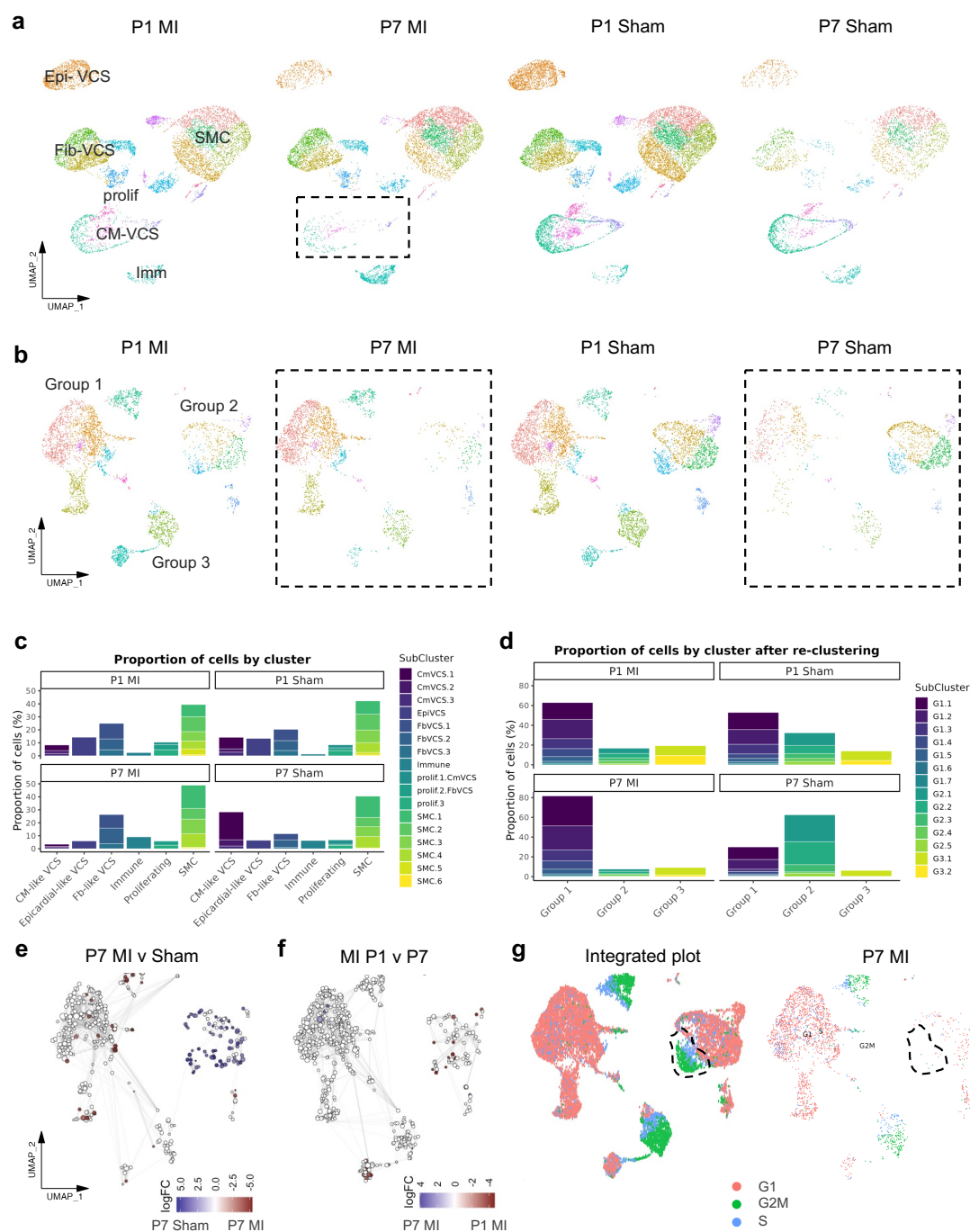


Figure 5.12: Shift in ventricular conduction system composition and loss of a cycling conduction system cluster in regenerating versus non-regenerating hearts

a. Uniform Manifold Approximation and Projection (UMAP) visualisation of the CX40+ CD31- single-cell RNA-sequencing data, split by condition. Boxed region highlights relative decrease in the cardiomyocyte-like VCS cluster in the P7 MI dataset. **b.** UMAP visualisation of re-clustered data following removal of smooth muscle, immune and epicardial-like cell populations, split by condition. Boxed regions highlight shift in dataset composition at P7 from group 2 cardiomyocyte-like VCS profiles in sham hearts to group 1 fibroblast-like VCS transcriptomic profiles following MI. **c-d:** Stacked bar plot of cell proportions by cluster before (**c**) and after (**d**) re-clustering. **e-f:** Milo differential abundance testing identifies significantly enriched or reduced neighbourhoods between non-regenerative P7 sham versus MI hearts (**e**) and infarcted regenerative P1 versus non-regenerative P7 hearts (**f**). **g:** Cell cycle analysis identifies 3 cycling populations in the re-clustered dataset. The cardiomyocyte-like VCS cycling population is missing in the P7 MI dataset, as highlighted by the dashed box region.

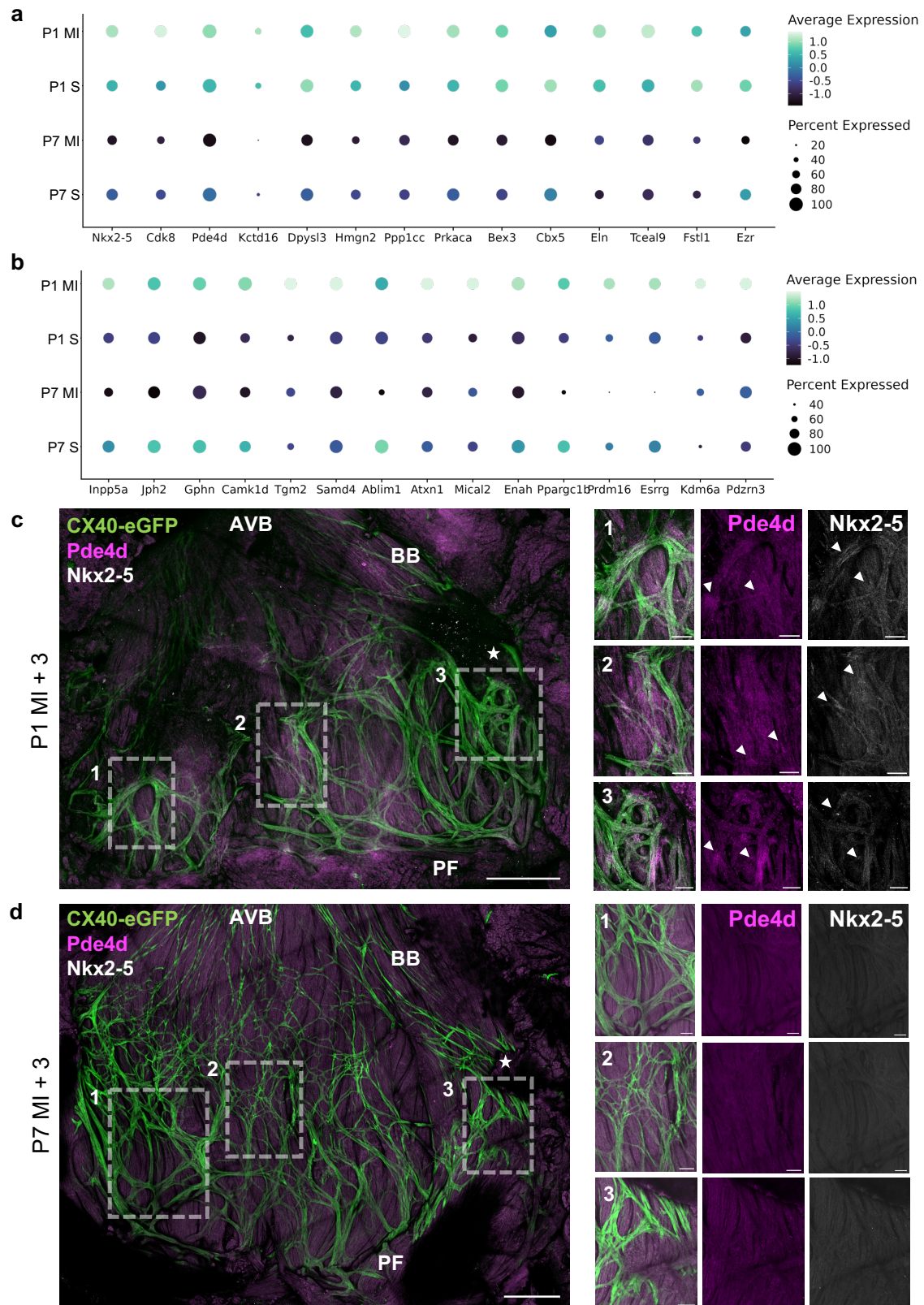


Figure 5.13: Differential expression of gene sets in the regenerative versus non-regenerative His/Purkinje network after myocardial infarction

a. Dot-plot showing average expression of markers with significant differential expression between P1 versus P7 MI hearts within the cluster with most conserved VCS marker expression. **b.** Dot-plot showing average expression of markers with significant differential expression within the proliferating cluster, which are greatly reduced in P7 MI hearts. **c-d.** Hybridisation Chain Reaction (HCR) gene expression

analysis of *Pde4d* (magenta) and *Nkx2-5* (grays) on the dissected left ventricular His/Purkinje network of P1 (**c**) and P7 (**d**) hearts, 3-days post-MI. Numbered inserts on the right-hand side show magnified panels from the corresponding dashed box regions. Arrows in (**c**) highlight HCR probe expression overlapping with CX40-eGFP-positive conduction fibres in the P1 heart. Very little HCR probe expression was detected in P7 hearts in (**d**), in accordance with the single-cell RNA-sequencing results. Stars indicate location of the LAD ligation. Labels indicate atrioventricular bundle (AVB), bundle branches (BB), Purkinje fibres (PF). Scale bars: 500µm for whole heart images, 100µm for magnified inserts.

knock-out studies (e.g., *Atxn1*, *Mical2*, *Ppargc1b*, *Fstl1*); and/or involved in intracellular calcium mobilisation or synaptic signalling (e.g., *Pde4d*, *Inpp5a*, *Jph2*, *Gphn*).

In agreement with my scRNA-seq expression analysis, hybridisation chain reaction (HCR) analyses found that while *Pde4d* and *Nkx2-5* were expressed in CX40-eGFP+ Purkinje fibres in infarcted P1 hearts, there was no detectable expression in the infarcted P7 network (**Figure 5.13c-d**). *Nkx2-5* has previously been implicated in maintenance of the conductive phenotype in the VCS¹³⁹ (see **Chapter 3.1.4**). To validate whether the higher level of *Nkx2-5* expression in the infarcted P1 heart plays a functional role in the regenerating neonatal VCS, I next performed MI and sham surgeries on *Nkx2-5*^{Cre/+} haploinsufficient neonates, which have a hypomorphic conduction system from P4 onwards due to the low dosage of NKX2-5 (**Chapter 3.3.3.2**). The non-regenerative P7 *Nkx2-5* heterozygotes' ability to repair following MI was comparable to *Nkx2-5*^{+/+} littermates despite the former having a hypomorphic VCS network by the time of surgery (**Figure 5.14g-l**). This was as predicted due to the low VCS *Nkx2-5* expression in P7 MI hearts in the days following injury, which was identified by scRNA-seq. Contrastingly, P1 *Nkx2-5*^{Cre/+} heterozygotes had severely reduced ability to regenerate a normal network in the low dose NKX2-5 conditions as compared to littermates with wild-type *Nkx2-5* levels (**Figure 5.14a-f**). This suggests a requirement for an injury-induced VCS gene-set and a specific functional requirement for *Nkx2-5* in the regeneration of the VCS network after MI at P1.

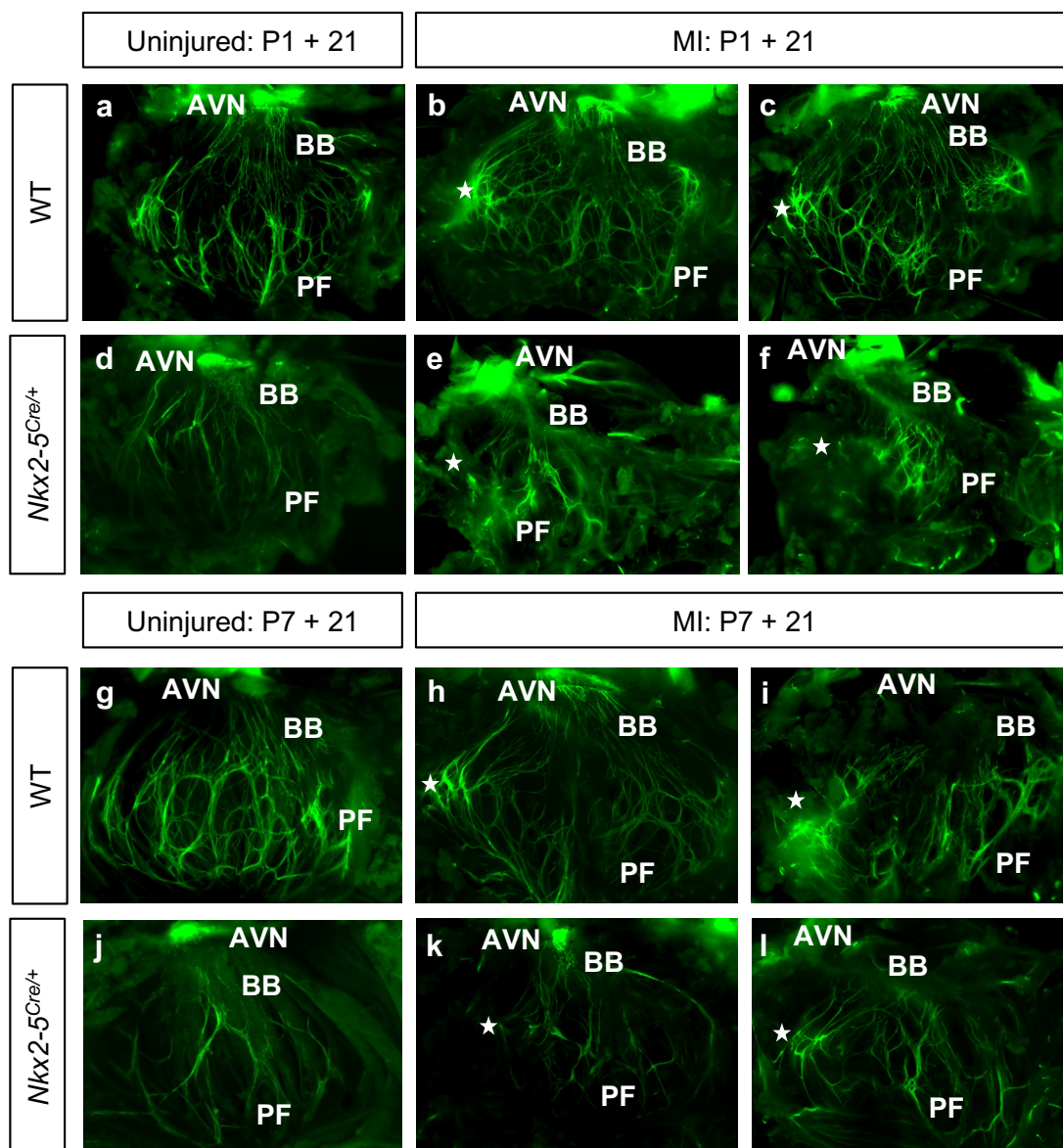


Figure 5.14: *Nkx2-5* regulates regeneration of the His/Purkinje network after myocardial infarction

The dissected left ventricular His/Purkinje network of *Cx40eGFP/+* hearts. **a-c.** Hearts that underwent MI surgery at P1, 21 days after injury (**b-c**) together with the stage-matched uninjured control (**a**). Overall regeneration of the His/Purkinje network is seen following MI at P1. **d-f.** Hearts from *Nkx2-5^{Cre/+}* haploinsufficient animals with a hypomorphic conduction system, that underwent MI surgery at P1, 21 days after injury (**e-f**) together with the stage-matched uninjured control (**d**). The reduced levels of *Nkx2-5* lead to very disrupted His/Purkinje network morphology as compared to the uninjured hypomorphic control. In all cases each image shows a separate individual's heart. **g-i.** Hearts that underwent MI surgery at P7, 21 days after injury (**h-i**) together with the stage-matched uninjured control (**g**). The His/Purkinje network has sustained alterations following MI at P7. **j-l.** Hearts from *Nkx2-5^{Cre/+}* haploinsufficient animals with a hypomorphic conduction system, that underwent MI surgery at P7, 21 days after injury (**k-l**) together with the stage-matched uninjured control (**j**). The hearts recover after P7 MI to a similar extent to the wild-type hearts despite reduced dosage of *Nkx2-5*. Stars indicate location of the LAD ligation. Labels indicate atrioventricular bundle (AVB), bundle branches (BB), Purkinje fibres (PF).

Strikingly, cell-cell adhesion genes required for fast conduction and ion channels involved in the depolarisation phase were down-regulated in P7 MI hearts across both the fibroblast-like and cardiomyocyte-like VCS populations but were maintained or up-regulated in the infarcted P1 VCS (**Figure 5.15a-b**). HCR analysis validated this expression profile within the CX40-eGFP+ network of P1 versus P7 hearts: eGFP+ VCS cells co-localised with *Slc8a1* (encoding the sodium-calcium exchanger NCX1) and *Ctnna3* (encoding alpha-Catenin) expression in the infarcted P1 VCS but not in the infarcted P7 VCS (**Figure 5.15c-d**). However, this spatial analysis of gene expression also uncovered up-regulation of these same gene sets in CX40-eGFP-negative gaps in the infarcted P7 network (**Figure 5.15d**). In this way, I identified genes involved in maintaining a functioning electrical network at P1, including *Slc8a1*, that were not only down-regulated in the non-regenerative CX40+ network, but also ectopically expressed in gaps in the network which lacked CX40 expression.

Given the sustained loss of CX40 in broad regions of the left His/Purkinje network identified in non-regenerative infarcted P7 hearts in Chapter 4 (**Figure 4.5**), I next wanted to determine whether the specific and ectopic expression of *Slc8a1* and *Ctnna3* in CX40-negative gaps at 3 days after P7 MI manifested as continued broad overexpression of these factors throughout the CX40-negative areas of the network by 21 days. I, therefore, repeated my analyses of *Slc8a1* and *Ctnna3* expression within the P1 versus P7 VCS, but this time I allowed 21 days for recovery before harvesting the hearts for HCR. HCR staining of regenerative P1 MI hearts 21 days post-MI did not detect VCS expression of either gene (**Figure 5.16a**). Contrastingly, 21 days after MI in P7 hearts I identified localised puncta of *Slc8a1* and *Ctnna3* signal in the regions negative for CX40-eGFP where the network was otherwise broadly CX40-positive (**Figure 5.16b**). This suggests a high specificity of *Slc8a1* and *Ctnna3* upregulation in the VCS network following MI, and moreover that small slow-conductive nodes within the CX40+ network may undergo particularly extensive electrical remodelling at acute-chronic stages in the non-regenerative infarcted heart.

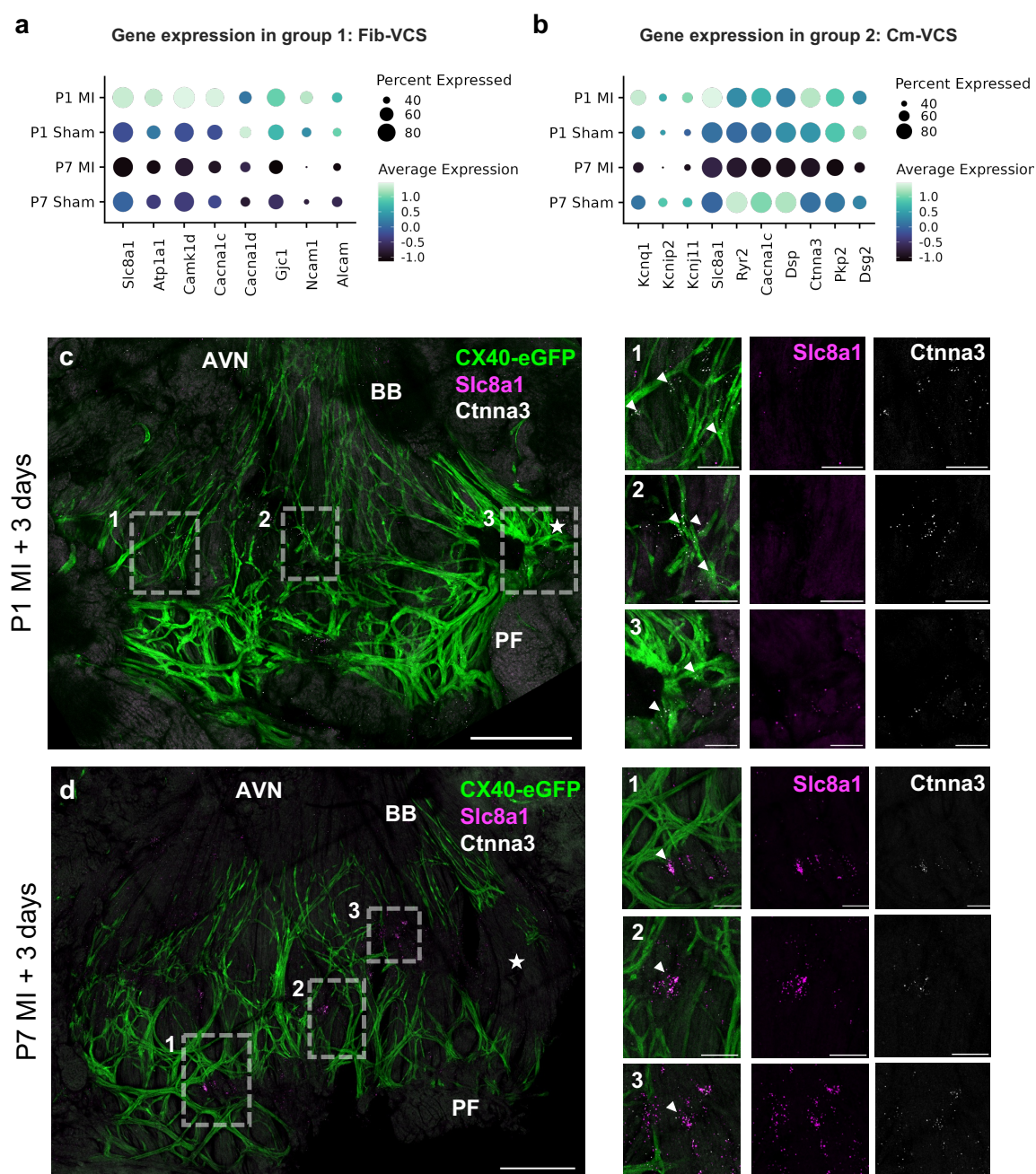


Figure 5.15: Electrical remodelling of His/Purkinje subpopulations after myocardial infarction in the non-regenerative heart

a. Dot-plot showing average expression of ion channel and cell-cell adhesion markers with significant differential expression between P1 versus P7 MI hearts within the fibroblast-like ventricular conduction system group 1 cell populations. These markers are all upregulated in P1 MI hearts, but downregulated in P7 MI hearts. **b.** Dot-plot showing average expression of ion channel and cell-cell adhesion markers with significant differential expression between P1 versus P7 MI hearts within the cardiomyocyte-like ventricular conduction system group 2 cell populations. These markers are all upregulated in P1 MI hearts, but downregulated in P7 MI hearts. **c-d.** Hybridisation Chain Reaction (HCR) gene expression analysis of *Slc8a1* (magenta) encoding the NCX1 sodium-calcium exchanger, and *Ctnna3* (grays) encoding α -Catenin on the dissected left ventricular His/Purkinje network of P1 (**c**) and P7 (**d**) hearts, 3-days post-MI. Numbered inserts below the main image show magnified panels from the

corresponding boxed regions. Arrows in (c) highlight HCR probe expression overlapping with CX40-eGFP-positive conduction fibres in the P1 heart. Arrows in (d) highlight HCR probe expression in the CX40-eGFP-negative gaps in the conductive network. Stars indicate level of the LAD ligation. Labels indicate bundle branches (BB), Purkinje fibres (PF). Scale bars: 500µm for whole heart images, 100µm for magnified inserts.

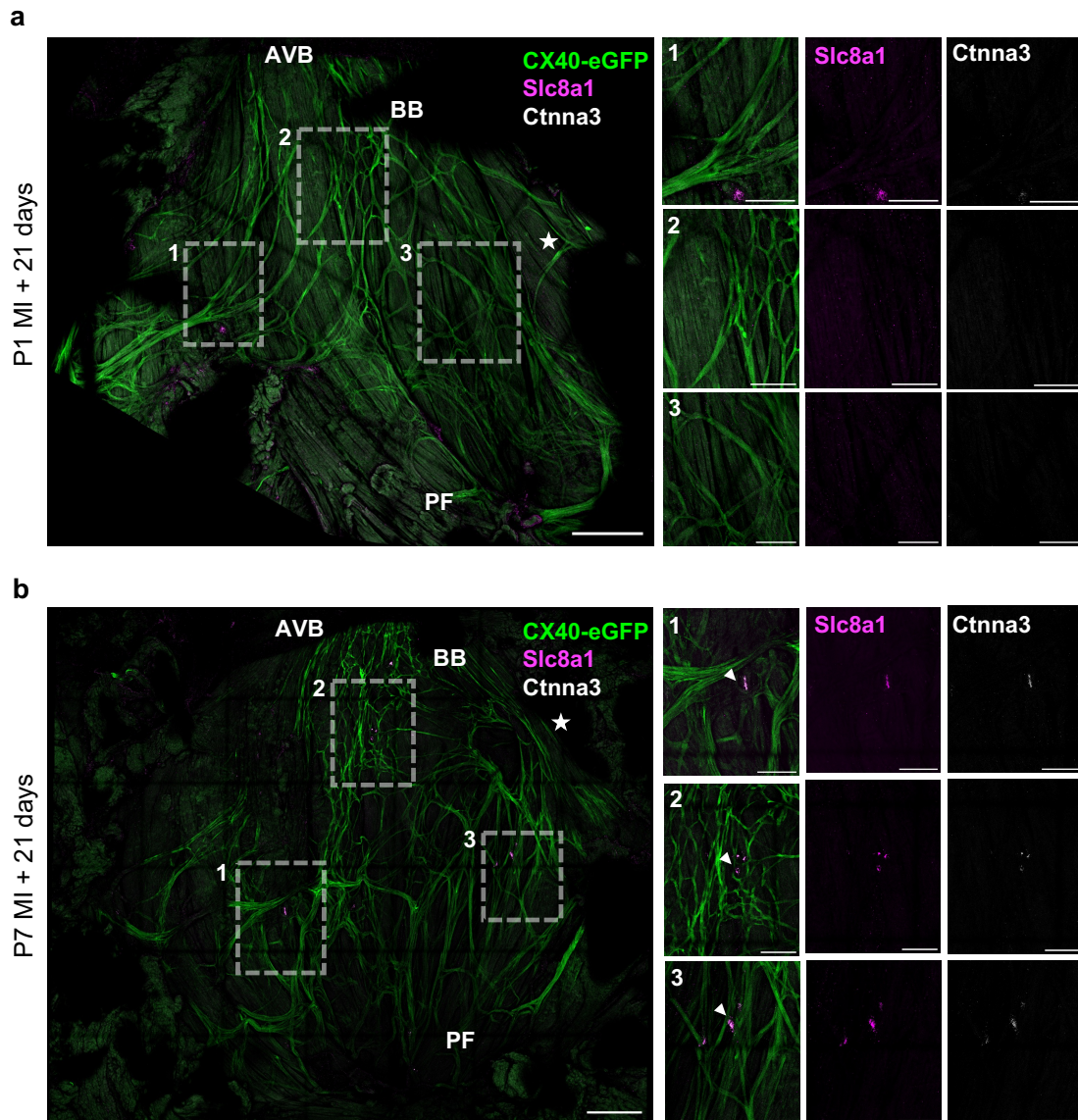


Figure 5.16: *Slc8a1* and *Ctnna3* are expressed in localized CX40-negative puncta in the non-regenerative infarcted His/Purkinje network 21 days following myocardial infarction Hybridisation Chain Reaction (HCR) gene expression analysis of *Slc8a1* (magenta) and *Ctnna3* (grays) on the dissected left ventricular His/Purkinje network of P1 (a) and P7 (b) hearts, 21 days post-MI. Numbered inserts on the right-hand side show magnified panels from the corresponding boxed regions. Arrows in (b) highlight HCR probe expression overlapping with small CX40-eGFP-negative punctate regions in the P7 network. Stars indicate location of the LAD ligation. Labels indicate atrioventricular bundle (AVB), bundle branches (BB), Purkinje fibres (PF). Scale bars: 500µm for whole heart images, 200µm for magnified inserts.

Overall, profiling by scRNA-seq identified greater transcriptional heterogeneity than previously elucidated across the post-natal mammalian VCS. Analysis of the regenerating versus non-regenerating VCS identified a shift in cell-type composition within the His/Purkinje network and a loss of proper electrical identity in the transition to non-regenerative stages; evident at the level of transcriptional regulation, and also in connexin, ion channel and cell-cell adhesion gene expression.

5.3.2.4 Sex-linked differences in the regenerating and non-regenerating conduction system response to myocardial infarction

There are significant differences observed in prevalence, mortality risk, time of onset and variety of arrhythmias suffered between male versus female patients, including after MI²¹⁸⁻²²⁰. Consequently, I set out to establish whether or not there are differences in the VCS transcriptional response post-MI between male versus female neonatal mice by leveraging my multiplexed VCS-enriched scRNA-seq dataset.

First, I needed to determine the sex of each individual in my dataset computationally. To this end, I defined an X-linked gene module (*Xist*, *Tsix*) and a Y-linked module (*Ddx3y*, *Kdm5d*, *Uty*) and plotted these for each individual in the dataset (**Figure 5.17a**). In this way, I established that at least 2 hearts of each sex were included in my dataset, for each stage and condition (**Figure 5.17b**). This correlated with a minimum of 905 cells per sex, per condition following quality control (**Figure 5.17c**).

With the dataset split by sex in this way, I ascertained whether the regenerative transcriptional signature differed in the His/Purkinje network of male versus female hearts, and whether there were sex-specific differences in VCS response following myocardial infarction. I began, therefore, by performing differential gene expression analyses on the re-clustered VCS-enriched scRNA-seq dataset following separation according to sex. After performing differential expression analysis in Seurat, I compared the differentially expressed

genes within the male versus the female datasets to establish whether the same gene sets were up-regulated in the VCS regardless of sex (**Figure 5.18**).

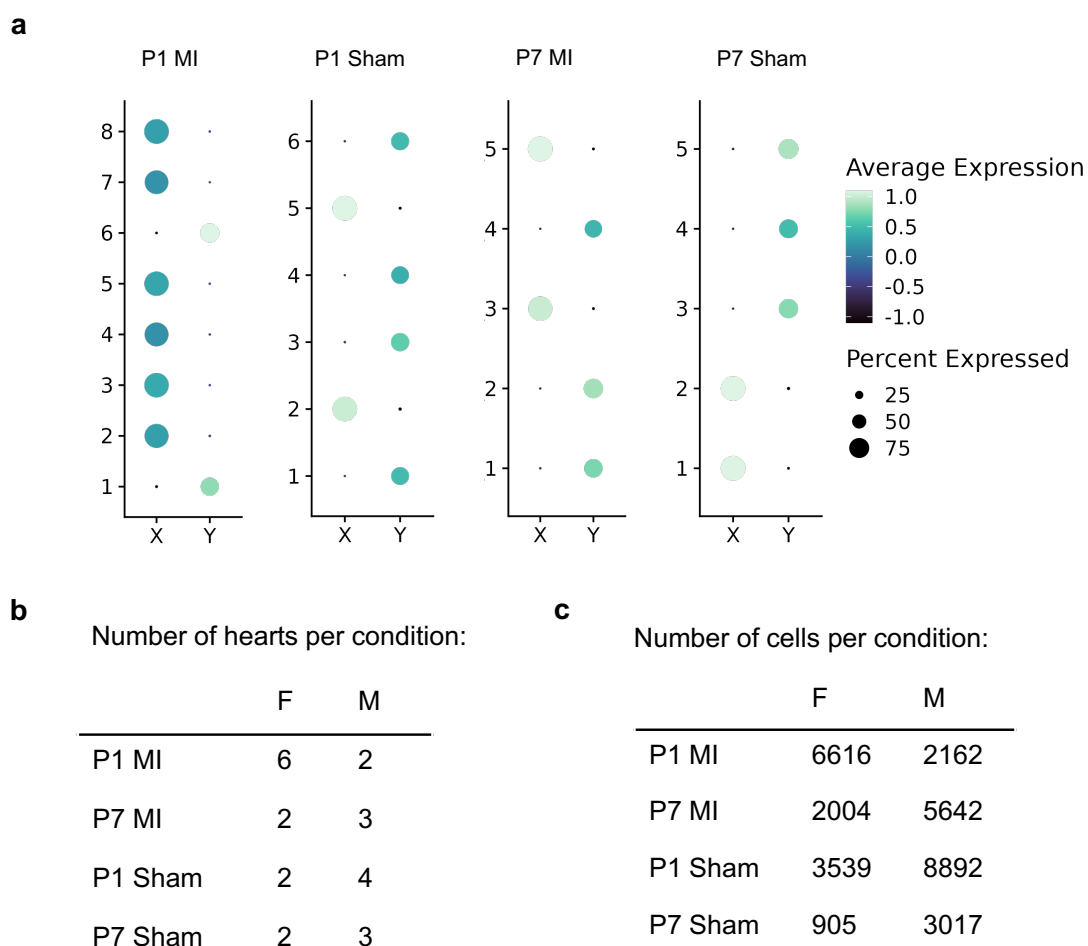


Figure 5.17: Computational determination of sex within multiplexed single cell RNA-sequencing data

a. Dot-plots showing average expression of an X-linked gene module (Xist, Tsix) and a Y-linked module (Ddx3y, Kdm5d, Uty) for each heart in the dataset, split by time of surgery (P1 v P7) and treatment (MI v sham). The sex of every heart was evident from the X/Y gene module expression patterns. **b.** Table listing number of individual hearts of each sex in my enriched single-cell RNA-sequencing dataset, separated by time of surgery (P1 v P7) and treatment (MI v sham). **c.** Table listing number of cells from hearts of each sex in my enriched single-cell RNA-sequencing dataset, separated by time of surgery (P1 v P7) and treatment (MI v sham).

I first analysed differentially expressed genes following MI at either regenerative P1 or non-regenerative P7 stages. I found that, while a majority of genes upregulated in the infarcted P1 versus P7 VCS were shared between male and female hearts, there was nevertheless a high amount of sex-dependent divergence in responding gene programmes, most especially in female hearts: 634 of 2302 differentially expressed genes between P1 versus P7 MI conditions were unique to the female VCS, whereas only 275 genes were unique to the male VCS (**Figure 5.18a**). Even more strikingly, when comparing genes significantly up-regulated in MI versus sham conditions (across both P1 and P7 stages combined) in male versus female hearts, I found that over 80% of genes upregulated in MI versus sham VCS cells were not shared between male and female hearts (only 181 of 916 genes in common). Interestingly, again a much greater proportion of genes were upregulated after MI in female than in male VCS cells (**Figure 5.18b**). This, therefore, indicates a substantial divergence in VCS response following MI between the sexes, and a more dramatic response to MI at the level of gene expression in the conduction cells of female than male hearts.

Analysing MI versus sham differential expression separately for P1 and P7 stages by sex found discordance between the gene sets being up-regulated in male versus female hearts both during and beyond the regenerative window (**Figure 5.18c-d**). However, surprisingly there was a much stronger sex-specific response in the regenerative than the non-regenerative context: 68% of all genes upregulated in MI versus sham hearts at P7 were differentially expressed in both male and female hearts, whereas only 17% of genes upregulated in MI versus sham hearts at P1 were common to both male and female hearts. This finding suggests, therefore, that there is a particularly strong sex-dependence in the regenerative response to MI in the neonatal VCS.

Having determined clear sex-dependent differences in VCS response following MI, I next analysed the key genes responsible for driving these male-specific and female-specific responses. I performed gene ontology (GO) enrichment analysis to determine what gene groups were differentially upregulated in regenerative and non-regenerative

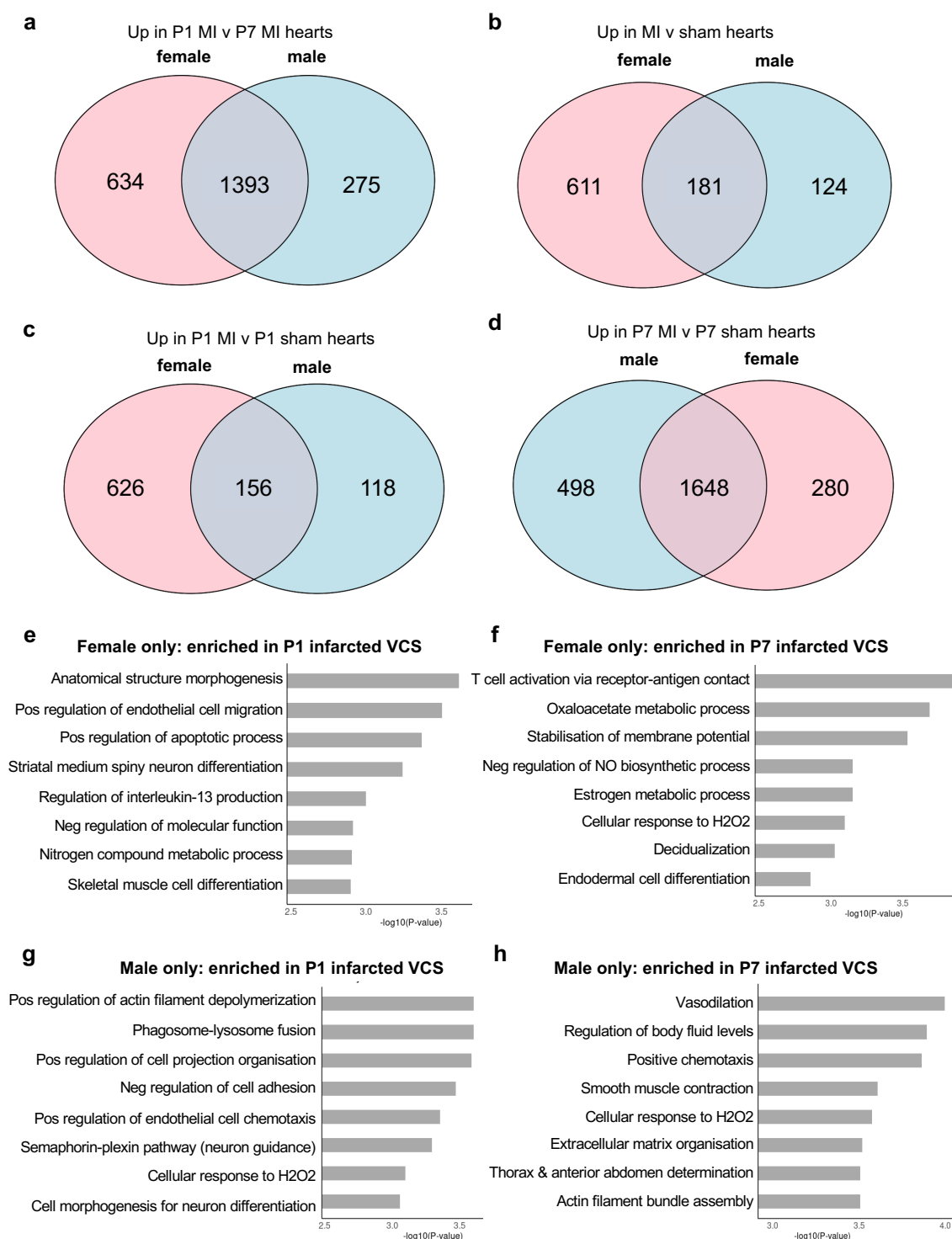


Figure 5.18: Sex-specific gene expression differences in the regenerating and non-regenerating ventricular conduction system after myocardial infarction

a-d. Venn diagrams to illustrate the extent of overlap in genes differentially upregulated in the re-clustered P1 MI versus P7 MI CX40+ CD31- single-cell RNA sequencing dataset, between male versus female hearts. Pink set represents female expression and blue set represents male expression. **a:** Genes differentially upregulated in P1 MI versus P7 MI groups; **b:** genes differentially upregulated in MI (all stages) versus sham (all stages) groups; **c:** genes differentially upregulated in P1 MI versus P1 sham groups; **d:** genes differentially upregulated in P7 MI versus P7 sham groups. **e-h.** Gene ontology terms enriched in: female but not male P1 MI cells (**e**); female but not male P7 MI cells (**f**); male but not female P1 MI cells (**g**); male but not female P7 MI cells (**h**).

female or male hearts (**Figure 5.18e-h**). Interestingly, I found that GO terms associated with neuron differentiation and guidance were upregulated in the P1 VCS but not the P7 VCS after MI, suggesting the fast-conductive neuronal-like gene sets important for restoration of VCS function are preferentially re-expressed in the regenerative context regardless of sex. However, in the regenerative context developmental morphogenesis pathways were upregulated in female hearts (muscle cell differentiation, anatomical structure formation, regulation of apoptosis) whereas there was male-specific enrichment of terms linked to cellular organisation (actin filament depolymerisation, cell projection organisation, negative regulation of cell adhesion) (**Figure 5.18e, g**). In the non-regenerative P7 context, while female VCS cells upregulated genes associated with stabilising membrane potential and control of metabolism, contrastingly male P7 VCS cells upregulated a more similar profile to the male P1-enriched terms, including genes involved in matrix organisation and actin filament assembly (**Figure 5.18f, h**). This finding suggests that the male VCS retains better reparative/regenerative competency than the female VCS does, beyond the neonatal regenerative window. Overall, therefore, the substantial divergence in the VCS gene sets upregulated following MI to regenerative and non-regenerative male and female hearts correlates with different functional pathways and suggests that there are sex-dependent differences in the electrical regenerative response versus pathological conductive remodelling beyond the regenerative window.

Given the striking differences at the level of gene expression that I identified in the infarcted VCS of male versus female P7 mice, I determined whether there were corresponding differences in functional outcome after MI in terms of ability to maintain normal cardiac rhythm. To this end, I performed electrocardiogram (ECG) recordings 21 days after MI surgery to P7 mice. I recorded lead II and lead III surface ECGs under isoflurane-induced general anaesthesia from female and male mice. Interval analysis identified a clear trend towards a prolonged PR interval in female as compared to male animals ($p = 0.0769$, unpaired t test, n (female) = 4, n (male) = 2), suggesting more severe conduction delays in female hearts following MI surgery (**Figure 5.19**). Moreover, taking the

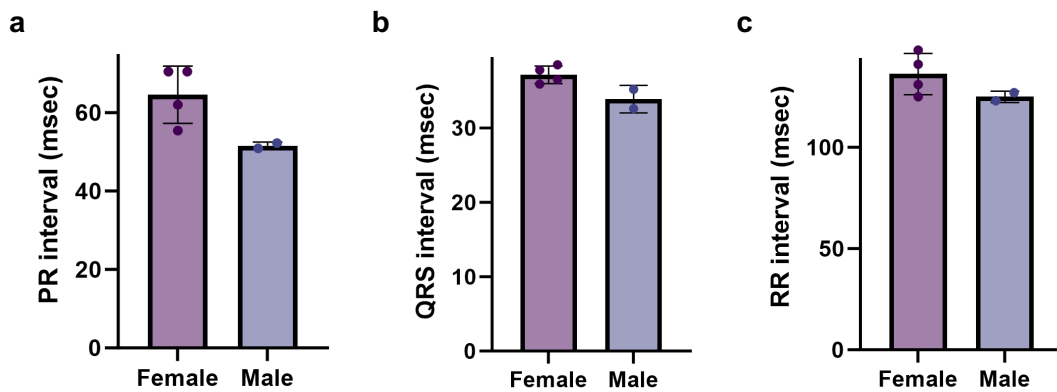


Figure 5.19: Sex-dependent incidence of pathological conduction delay following MI to non-regenerative hearts

Interval measurements were made from lead II and III surface electrocardiogram (ECG) recordings, 21 days following P7 MI surgery. A trend towards a more prolonged PR interval (a) was detected in female versus male animals, although due to small sample sizes this trend was not statistically significant ($p = 0.0769$, n (female) = 4, n (male) = 2). 3 of 4 female animals had a PR interval > 60msec, indicating 1st degree heart block. No strong trend was evident in QRS (b) and RR (c) intervals between male versus female animals. Statistics: unpaired two-tailed t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

previously reported “normal” PR interval range of 30 - 56msec for mice²²¹ and the previously suggested threshold of > 60msec as a cut-off indicator of 1st degree AV block²²², 75% of female mice had 1st degree AV block whereas no male animals displayed this pathology. These results support the hypothesis that pathological electrical remodelling in the BBs is more severe in female than male mice following MI, leading to a greater incidence of pathological conduction delay in female infarcted hearts.

Overall, therefore, in this analysis I determined that the response of the regenerating and non-regenerating conduction system following MI differs dependent on sex. Furthermore, I identified the sex-specific gene-sets most upregulated in males and most up-regulated in females, many of which have known functions in cardiac biology. These gene expression changes indicate an improved VCS reparative response beyond the regenerative neonatal window in male than female hearts. Moreover, these findings provide functional insights into the importance of tailoring treatment strategy for male or female patients, to reduce arrhythmic risk following MI.

5.4 Discussion

The findings presented in this Chapter elucidate the core transcriptional networks of the healthy, regenerating and non-regenerating VCS. Re-analysis of a published cardiomyocyte snRNA-seq dataset identified a suite of novel VCS markers. By performing enriched scRNA-seq of CX40-eGFP⁺ CD31⁻ cells I identified significant transcriptional heterogeneity at single-cell resolution across the VCS, including a previously undescribed fibroblast-like VCS population characterised by expression of collagens, cell-cell adhesion and synaptic markers.

Following MI, I identified the gene networks driving electrical regeneration versus those underpinning pathological modification of the conduction system during fibrotic repair beyond the neonatal regenerative window. I found a major shift in VCS cell type composition in infarcted P7 hearts, including significant reduction in the cardiomyocyte-like VCS populations but maintenance of the fibroblast-like VCS cells. Furthermore, there were substantial changes in gene expression between P1 versus P7 infarcted VCS populations. These included differences in transcriptional regulation, as well as downregulation of ion channels and conduction-associated cell-cell adhesion gene sets in non-regenerative P7 MI hearts that conversely were maintained or upregulated in regenerating P1 MI hearts. HCR analysis validated these transcriptional changes and identified spatially dysregulated expression of genes important for conduction and ion handling in CX40-negative nodes of the VCS network. Finally, sex-specific analysis of the scRNA-seq dataset identified differences in the response of the regenerating and non-regenerating female versus male VCS which correlated with different functional outcomes manifesting by ECG to reveal a worse arrhythmia prognosis in females.

5.4.1 Transcriptional heterogeneity in the ventricular conduction system

Through generating a scRNA-seq dataset enriched specifically for CX40+ VCS cells, in this Chapter I performed the first gene expression analysis of the post-natal mammalian conduction system with single-cell resolution. I identified a more heterogeneous transcriptional signature across the VCS than had previously been reported. The cardiomyocyte-like expression profile of cells in group 2 of the re-clustered dataset was in line with my expectation for VCS transcriptional networks. Indeed, I had predicted that the scRNA-seq would reveal cells expressing working myocyte gene sets, but with additional enrichment for ion channels, calcium handling machinery, and other conduction-related genes. These expectations were based not only upon previous studies of VCS gene expression, but also on the shared cellular origins of the VCS with the working myocardium and the extent of morphological similarity between these cell types in the mouse heart¹⁶. However, VCS cells with fibroblast-like signatures such as those I found in group 1 cells of the re-clustered dataset have not previously been characterised.

Given the extensive collagen/elastin sheath and the complex fibrous bundling arrangement of the His bundle, BBs and Purkinje fibres (see **Chapter 4.4.2**), it seems likely that these fibroblast-like group 1 VCS cells may be responsible for laying down and organising the connective sheath that insulates the VCS from the working myocardium. Moreover, given the high expression of synaptic genes and cell-cell adhesion markers within this fibroblast-like population (see **Supplementary Table 3**), it is possible this population mediates cell-cell coupling within the electrical bundles to ensure appropriate, fast conduction through the network. It is further interesting to note that this population shares some similarities in gene expression with the myelin-producing oligodendrocyte/Schwann populations of the central and peripheral nervous systems, and with cardiac glial populations. Despite this, these cells do not show conserved expression of canonical markers for glial cells (including *Plp1*, *Kcna1*), so they appear to represent a unique and specialised VCS population.

The presence of a population of cells with epicardial-like expression patterns in the scRNA-seq dataset was surprising. This cell cluster had highly conserved expression of many common epicardial markers, including *Upk3b*, *Wt1* and *Tcf21*. However, despite early reports from the chick suggesting important roles for epicardial-derived progenitors in patterning the Purkinje fibres during CCS development^{223,224}, and a reported contribution of *Tbx18*⁺ epicardium to the connective tissue surrounding AVN cells¹¹⁷, there is no well-established epicardial contribution to the VCS. Moreover, the epicardium is not known to express CX40, and correspondingly in my imaging analyses I did not identify CX40-eGFP expression in the epicardial layer of the heart (**Chapters 3-4**). It seems likely, therefore, that this population may have been carried over during the FACS sort despite being CX40-negative, perhaps due to autofluorescence in the 488-channel. However, this population was present in all four treatment groups and relatively significant in terms of cluster size, which makes it surprising that these cells would have been incorrectly sorted so reproducibly. It should further be noted that while expression of epicardial markers is conserved in these cells relative to the other cells in the dataset, given the extent of enrichment within the dataset these cells may still have low epicardial expression as compared to “true” epicardial cells. Currently, therefore, it remains unclear to what extent this population is biologically interesting and whether it has relevance to CCS functionality. Overall, the epicardial-like population identified by scRNA-seq warrants further investigation that was beyond the scope of this thesis.

Finally, it is also worth noting that despite the extensive heterogeneity identified between VCS cells in the scRNA-seq data, there was no distinctive transitional CCS cell population in the dataset. Indeed, analysis of the “transitional” markers from Goodyer et al.’s E16.5 CCS dataset (including *Ntm*, *Cpne1*, *Gja1*)⁷⁶ did not find a unique cluster with conserved expression of these markers. This was perhaps to be expected given the transitional cells (suggested to link Purkinje cells to working myocardium) showed low or no expression of *Gja5* (encoding CX40) in the embryonic CCS dataset. These cells, even if present and

transcriptionally similar at post-natal stages, would consequently not have been retained in my FACS gating for eGFP+ cells.

Overall, this rich dataset provided unprecedented insight into the heterogeneity of the VCS network, including within the His bundle, BBs and Purkinje fibres. Going forwards, spatial transcriptomic and multiplexed fluorescent *in situ* hybridisation analyses would be helpful in order to locate the fibroblast-like versus cardiomyocyte-like conduction cells and ascertain their cytological arrangement within the VCS.

5.4.2 The distinct transcriptional response of regenerating versus non-regenerating conduction cells in the infarcted heart

My scRNA-seq analyses identified distinct gene expression changes in the conduction cells of P1 versus P7 infarcted hearts, and significant changes to cellular composition following MI. Understanding of the gene networks driving the different responses in the regenerative versus non-regenerative setting may inform novel strategies for restoration of normal heart rhythm in MI patients with clinical arrhythmias.

The decrease in the cardiomyocyte-like VCS population in non-regenerative infarcted hearts was particularly notable. This might to some extent result from reduced CX40 expression in some of the P7 VCS cells following injury, which may have led them to be de-selected during the FACS enrichment step. However, my imaging analyses in Chapter 4 found that the regional CX40 downregulation 21 days after MI in P7 hearts does not generally manifest at the early 3-day time-point at which I selected to harvest cells for scRNA-seq (**Figures 4.2-4.5**). It seems likely, therefore, that some cardiomyocyte-like cells in the P7 infarcted VCS were lost because they were relatively less resistant to the ischaemic stress that resulted from the MI event than the fibroblast-like cells. Future work will be needed to determine the extent to which cell death contributes to the significant

reduction in this core Purkinje population, and consequently to the inability of the P7 VCS to recover normal electrical function.

The reduction in the cardiomyocyte-like VCS population within P7 MI hearts and transcriptional changes within the fibroblast-like VCS population, including reduced expression of cell-cell adhesion markers, may explain the altered bundle morphology observed in the non-regenerative heart, in which the fibrous sheath failed to hold bundles together as is normally seen in healthy, sham control and regenerative hearts (see **Figure 4.2**). Given the importance of the insulative sheath in achieving electrical isolation between the fast-conductance cells of the CCS and the working myocardium, restoring these transcriptional networks to promote repair of bundle architecture may ultimately be important to achieve regeneration of heart rhythm. Moreover, the loss of a cycling VCS population in the P7 MI heart, characterised transcriptionally by expression of both the cardiomyocyte-like VCS signature and a G2M/S signature, is significant. Absence of these cycling cells is a likely contributing factor resulting in the lost ability to repair and repopulate the cardiac conduction network in non-regenerative hearts.

Differential expression analysis identified a requirement for an injury-responsive gene set in the regenerative P1 heart. A number of the upregulated genes play known roles in transcriptional regulation, including *Cdk8* (component of the mediator complex), *Hmgn2* (regulator of the interaction between DNA and histone octamers), *Cbx5* (heterochromatin component), *Tcea19* (regulator of Yap localisation). The injury-responsive gene set also included many synaptic signalling genes (e.g., *Dpysl3*, *Kctd16*, *Ppp1cc*). These genes likely act in concert, therefore, to enact a regeneration-competent chromatin landscape and remodel the cell. Finally, the identification of a specific functional requirement for appropriate dosage of transcription factor NKX2-5 in regeneration of the VCS network after MI at P1, validated the functional relevance of this injury-responsive gene set for successful restoration of the conduction system during neonatal heart regeneration.

5.4.3 Changes in expression of ion channels and calcium handling machinery in the non-regenerative heart

One of the most notable transcriptional signatures of the infarcted VCS was the downregulation of ion channels and calcium handling machinery in non-regenerative P7 conduction cells. The reduced expression of delayed rectifier potassium channels (including *Kcnq1* – see **Figure 5.15b**), together with loss of KChIPs (including *Kcnip2*) that regulate the outwards current I_{to} , is likely to lead to a lengthening of the duration of the ventricular action potential, with potential arrhythmic consequences^{225,226}. Moreover, these cells also showed reduced expression of proteins required for normal calcium handling including *Kcnj11*, *Slc8a1*, *Ryr2*, *Cacna1c*, *Cacna1d* (**Figure 5.15a-b**), and consequently may be at increased risk of becoming highly arrhythmogenic²²⁷.

Alongside dysregulation of ion handling, the infarcted P7 VCS cells downregulated genes involved in cell-cell coupling at the desmosome, and in cell-cell adhesion. Most notably, *Dsp*, *Ctnna3*, *Pkp2*, *Dsg2* (desmosome components), and *Ncam1*, *Alcam* (cell-cell adhesion molecules) were all dramatically downregulated at P7 (**Figure 5.15a-b**). Previous studies have reported a causal link between reduced *Pkp2/Dsg2* and delayed cardiac conduction, alongside reduced sodium current and altered cellular calcium handling in cells with reduced or impaired PKP2/DSG2²²⁸⁻²³⁰. This, therefore, supports the hypothesis that the gene expression changes identified here in the P7 VCS may promote arrhythmogenesis after MI.

Loss of *Ncam1* and *Alcam*, known Purkinje markers with important roles in cell adhesion, is likely to affect network architecture and further to lead to delayed conduction¹⁶⁹. Moreover, it has been found that *Dsp*-encoded desmoplakin is required to stabilise connexin integrity at the desmosome: in conditional *Dsp* knock-out mice, CX40 protein expression was dramatically downregulated in the VCS despite no detected decrease in *Gja5* transcription²³¹. The authors correlated the reduction in *Dsp* and post-transcriptional loss of CX40 with development of significantly prolonged activation time and AP duration, and with

right bundle branch block. This is especially interesting because it provides a potential mechanism at the level of genetic regulation for the severe downregulation of CX40 that I identified across regions of the infarcted P7 VCS by 21 days post-MI in Chapter 4 (**Figures 4.5-4.6**). Whether defective coupling at the desmosome due to these changes in gene expression following MI also leads to fibro-adipose replacement, pro-apoptotic signalling and/or abnormal mechanical coupling between cells as is seen in arrhythmogenic cardiomyopathy, warrants further investigation²²⁸.

The comparative nature of my scRNA-seq analysis between regenerative P1 versus non-regenerative P7 hearts, allowed me to identify upregulation of these desmosome and cell-cell adhesion gene sets in the P1 VCS as compared to sham control hearts. It should be noted, however, that my experiments were undertaken in a CX40 heterozygous background, with potential consequences for the observed gene expression changes. Nevertheless, the divergent responses of the P1 versus the P7 VCS provide a platform for strategies to restore CX40 levels and fast conductivity to the mature His/Purkinje network following MI.

Overall, therefore, the identification of core calcium handling components, of ion channels/exchangers, and of desmosome and cell-cell coupling proteins which are downregulated in the infarcted P7 VCS but maintained in the regenerating P1 VCS provides potential targets to reduce pathophysiological remodelling in the non-regenerative context and support reconstitution of a functional electrical network.

5.4.4 The implications of spatially dysregulated expression of NCX1

The finding that the sodium-calcium exchanger NCX1 is ectopically expressed in a highly specific punctate pattern around CX40-negative regions of the infarcted P7 network, both in the days immediately following MI but also in the chronic MI heart, was unexpected and interesting (**Figure 5.15-5.16**). In normal cardiac physiology, NCX1 is responsible for regulating intracellular Ca^{2+} concentration by mediating the removal of the trigger Ca^{2+}

during the repolarisation phase of the cardiac AP²³². When cytosolic calcium levels are high during systole, Ca^{2+} is extruded from the cytoplasm into the extracellular space by NCX1; 1 Ca^{2+} ion is extruded in exchange for 3 Na^{+} ions, generating an inwardly-directed membrane current I_{NCX} . Additionally, NCX1 can mediate cardiac repolarisation indirectly by modulating the calcium current I_{Ca} , through a functional interaction with L-type calcium channels: an increase in I_{NCX} causes increased I_{Ca} , overall combining to cause prolonged cardiac repolarisation²³². Contrastingly, during conditions of high intracellular Ca load, the NCX current can reverse leading to a transient inward current I_{ti} which contributes significantly to cytoplasmic Ca^{2+} overload. Hence, localised increase of NCX1 affecting AP dynamics in the slower-conductance regions of the infarcted heart where CX40 expression has been lost, is likely to have important functional consequences for local impulse propagation, AP duration and likelihood of arrhythmogenic activity.

Importantly, slowed AP repolarisation has been hypothesised to induce afterdepolarisations that might go on to trigger tachyarrhythmias^{232,233}. These may be in the form of early afterdepolarisations (EADs) (often linked to enhanced Ca loading during the prolonged AP) or delayed afterdepolarisations (DADs) (often linked to the NCX-mediated transient inward current I_{ti}). Moreover, increased NCX1 experimentally has been shown to promote arrhythmias, whereas genetic or pharmacological strategies to inhibit NCX1 activity can protect from arrhythmic activity²³⁴. Consequently, NCX1 inhibition has previously been suggested to have substantial therapeutic potential²³². The specific and local upregulation of NCX1 identified here in the infarcted heart, suggests that therapies aiming to inhibit NCX1 to protect from arrhythmia may need to consider how to achieve highly targeted delivery to affected nodes, rather than systemic inhibition, to improve drug efficacy. Moreover, it is possible that these results may have implications for identifying the source of automatic foci in patients with ventricular arrhythmia, and for directing catheter/ laser ablation therapy in these patients after MI. Future experiments will be needed in order firstly to determine the specific cell type(s) within which the identified NCX1 overexpression occurs, and secondly the functional consequences of this overexpression.

5.4.5 The gene expression changes underpinning increased incidence of conduction block following myocardial infarction in female versus male hearts

While clinical studies conducted over several decades have noted extensive differences in the prevalence and severity of arrhythmias that present in male versus female MI patients^{219,235-237}, the mechanisms underpinning these sex differences have largely been elusive. The results presented in the final section of this Chapter elucidated a strong sex-dependence in VCS transcriptional response to MI and identified the gene-sets underpinning male versus female electrical repair following conduction system damage.

It is especially interesting that I uncovered such extensive VCS gene expression divergence in male versus female infarcted hearts given that these experiments were performed in the neonatal context, prior to sexual maturity. Although the timing of puberty onset in mice is strain-dependent, puberty is not reached before P26-30²³⁸, significantly later than the P1 and P7 post-natal stages examined experimentally in this work. Moreover, prior analysis has shown that levels of the gonadal hormones estradiol, progesterone and testosterone are low at both P1 and P7 stages²³⁹. This has important implications, since the differences identified in my scRNA-seq analysis can consequently be attributed primarily to genetic factors (i.e., linked to the sex chromosomes and gene dosage) rather than to hormone levels.

It is noteworthy that the regenerative response in P1 hearts involved many more genes in the female than the male VCS, and that a much greater proportion of genes were differentially upregulated in female than in male VCS cells following MI versus sham surgery. Furthermore, the male VCS cells upregulated genes involved in similar pathways following MI at regenerative or at non-regenerative stages, whereas the non-regenerative female VCS response differed substantially from the regenerative response, suggesting worse regenerative competence in the P7 female VCS. This correlated with an increased incidence of 1st degree AV block in female versus male mouse hearts 21 days following P7 MI. This is

directly comparable to the increased AV block which has been seen in female acute MI patients clinically; contrastingly, men have been found to have increased incidence of life-threatening ventricular arrhythmias^{235,240}. Consequently, this analysis highlights the need for future research focused on elucidating the mechanisms driving these sex-linked differences in arrhythmia presentation, so as to diagnose and treat these conditions more effectively in the clinic.

Overall, the sex-dependent analysis presented in this Chapter uncovered a significant divergence between the VCS injury response of male versus female hearts which correlated with increased incidence of 1st degree AV block in non-regenerative female hearts after MI. Going forwards, it will be valuable to perform equivalent analyses following MI in adult mice, after onset of full sexual maturity. Indeed, a full comparison of differentially expressed gene-sets between non-regenerative P7 versus adult contexts would not only help in identification of the key drivers of electrical regeneration versus pathological VCS remodelling in female versus male hearts, but would also enable identification of the extent to which mature sex-dependent responses are hormonally versus genetically determined.

5.5 Conclusions

The results of this Chapter elucidate the transcriptional signature of the healthy, regenerating and non-regenerating VCS. I identified extensive heterogeneity within the post-natal mouse VCS, including a fibroblast-like conduction cell population with a distinct gene expression profile that has not been characterised previously. Analysis of regenerating versus non-regenerating VCS cells found a requirement for an injury-induced gene-set and a specific functional requirement for *Nkx2-5* in restoration of the His/Purkinje network during neonatal heart regeneration after MI at P1. After MI at P7, the aberrant changes detected included a shift in cell composition combined with significant down-regulation of ion channels, calcium handling machinery, desmosome and cell-cell adhesion markers. Finally, I identified divergent gene expression profiles in the male versus female VCS which correlated with functional outcome, manifesting as conduction delay via surface ECG recording. Collectively, the results presented in this Chapter inform our molecular understanding of the healthy and infarcted VCS and reveal the gene networks responsible for driving electrical restoration during neonatal heart regeneration, versus pathological remodelling during fibrotic repair.

6 The consequences of His/Purkinje electrical remodelling for arrhythmogenesis

6.1 Background

6.1.1 The murine and human electrocardiogram

The electrocardiogram (ECG) has been a primary diagnostic tool for cardiac patients since the first decades of the 20th century²⁴¹. ECGs provide a graphic representation of the electrical potentials that are generated during the heartbeat. When an electrical impulse is initiated in the SAN and conducted through the CCS, further propagation causes weak electric currents to spread throughout the body. Electrocardiography capitalises upon this by positioning electrodes in particular locations on the body in order to measure the relative electric potentials and so to provide a stereotyped read-out of cardiac electrical activity. ECGs recorded in this way were first used to diagnose arrhythmias in the 1900s, and shortly afterwards were applied to recognise and confirm diagnosis of MI^{242,243}. In the intervening decades, the ECG has become a fundamental component of the clinician's toolkit in order to diagnose and treat many cardiac conditions including acute MI and associated arrhythmias.

The first detailed murine ECG analysis was performed on C57 BL/10 and SEC/1 healthy mice under ether anaesthesia in 1968, over 70 years after the equivalent analyses had been performed on human subjects²⁴⁴. Indeed, the mouse has only become a commonly-used model for studying cardiac conduction in the last twenty years^{244,245}. There are several key differences between the human and mouse surface ECG that require careful consideration for correct interpretation of murine ECG recordings.

Firstly, due to the 10-fold higher resting heart rate of mice as compared to humans, the mouse ventricular action potential (AP) is much more rapid. This makes studying ventricular conduction velocity challenging using mouse surface ECG recordings. In humans, QRS duration provides a useful read-out for determining the origin of each QRS complex; extended QRS intervals are a reliable indicator of bundle branch block (BBB) or of non-specific conduction delay. However, in the mouse a delayed ventricular conduction velocity results in the QRS complex ending prior to completion of ventricular activation²⁴⁶, partly on account of the rapid heart rate, and also due to earlier repolarisation in the mouse as compared to the human. The early murine repolarisation event can also result in changes to QRS duration independently from any change to ventricular conduction velocity, confounding ECG analysis. Moreover, in mice the activation time of the left ventricle is shorter than that of the right ventricle; consequently, any delay in left ventricle activation does not correlate linearly with increased QRS duration²⁴⁶. As a result of these combined factors, increased QRS interval length cannot be used reliably for diagnosis of BBB or delayed ventricular conduction in mice. However, a prolongation of the PR [PQ] interval is generally considered a dependable indicator of 1st degree atrioventricular block that manifests more consistently via the murine ECG.

Secondly, it is important to note that mice and humans display different AP profiles: the murine AP is triangular and does not have an equivalent plateau phase to that seen in humans²⁴⁵. Since differences in ion currents bring about these distinctions between the mouse and human AP, the impact of changes in particular ion channels affecting either depolarisation or repolarisation phases show up differently on mouse versus human ECGs²⁴⁷.

Finally, the overall mouse ECG morphology differs from that of humans (**Figure 6.1**). Most strikingly, the mouse has a non-isoelectric ST segment with an additional J wave that occurs due to early repolarisation and can merge into a negative T wave. The presence of the J wave significantly alters ECG patterns under different drug treatments or pathologies as compared to human ECG morphologies. Moreover, determining the end of the QRS is

challenging in mice on account of the immediate transition into the J wave. While the end of the J wave used to be taken as the end-point of the QRS complex in interval measurements, many studies now measure the QRS complex up until the point at which the S wave crosses the isoelectric line, in order to exclude early repolarisation from the measure of ventricular depolarisation^{245,246}.

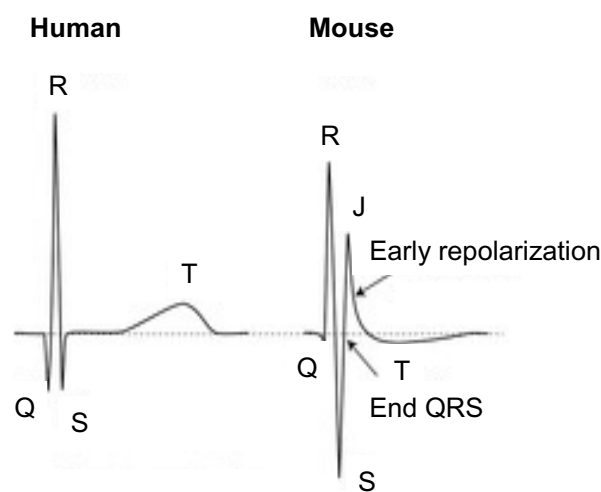


Figure 6.1: The human and murine ECG

A schematic to illustrate the morphology of the human and mouse ECG. Whereas the human ECG has an isoelectric ST segment and clearly defined T wave, due to early repolarisation in the mouse a characteristic J wave follows immediately after the murine QRS complex. *Figure adapted with permission from Boukens et al., 2014, the Journal of Physiology, copyright of John Wiley and Sons.*

Overall, though notwithstanding the species differences noted above, the mouse has become a very useful model for studies of cardiac conduction. We have developed an increasingly good understanding of the differences between mouse and human ECG. While care must be taken in the interpretation of changes to mouse ECG complex morphology and interval measurements, mouse ECG recording allows for successful identification of arrhythmias and normal versus pathological electrical function.

6.1.2 Computational approaches to modelling and simulation of the infarcted human heart: from ionic currents to electrocardiographic signal

Performing experimental investigations with human subjects is challenging, and in many cases, it is not possible to generate appropriate and relevant cardiac electrophysiology data. However, progress in human-based cardiac modelling and simulation methods is supporting increasingly sophisticated investigations of the healthy and diseased heart. In particular, the ability to incorporate cell-, tissue- and organ-level information into a unified computational framework allows for granular analysis of the impact of genetic mutations, drug treatments and disease states for cardiac electrophysiology. Such modelling can produce instructive ECG and whole-organ simulation results that help to refine our theoretical understanding and enable testing of hypotheses arising from experiments with animal models, in a human setting. Ultimately, by building towards development of patient-specific simulations (so-called cardiac “digital twins”) built using a patient’s own MRI-derived anatomy and electrophysiology data, the hope is that these models will inform diagnosis in the clinic and aid in selection of tailored treatments for cardiovascular diseases²⁴⁸.

Incorporating the His/Purkinje system into cardiac computational models has been especially challenging, largely on account of the high morphological complexity of the Purkinje network⁸⁴. Due to difficulties in identifying VCS cells as distinct from the working myocardium, and in imaging the intact network’s structure (see **Chapter 3.1.3**), understanding the 3D complexity of the VCS network has been held back by experimental and technological limitations. Consequently, numerous methods have been developed to derive realistic VCS networks. First, image-based approaches try to circumvent the need for imaging the intact VCS network with high resolution, by segmenting Purkinje networks from 2D imaging data and projecting these onto anatomically-accurate 3D heart models²⁴⁹. However, this methodology cannot practically be applied to incorporate subject-subject

variability and so to generate human patient-specific His/Purkinje systems⁸⁴. Secondly, and sometimes in combination with image-based segmentation approaches, fractal trees can be built in order to simulate the Purkinje network and grow out regions of the network that cannot be identified via image processing²⁵⁰⁻²⁵². Thirdly, methods based on optimisation principles have been successfully applied to generate realistic Purkinje networks that include a large number of morphological and electrophysiological inputs⁸⁴. Taken together, recent progress has therefore resulted in several alternative approaches to make electrically and topologically accurate VCS networks, that can be incorporated into multiscale computational frameworks for modelling human cardiac electrophysiology.

In this Chapter, a human-based multiscale modelling and simulation framework was used to investigate the consequences of the His/Purkinje remodelling I identified in the neonatal mouse LAD MI model, for arrhythmogenesis in infarcted human hearts. The computational framework was calibrated and evaluated using extensive experimental and clinical data from ionic to ECG levels, and the biventricular anatomy was obtained from clinical MRI data⁸⁵. The model includes an integrated *in silico* Purkinje network constructed using optimisation principles⁸⁴. Modelling was performed in both healthy and MI contexts, building on previous studies from the Rodriguez group. Simulations of electrical AP propagation in both the ventricular tissue and the integrated *in silico* Purkinje network were performed using the MonoAlg3D software, an efficient parallel cardiac solver that has been described previously²⁵³. Overall, therefore, employing a biophysically-detailed computational framework to simulate human cardiac electrophysiology provided a means by which to interrogate the effects of the His/Purkinje pathological remodelling that I previously identified in this thesis, for arrhythmogenesis in human MI patients.

6.2 Aims and Objectives

The aims of this chapter were to:

1. Determine whether there is functional electrical regeneration following myocardial infarction in the neonatal mouse heart
2. Assess the consequences of the non-regenerative electrical remodelling identified in Chapters 4 and 5 for arrhythmogenesis in the *in silico* infarcted human heart

6.3 Results

6.3.1 Electrophysiological analysis following neonatal heart regeneration

The results from Chapters 4-5 of this thesis indicated that while the P1 heart can reconstitute a full His/Purkinje network following MI, the P7 heart fails to do so. Through a combination of wholemount immunostaining, fluorescence imaging, RNA fluorescence in situ hybridisation and scRNA-seq analyses, I identified sustained loss of electrical identity in regions of the non-regenerative infarcted VCS, as evidenced by downregulation of CX40, reduced sodium and potassium channel expression, altered calcium handling machinery, downregulation of Purkinje cell adhesion proteins, as well as altered expression of ion exchangers including NCX1 and HCN4.

Collectively, this body of evidence supports the hypothesis that synchronous propagation of the electrical impulse through the ventricles becomes disrupted following MI in the non-regenerative P7 heart but is maintained in the P1 regenerative setting. Here, I

trialled two approaches in order to test this hypothesis and functionally validate the extent of electrical regeneration in the neonatal mouse heart.

6.3.1.1 Proof of concept: reading cardiac action potentials from infarcted mouse hearts using microelectrode array recordings

I first set out to establish a means by which to perform electrophysiological recordings directly from the infarcted ventricle of the neonatal mouse heart. Initially, I selected a Microelectrode Array (MEA) approach. MEA devices are commonly used to record cardiac APs from cardiomyocyte cultures, and have also been applied to record from cardiac slices of the embryonic and adult mouse heart²⁵⁴⁻²⁵⁶. The strength of the MEA approach lies in the ability to take simultaneous high-quality recordings from 60-120 electrodes arranged in a grid. While patch clamping remains the gold standard for signal quality, the ability to record cardiac APs from a broader area of tissue facilitates mapping of signal propagation across the array which cannot be achieved using patch clamp techniques. However, MEA devices have not previously been used to record from a cardiac ventricle preparation directly without histological slicing. A slice preparation would have been uninformative for my purposes, though, since slicing the heart prevents any interpretation across the extent of the 3D His/Purkinje network to correlate with my whole-mount imaging and phenotyping. I hoped that by designing a novel wholemount recording set-up from the whole left ventricle, this might ultimately enable me to build a complete left ventricular propagation map, to assess the impact of regional conductivity loss within the VCS on electrical impulse propagation and wider cardiac electrophysiology. I first planned, therefore, to test whether it would be feasible to establish MEA recordings from the whole mouse ventricle.

To this end, following MI and sham surgeries on neonatal *Cx40-eGFP/+* mouse hearts, I dissected open the left ventricular chamber to expose the VCS network under a

fluorescent dissection scope, dissecting as quickly as possible so as to keep the live tissue healthy (as described in **Chapter 2.4.4.1** and **Chapter 2.8.1**), but with minimal disturbance of the atrial chambers. A 60-electrode MEA2100 recording chamber was next filled with CO₂-independent medium at 37°C. The exposed endocardial inner surface of the ventricle was positioned face down onto the MEA such that the ventricle was localised centrally over the electrodes, and the heart weighted down to ensure good contact with the individual electrodes using a circular metal weight.

In this way, following trial rounds performed with technical input from Multichannel Systems, Harvard Bioscience, I was successfully able to collect cardiac AP recordings directly from the inner ventricular chamber using the Multichannel Systems 2D software (**Figure 6.2**). This, therefore, provided proof of concept confirming the applicability of this method as a novel means by which to record APs from the ventricle of the wholemount mouse heart. However, the recordings were not consistent between samples. Firstly, heart rate was very variable between samples with rates varying 4-fold even between individual hearts of equivalent treatment type, and heart rate was always much lower than expected from a healthy heart recorded *in vivo* (**Figure 6.2a, c**). This is probably on account of suboptimal temperature and nutrient conditions during incubation of the heart, and further due to the tissue becoming hypoxic over time. Use of a perfusion cannula with temperature control and optimised buffer conditions might ameliorate these issues going forwards. Secondly, I observed variability in the amplitude of the signal recorded from one heart to the next. This is most probably due to unreliable contact between the ventricle and the electrodes. Optimisation of the set-up, including use of a heavier weight pressing down from above, or recording with a 3D electrode array instead of a 2D array, might improve signal amplitude for future recordings. Thirdly, very likely for the same reason, the signals I recorded were noisy and it was consequently not possible to identify P or T waves, or to measure QRS durations from the resulting traces. Finally, using the standard-sized 60-electrode MEA, propagation was almost instantaneous across the array (**Figure 6.2b, d**). This is perhaps unsurprising given the small area of the standard 60-electrode MEA. Use of

a 120-electrode wide-spaced MEA which can cover a 1-1.5cm diameter area will consequently be necessary in future recordings in order to map propagation patterns across the full left ventricular chamber.

Overall, therefore, using MEA recording to read APs directly from infarcted mouse hearts seems to be a promising approach for future studies (see **Chapter 7**). However, due to the extent of optimisation which would have been required to apply this technique reliably for my purposes, I decided instead to proceed by adapting conventional rodent ECG in collaboration with Professor Neil Herring, to collect recordings from the neonatal mouse following MI.

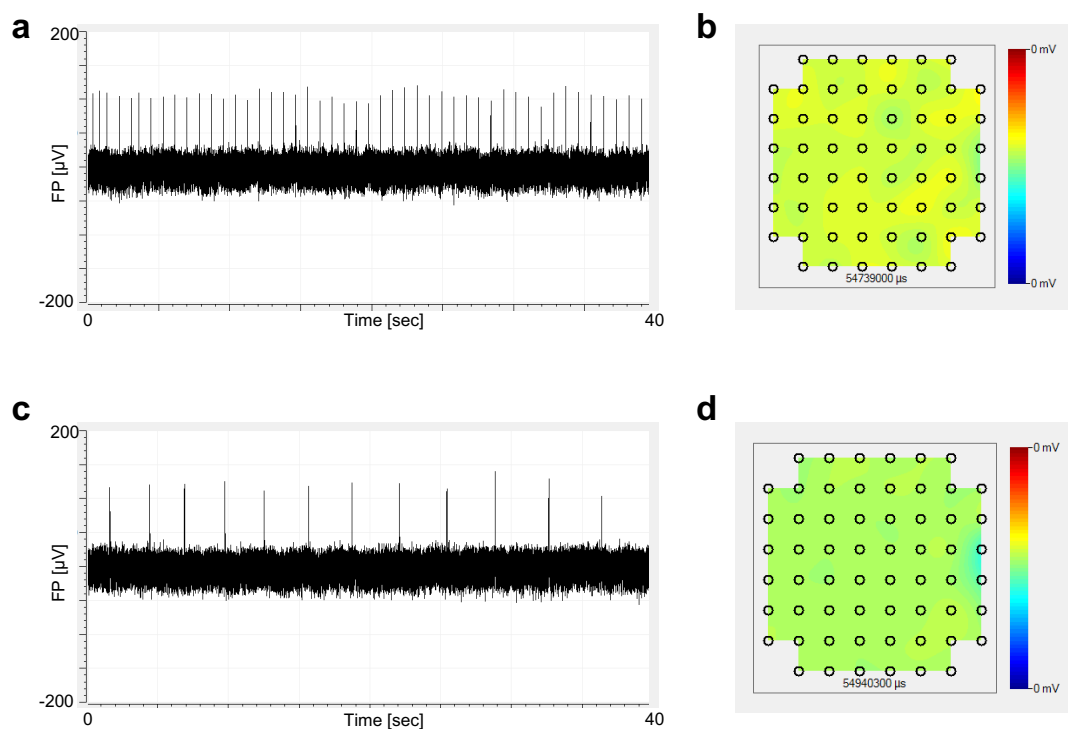


Figure 6.2: Cardiac action potential microelectrode array recordings from a wholemount cardiac ventricle preparation

Recordings from the inner chamber of dissected wholemount ventricles, 21 days following MI surgery at post-natal day 1. **a-b** and **c-d** show representative traces from 2 different mouse hearts. **a, c**: example MEA 40-second traces. **b, d**: false colour maps showing the spatial distribution of local field potentials across the 60-electrode array.

6.3.1.2 Demonstration of electrical regeneration in the neonatal mouse versus sustained conduction delays beyond the regenerative window

To determine the functional impact of putative electrical regeneration versus sustained VCS remodelling that I observed over the neonatal regenerative window in Chapters 4-5, I next determined to perform ECG recordings 21 days after MI or sham surgery to regenerative P1 and non-regenerative P7 mice. I recorded lead II and lead III surface ECGs under isoflurane-induced general anaesthesia and collected ECGs from between 4 and 6 mice per treatment group to power statistical analysis of the resulting datasets.

I found no significant detectable differences between MI versus sham control groups following P1 surgery in terms of ECG traces or interval analysis (**Figure 6.3**). However, following MI at P7 mice had altered ECGs including merged T and P waves, a greater negative deflection at the end of the QRS / J wave and a biphasic P wave (**Figure 6.3a**). Moreover, interval analysis identified a significantly prolonged PR interval in the P7 MI condition as compared to all other groups, evidencing conduction delay (ANOVA with Tukey-Kramer test for multiple comparisons: p-values of 0.0113 (P1 sham v P7 MI), 0.0014 (P1 MI v P7 MI) and 0.0161 (P7 sham v P7 MI)) (**Figure 6.3b**). Taking the previously reported “normal” PR interval range of 30 - 56msec for mice²²¹ and the previously suggested threshold of > 60msec as a cut-off indicator of 1st degree AV block²²², in this study 50% of P7 MI animals (n=6) had 1st degree AV block whereas no animals in any of the other treatment groups displayed this pathology. These data therefore indicate normal electrical function in the regenerated VCS following MI at P1, versus substantial delays to atrioventricular conduction due to electrical remodelling following MI at P7.

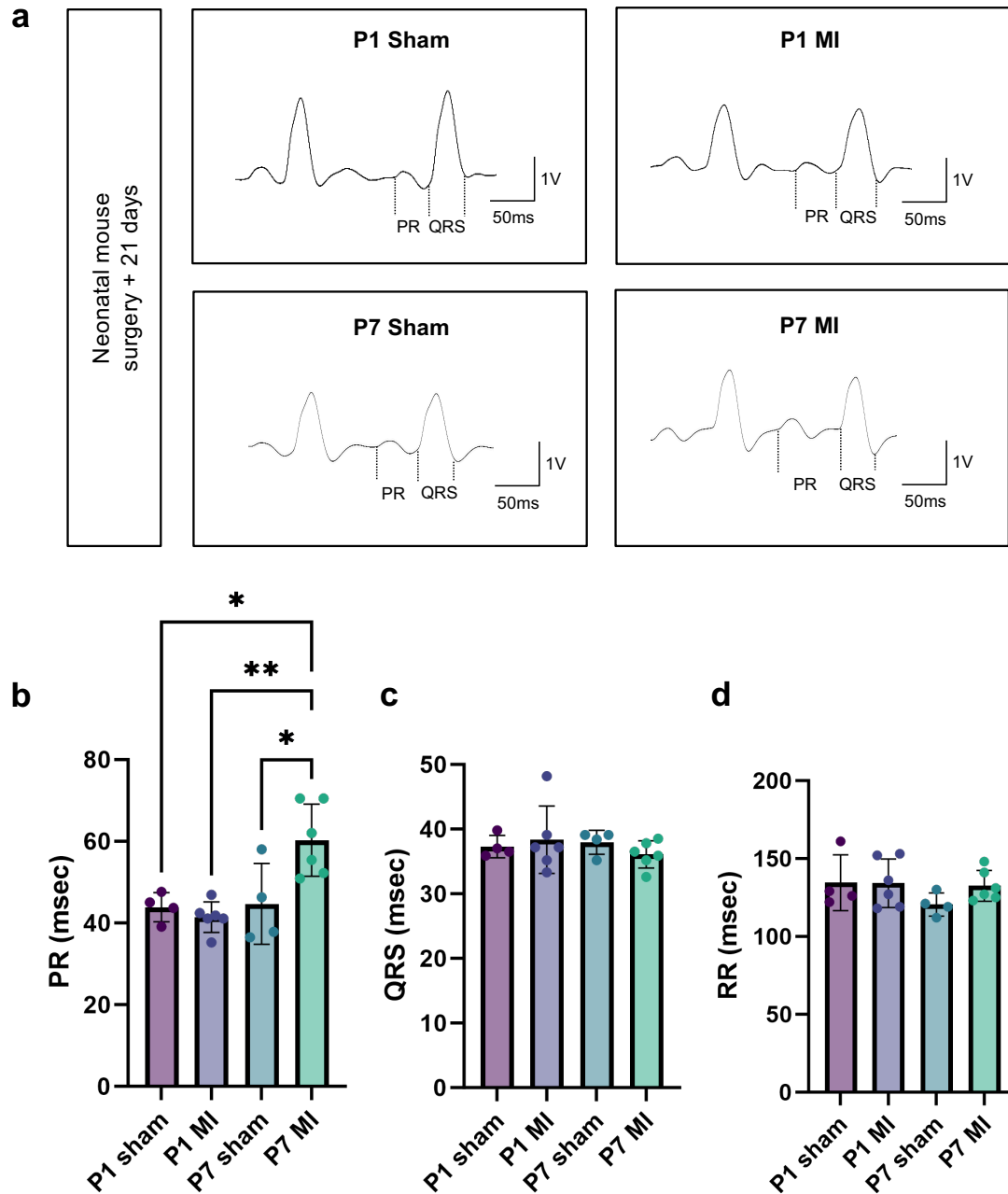


Figure 6.3: Regeneration of cardiac rhythm during neonatal heart regeneration versus significant conduction delay following non-regenerative fibrotic repair

a. Representative lead II surface ECGs from mice 21 days following MI or sham surgery at P1 and P7 stages. ECGs from P7 MI mice had prolonged PR intervals and biphasic P waves whereas ECGs from P1 MI and P1/P7 sham mice were comparably normal. **b.** Graphs quantifying PR, QRS and RR intervals in mice 21 days following P1 sham, P1 MI, P7 sham and P7 MI surgeries. The PR interval was significantly longer in P7 MI mice as compared to all other groups but no significant differences in QRS or RR intervals were detected, indicating an overall delayed atrioventricular conduction in the non-regenerative infarcted heart. Statistics: ANOVA with Tukey-Kramer test for multiple comparisons (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

6.3.2 Modelling and simulation of electrical remodelling in the His/Purkinje network of the human heart following myocardial infarction

The results of the following section of this Chapter contain work completed in collaboration with Dr. Hector Martinez-Navarro in the Department of Computer Science, University of Oxford. All modelling and simulation experiments were executed by Hector; I conceptualised the project and hypotheses to be tested, aided in the overall model design, and we together performed downstream data analysis of the resulting simulated ECG recordings.

6.3.2.1 Regionally reduced conductivity within the Purkinje system produces ECG signatures of delayed conduction

The major unknown following the results of Chapter 4-5 and the ECG analyses above, was the consequence of regional downregulation of CX40 and loss of His/Purkinje conductivity at non-regenerative stages for arrhythmogenesis in human patients following MI. To address this question, I designed a series of modelling experiments in collaboration with Hector Martinez-Navarro. We set out to develop a computational framework and to conduct simulations, using an *in silico* human ventricular model with Purkinje and chronic LAD MI (first weeks post occlusion). My aim was to use this methodology in order to predict the clinical consequences of the pathological His/Purkinje remodelling identified in Chapters 4-5 of this thesis.

I hypothesised that CX40 loss would lead to a regional reduction in conduction velocity in the affected area of the VCS network, resulting in delays in activation sequence and abnormalities in the QRS complex in the ECG. Due to the lack of experimental data in human Purkinje systems post-MI, it was first necessary to run a sensitivity analysis to assess the range of possible conductivity values in the His/Purkinje regions suffering from

CX40 loss. For this, antero-septal MI was simulated co-existing with a variable conduction delay in the LBB, in a region defined to match the CX40 loss that I identified in non-regenerative P7 mouse hearts following MI (**Figure 4.5**). Mild conduction delays led to negligible alterations in activation sequences, whereas severe and extreme conduction delays reproduced the ECG phenotype of left bundle branch block (LBBB) patients, and both severe and extreme delay showed very similar results. The CX40 loss in our in-silico experiments was modelled, therefore, by attributing the severe delay condition of ventricular conductivities to the affected VCS network segments, resulting in conduction velocities similar to those measured experimentally in human ventricular myocardium, namely ~ 65 cm/s²⁵⁷.

We found that the placement of ventricular-like conductivities in small patches of the His/Purkinje network within the infarcted human heart was sufficient to disrupt the normal ECG signature (**Figure 6.4**). Reduction of conductivity in the LBB patch designed to match the experimental findings in the murine LBB resulted in an ECG signature typical of a LBBB patient (**Figure 6.4b-b'**). To test the impact of equivalent remodelling in the RBB, we next modelled reduced conductivity in a patch of RBB network segments and found that this resulted in the stereotypic ECG signature of right bundle branch block (RBBB) patients (**Figure 6.4c-c'**). Furthermore, since my experimental data found reduced CX40 not only in the BB but also in more distal regions of the left His/Purkinje network (**Figure 4.5**), we modelled reduced conductivity in a left ventricular free wall His/Purkinje patch (**Figure 6.4d**). Placement of ventricular-like conductivity in a small patch of free wall network segments resulted in ECG signatures similar to those seen in patients with non-specific intraventricular conduction delay (NSIVCD) (**Figure 6.4d'**). Overall, therefore, these results indicated that the mechanism of reduced His/Purkinje conductivity following MI identified in the non-regenerative mouse heart in Chapters 4-5 of this thesis, is sufficient to reproduce the classic ECG patterns of MI patients with clinical arrhythmia.

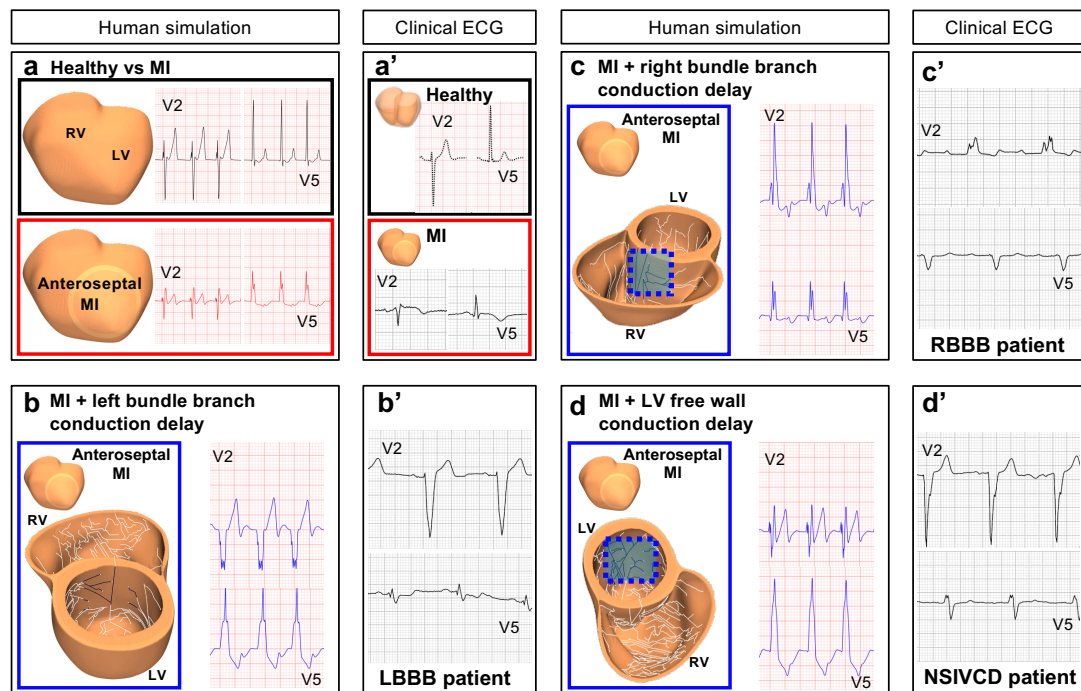


Figure 6.4: Regionally reduced conductivity within the His/Purkinje network results in ECG signatures of pathological conduction delay

a-d. Simulated human ECGs presented alongside analogous clinical patient ECGs (corresponding panels **a'-d'**). Leads V2 and V5 are presented in all cases. **a/a'**. The simulated ECG under healthy conditions closely mirrors the morphology of the ECG from the individual whose anatomy was used to construct the model (black boxes); incorporating an anteroseptal chronic infarct from LAD MI into the model produces an ECG with an inverted T wave, fractured and lengthened QRS and ST deviation (red boxes). ECG alterations are consistent with the clinical ECG in the **a'** red box, obtained from a 70-year-old male with hypertension presenting with angina and out of hospital VF arrest (treated with CPR and 1 successful AED shock), who had occlusion of the proximal LAD and reduced ejection fraction (42%). The patient's ECG shows T wave inversion and anterior ST elevation. **b.** Regionally reduced conductivity in a defined patch within the left bundle branch in the simulated anteroseptal MI heart results in an ECG signature typical of left bundle branch block (LBBB), characterised by prolonged QRS, and QRS morphology alterations (leading to QS or RS complexes and notched R waves in lateral leads). These alterations are recognisable in the clinical ECG **b'**, obtained from a 69-year-old male with hypertension, type II diabetes mellitus and end stage renal failure presenting with heart failure symptoms. Patient had large anterior MI with no inducible ischaemia on myocardial perfusion scan. Patient had severely impaired LV systolic function (ejection fraction 30%) and LBBB (QRS duration 173 ms). **c.** Regionally reduced conductivity in a defined patch within the right bundle branch in the simulated anteroseptal MI heart results in an ECG signature typical of right bundle branch block (RBBB), including QRS prolongation and morphological changes (RSR' complex

in V1/V2, slurred S wave in lateral leads). This is comparable to the clinical ECG in **e'**, obtained from a 51-year-old man with type II diabetes mellitus presenting with heart failure symptoms 2 years following coronary artery by-pass grafting. Patient had severely impaired LV systolic function on transthoracic echocardiography (previously normal), 1st degree heart block (277 ms) and RBBB (QRS duration 161 ms) despite medical optimisation. A myocardial perfusion scan showed a fixed apical perfusion defect, further apical to mid inferior lateral defect, with minimal inducible ischaemia. **d.** Regionally reduced conductivity in a defined patch of Purkinje fibres in the left ventricular free wall in the simulated anteroseptal MI heart results in an ECG signature typical of non-specific intraventricular conduction delay (NSIVCD) (including wide QRS and slurred S wave). Comparable changes are seen in the clinical ECG **f'**, obtained from a 63-year-old male with 2 previous MI events and subsequent coronary artery bypass grafting presenting with worsening heart failure symptoms. Severely impaired function (ejection fraction 15%) and primary prevention implantable cardioverter defibrillator (ICD). Broadening of QRS (160msec) with NSIVCD.

6.4 Discussion

In this Chapter, I analysed the impact of electrical remodelling in the His/Purkinje system, on arrhythmogenesis following acute MI. Using surface ECG recording with Dr Neil Herring, I identified functional electrophysiological anomalies associated with the structural and molecular changes identified in the infarcted VCS in Chapters 4-5 across the neonatal regenerative window. This manifested as transition from normal ECG recordings following MI at P1, to pathological conduction block as indicated by biphasic P waves and significantly lengthened PR intervals after MI at P7. In collaboration with Dr Hector Martinez-Navarro, I subsequently incorporated the identified mechanism of regionally reduced conductivity in the His/Purkinje network into a human modelling and simulation framework. The computational analysis implicated these changes as causative for BBB and greatly increased dyssynchrony of cardiac contraction. The mechanism of reduced Purkinje conductivity was sufficient to disrupt normal ventricular activation sequence, ventricular repolarisation sequence and ECG signature. Collectively, these results establish functional regeneration of the conduction system at neonatal stages in the mouse, versus pathological conduction delay mirroring

clinical arrhythmias seen in patients beyond the regenerative window and extending into the human context.

6.4.1 Functional electrical regeneration during neonatal heart regeneration

While it has previously been established that there is recovery of ventricular function during neonatal heart regeneration¹⁸⁶, whether and how this translates to functional electrical and conductive maintenance or restoration had not previously been investigated. In this Chapter, I performed an electrophysiology study which demonstrated recovery of normal electrical function during neonatal mouse heart regeneration following MI at P1, versus pathological conduction delays including 1st degree AV block during fibrotic repair following MI at P7.

Interestingly, a prior study using comparative epicardial electrophysiologic assessment of neonatal pigs found similar epicardial activation patterns and electrophysiology between P1 MI and healthy control hearts, but slower conduction velocity, delayed apex activation and isochrones showing left or right BBB in all P7 piglets post-MI²⁵⁸. This pig study relied exclusively on epicardial mapping data, possible given the larger size of the neonatal pig heart, and consequently did not interrogate cellular/molecular mechanism or regeneration in the His/Purkinje network. However, the electrical recovery of full function at P1 versus impairment at P7 in the piglets can be explained by the changes I have identified in Chapters 4-6 of this thesis: in His/Purkinje bundle morphology; gene and protein expression; and susceptibility to arrhythmia. Consequently, the molecular pathways I have elucidated underpinning VCS regeneration versus pathological remodelling, likely extend to larger mammals which in turn may provide an approach for restoring proper conductivity in infarcted human hearts.

6.4.2 Reduced Purkinje conductivity outside of the infarct area as a causative mechanism for conduction delays following myocardial infarction

It is striking that the mechanism of reduced conductivity in patches of the BBs or Purkinje fibres was sufficient to reproduce strong BBB or NSIVCD signatures in the *in silico* infarcted human heart. Previous computational models have incorporated cell (ionic current underpinning the cardiac AP) through organ (MRI-derived anatomical information, ECG signature output) levels, in the context of MI. However, while these studies have been very informative with regards to elucidating, for example, arrhythmic risk due to myocardial infarction²⁵⁹ and ischaemia²⁶⁰, and impact of remodelling in the border zone²⁶¹, to the best of my knowledge this is the first modelling study of its kind to use an *in silico* Purkinje network to identify a mechanism driving BBB and NSIVCD, producing simulated ECG signatures that closely match the morphology of patient ECG recordings. This is important given the relatively common presentation of patients in the clinic suffering from conduction block following acute MI event(s).

In the modelling study presented here, the addition of Purkinje network segments with reduced conductivity overwhelmed the otherwise consistent ECG hallmarks of MI: the dominant simulated ECG signature following incorporation of remodelled conductivity related to conduction delays, especially where we modelled MI with LBBB. This, therefore, reinforces the importance of the (modified) Sgarbossa criteria for MI diagnosis in the presence of LBBB^{262,263}. Importantly, there were identifiable differences between LBBB, RBBB and NSIVCD not just in diagnosis, but also in pathophysiology; the location of the conduction delay induced variability in myocardial activation and repolarisation synchrony. This helps to explain the observed differences in the proportion of MI patients with LBBB, RBBB and NSIVCD that go on to develop heart failure, arrhythmogenesis and early mortality²⁶⁴⁻²⁶⁶.

The results of this Chapter indicate that regionally reduced Purkinje conductivity in the BBs or Purkinje fibres outside of the ischaemic zone can be a causative mechanism for

conduction diseases including BBB that are commonly seen in patient cohorts. Going forwards, extending the human computational framework employed here in order to enable precise modelling of ionic current variations within the *in-silico* Purkinje network will allow for important follow-up investigations into MI-induced Purkinje remodelling. In particular, this would enable modelling to investigate the electrophysiological consequences of the complex ion channel expression changes identified in the scRNA-seq datasets from the regenerating and non-regenerating conduction system in Chapter 5. Investigations of this sort will refine our understanding of electrical changes in the conduction system following MI, with a view to intervening to limit MI-induced clinical arrhythmias.

6.5 Conclusions

The results of Chapter 6 demonstrate functional electrical restoration during neonatal mouse heart regeneration following MI at P1. Contrastingly, surface ECG recording identified significant conduction delays including 1st degree AV block following MI at P7, indicating an inability to restore proper electrical function beyond the neonatal regenerative window. Modelling the non-regenerative murine phenotype within an *in-silico* Purkinje network in the chronic MI human heart implicated these changes as causative for conduction pathologies including BBB and NSIVCD, with ECG signatures comparable to those that are seen in acute MI patients.

7 Final conclusions and future work

7.1 Main findings

In this thesis, I set out first to characterise the anatomy of the mammalian cardiac conduction system, and secondly to analyse the response of the conduction system to MI in the context of neonatal mouse heart regeneration.

In Chapter 3, by developing a tissue clearing, advanced whole-organ imaging and analysis pipeline, I performed the first systematic 3D-anatomical assessment of the intact murine ventricular conduction system across post-natal development. I quantified the maturation of the VCS after birth and determined that while the network grows significantly during these stages, much of the network complexity already exists by P2, and that the RV network's development is delayed as compared to the LV network. I identified close interactions between tissue-resident macrophages and the VCS network, and analysed the development of the vascularisation of conduction fibres during the first weeks of life. Finally, by applying my imaging pipeline to analyse the hypomorphic conduction system of the haploinsufficient *Nkx2-5*^{+/-} mutant heart, I found that normal network architecture is maintained in low-dose NKX2-5 conditions through until P2. Contrastingly, there was significant His/Purkinje network hypoplasia by P4, suggesting that while embryonic and very early post-natal development is unaffected in these mutants, proper dosage of NKX2-5 is required from P2 onwards for maintenance of the fast-conductance VCS phenotype.

In Chapter 4, I leveraged the wholemount imaging capabilities developed in Chapter 3 in order to analyse the response of the conduction system following acute MI in the neonatal mouse model of heart regeneration. In this way, I identified direct disruption to the His/Purkinje network in the days immediately following the MI event. Fibre bundling was altered with differences between P1 versus P7 hearts, and at 3 days following MI, CX40 was significantly upregulated in the distal part of the infarcted P1 network but not the infarcted P7 network. By 21 days after MI, whereas the P1 regenerative heart had reconstituted a full CX40+ VCS network, the P7 non-regenerative heart failed to do so. Strikingly, in P7 infarcted networks I identified loss of the fast-conductance CX40 in large patches of the left ventricular conductive network, including in the BBs remote from the ischaemic area. Finally, I found upregulation of the pace-maker HCN4 channel across the infarct of P7 post-MI hearts, versus maintained low-level HCN4 expression restricted to the VCS in the infarcted P1 heart following MI. These results indicated, therefore, that there is an electro-pathological response in the P7 heart, versus broad network regeneration after MI at P1.

In Chapter 5, I elucidated the molecular mechanisms underpinning the different response of the P1 versus the P7 VCS following MI, through combined snRNA-seq, scRNA-seq and HCR gene expression analyses. Transcriptomic profiling uncovered new VCS cell markers, and identified previously uncharacterised heterogeneity of cell types within the VCS including a novel fibroblast-like conductive population. Comparative analyses between P1 and P7 stages found: a shift in cell type composition after MI at P7, and a reduction in the number of cardiomyocyte-like conduction cells; an injury-responsive regenerative gene-set activated after MI at P1, including a functional requirement for *Nkx2-5*; and a major transition at the transcriptional level in infarcted P7 hearts, involving downregulation of a suite of ion channels, cell-cell adhesion and desmosome factors required for the fast-conductance phenotype. Separating the scRNA-seq dataset by sex identified a significant sex-dependence to VCS response after MI, that

correlated with different functional outcomes manifesting on ECG of male versus female mice. Overall, these results provided insight into the gene regulatory and expression changes supporting electrical regeneration in the neonatal heart versus pathophysiological remodelling beyond the regenerative window.

Finally, the results of Chapter 6 found that following MI at P1 there is functional electrical regeneration, whereas following MI at P7 cardiac conduction is defective. This presented as 1st degree AV block and extended PR interval on the murine ECG following P7 MI surgery, indicating a lack of functional electrical regenerative capacity beyond the neonatal regenerative window. Modelling and simulation of the non-regenerative neonatal mouse phenotype in the chronic human MI setting implicated the observed changes in Chapters 4-5 as causative for BBB and greatly increased dyssynchrony of ventricular contraction. The simulated ECG results were directly comparable to the outcome observed in MI patients with conduction disease, providing evidence for a novel mechanism (distinct from direct ischaemic damage local to the infarct) to explain the origin of pathological conduction delays in MI patients.

7.2 Future work

7.2.1 Elucidating the fast-conductance gene regulatory programme and origin of left-right differences during conduction system maturation

The results of Chapter 3 identified significant differences between the post-natal development of the left versus right VCS networks, and implicated *Nkx2-5* in controlling post-natal VCS maturation from P2 onwards. However, the precise regulatory mechanisms driving the establishment of left-right differences in the mammalian VCS, and the exact details of how *Nkx2-5* acts together with *Irx3*, *Etv1* and *Ncam1*, amongst other genes, to establish the fast-conductance VCS cell phenotype, remain elusive. Future studies will, therefore, be required in order to address these outstanding unknowns. In particular, it would be informative to apply the imaging and analysis pipeline developed in Chapter 3 to single and combined *Irx3*, *Etv1* and *Ncam1* mutants, known to have hypomorphic conduction systems^{40,106,168,169}. This will help ascertain how these genes act in concert over early post-natal development to drive VCS maturation, with differences between left and right networks. Moreover, these analyses should be supported by targeted deletion experiments using VCS-, trabecular- and cardiomyocyte-specific Cre lines to test whether the involvement of these genes in VCS maturation results from VCS-intrinsic expression or from neighbouring cell recruitment and patterning activity. Finally, performing RNA-seq analysis of left versus right VCS cells through a post-natal time-course will be needed in order to establish the regulatory differences in the fast-conductance gene expression profile through development in an unbiased way.

7.2.2 Characterisation of the adult murine cardiac conduction system following myocardial infarction

In this thesis, I investigated regeneration of the conduction system in the neonatal mouse model of heart regeneration, using a comparative approach to analyse regeneration versus maladaptive repair beyond the regenerative window. Future studies will be needed to extend this analysis into the infarcted adult mouse heart, so as to ascertain whether there are biologically important differences between non-regenerative post-natal (P7) and more mature adult hearts.

The P7 heart undergoes general pro-fibrotic repair analogous to the mature adult heart post-MI⁴⁷. Indeed, the identification of pathological electrical remodelling in the non-regenerative P7 VCS network, with clear differences in network morphology (Chapter 4), gene expression profile (Chapter 5) and functional recovery as assessed by ECG (Chapter 6), as compared to the regenerative P1 VCS, validate the P7 heart as an appropriate proxy for the adult MI response. However, continued post-natal growth and expansion of the heart, the onset of sexual maturity, and development of other comorbidities with age, may all have confounding elements which manifest in the adult context. It will be informative, therefore, to study the adult conduction system's structural integrity, transcriptional response, and functional outcome following myocardial infarction.

7.2.3 Exploring candidate targets for repairing the infarcted conduction system

The finding of Chapters 4-6 that electrical regeneration occurs successfully during neonatal mouse heart regeneration, offers the prospect of understanding how this comes about in order to inspire strategies to repair conduction system damage in MI patients. Moreover, the results of Chapter 5, and particularly the identification of an injury-

responsive regenerative gene-set activated in the VCS of P1 mice, provides a series of candidate targets for restoring the non-regenerative conduction system. With this in mind, future work could follow up on these findings using three complementary strategies to assess their suitability as functional targets for cardiac electrical repair.

Firstly, the development of a robust Purkinje differentiation protocol and assay would facilitate functional targeting and analysis of individual gene candidates. There has been significant recent progress in *in vitro* Purkinje differentiation strategies from human induced pluripotent stem cells (hiPSCs) including: via small molecule-mediated direct reprogramming⁴¹; by specific knockout of transcription factor NKX2-5²⁶⁷; and through 15 days of electrical stimulation during the hiPSC to cardiomyocyte differentiation process²⁶⁸. Improving upon these strategies – perhaps through combining electrical stimulation with optimised provision of small molecule cocktails – to produce a reliable Purkinje culture in a dish, that more faithfully recapitulates the *in vivo* VCS, will enable high throughput targeting of the differentially expressed genes identified in my scRNA-seq experiments. This will allow future experiments to assay for genes to take forwards as candidates for electrical reprogramming in the infarcted heart.

Secondly, alongside 2D *in vitro* cultures, it would be valuable to work towards integrating the differentiated conduction system cells into a 3D cardioid model. By analysing Purkinje cells within a 3D self-organised structure with mixed cardiac cell types and extracellular matrix, this would provide a more physiologically-relevant functional read-out. Moreover, this could be combined with 3D MEA mapping and functional imaging analyses of the cardioids over time.

Thirdly, while the *in vitro* strategies allow for high throughput analysis, these approaches could be complemented by generation of mouse models for targeted knock-out of the top candidates required for regeneration of the His/Purkinje network. This would provide important *in vivo* validation of the functional importance of candidate targets in VCS regeneration.

Finally, it will also be important in future studies to determine whether, and to what extent, regeneration of the VCS in the neonatal mouse heart is mediated by cellular repopulation from non-conductive lineages versus expansion of surviving conduction cells following the direct MI-induced damage identified in Chapter 4. Lineage tracing to assess the role of potential contributions from other cardiac cell types to the VCS following injury will be informative in order to establish where cells of the regenerated conduction system originally derive from.

7.2.4 Comprehensive spatial analysis of changes across the infarcted post-natal day 1 versus post-natal day 7 His/Purkinje network

The results of Chapters 4-5 demonstrate regional electrical remodelling within the non-regenerative infarcted heart including, for example: patches of reduced CX40 within the BBs and Purkinje fibres of the VCS network; punctate upregulation of the sodium-calcium exchanger NCX1 and of alpha-Catenin in CX40-negative nodes; ectopic expression of HCN4 across the infarct area. These findings signal the importance of understanding gene and protein level changes across the conduction network and surrounding tissue with high spatial resolution. Consequently, it would be highly informative to follow up on the transcriptomic and imaging analyses of Chapter 5 by performing spatial transcriptomics and/or spatial proteomics of the P1 versus P7 conduction system following MI and to expand this out to include the adult mouse post-MI. Moreover, these analyses would also aid in the identification of the cytological arrangement of the fibroblast-like versus cardiomyocyte-like conduction cells isolated in the enriched scRNAseq dataset. Finally, these experiments might help to localise the proliferating Purkinje cluster identified in Chapter 5, which is lost in the P7 infarcted heart. Spatial analyses of this sort would, therefore, provide important follow-up characterisation

of the cellular architecture of the mammalian VCS in healthy versus infarcted regenerating and non-regenerating hearts.

7.2.5 Dissection of the mechanism driving connexin and ion channel remodelling

Future work will be needed in order to provide mechanistic insight into the connexin and ion channel remodelling identified in the non-regenerative infarcted VCS in Chapters 4-5. As discussed in Chapter 4, it will be important going forwards to assess the sub-cellular distribution of CX40 as well as the other connexins including CX43, the major connexin of the working ventricular cardiomyocytes. Experiments should aim to determine whether CX40 is reduced at a protein level only, whether it is also re-distributed/internalised within the cell and/or whether it is replaced by slower-conductance CX43 hemi-channels at gap junctions. Moreover, future studies will be needed to establish whether the open/closed state of connexins is also changing after MI. Functionally, it will be necessary to test at a single-cell level what consequences these changes have upon speed of ion passage from one VCS cell to the next.

It was particularly notable that experiments in non-regenerative P7 hearts at chronic stages following MI identified CX40-eGFP loss in the BBs and Purkinje fibre regions remote from the ischaemic zone. Follow-up experiments will be required, therefore, in order to shed light upon the mechanism driving this CX40 downregulation distal from the infarct. In particular, it would be sensible to test whether this correlates with levels of regional inflammation, given that inflammatory cytokines can act to down-regulate expression of CX40 and CX43²⁰⁰. Moreover, given that desmosome factors (including, for example, desmoplakin) are required to stabilise CX40 expression²³¹, future efforts should assess whether or not loss of CX40 is a post-transcriptional phenomenon occurring downstream of desmosome dysregulation.

7.2.6 Electrophysiological follow-up to assess conduction system pathology following myocardial infarction

In Chapter 6 of this thesis, I performed a surface ECG electrophysiology study which demonstrated functional electrical recovery following neonatal mouse heart regeneration, versus pathological conduction delay beyond the regenerative window. Future work will be required in order to follow up on this finding and further elucidate the electrophysiological transition that takes place after MI across the neonatal regenerative window and potentially beyond into adult stages.

In particular, my early attempts using MEA recordings established the possibility of recording cardiac APs from across the wholemount murine ventricle (**Chapter 6.3.1.1**). Going forwards, it will be very informative to optimise this approach in order to design a set-up capable of generating reproducible and high-quality data from these whole-heart preparations before they become ischaemic and/or necrotic. This would enable propagation maps to be generated across the full ventricular chamber of hearts following MI surgery at P1 versus P7 stages. Moreover, since the MEA recordings can be combined with simultaneous fluorescence imaging on an inverted microscope, these propagation maps could be correlated to areas of reduced CX40 expression so as to determine the precise relationship between His/Purkinje remodelling and (disrupted) electrical propagation sequence across the ventricular myocardium.

Additionally, concurrent functional calcium imaging alongside MEA recording experiments would help to elucidate the consequences of electrical remodelling in the non-regenerative infarcted heart for calcium handling. Using fluorescence imaging together with functional GCaMP imaging, would enable cross-correlation of aberrant calcium handling with identified regions affected by reduced CX40 expression and/or ectopic over-expression of NCX1, as identified in Chapters 4 and 5 respectively. Finally,

future experiments might perform optical mapping and optogenetic experiments on Langendorff-perfused hearts following MI at P1 versus P7 stages, to enable perturbation of the VCS with a functional electrophysiological read-out. Overall, a full electrophysiological follow-up assessment of the infarcted P1 versus P7 mouse heart would be very useful in establishing more precisely what the consequences of poor VCS regeneration are for cardiac function.

7.2.7 Investigation of the His-Purkinje niche in regeneration versus fibrotic repair

My characterisation of the intact conduction system in Chapter 3 identified close interactions between Purkinje cells, macrophages and coronary arteries, that to my knowledge have not previously been studied extensively. Moreover, as discussed in Chapters 4-5 of this thesis, the VCS bundles are encased in an insulative sheath. My scRNAseq experiments established that the VCS is made up of fibroblast-like conduction cells that may be responsible in part for the deposition of the collagens, elastin, et cetera, that wrap around the conductive bundles. Whether or not dedicated cardiac fibroblast cells also interact with the His/Purkinje network to support sheath deposition warrants investigation, especially in light of recent evidence that fibroblasts can couple electrically to cardiomyocytes and regulate cardiac excitability *in vivo*³⁴.

Collectively, therefore, the results of this thesis have highlighted the complexity of the His-Purkinje niche and its interactions with varied cell types. Future studies will be required to elucidate and disentangle the interactions of His/Purkinje cells with immune, vascular, fibroblast and nerve populations in healthy, diseased and regenerating hearts. In particular, an *in vitro* reductionist approach, performing mixed culture experiments with hiPSC-derived Purkinje-like cells (see **Chapter 7.2.2**), combined with other cardiac cell types, might enable identification of cell-cell interactions required for mature VCS

formation. Niche-net analysis of spatial transcriptomic and spatial proteomic data (see **Chapter 7.2.3**), might elucidate specific cell-cell interactions in an unbiased way.

Moreover, high-resolution tissue-cleared imaging of CX40-eGFP⁺ hearts stained for nerve (e.g., Tuj1), fibroblast (e.g., Tcf21, Wt1), vascular (e.g., Pecam) and immune (e.g., CD68, Lyve-1 for macrophages) markers would be informative. Finally, targeted deletion of these cell types in turn and subsequent analysis of how this impacts ability to regenerate electrical function following MI (with read-outs including ECG recording) would provide insight into the role of the His/Purkinje niche in regeneration versus fibrotic repair.

7.3 Final remarks

Taken as a whole, the results of this thesis provide a comprehensive characterisation of the 3D architecture of the mammalian VCS during post-natal development and maturation, and in response to ischaemic injury. Through a combination of imaging, single-cell RNA-sequencing, electrophysiology, modelling and simulation experiments, this thesis demonstrates reconstitution of a functional His/Purkinje network during heart regeneration, versus pathological electrical remodelling in fibrotic repair. The identified phenotype of regionally reduced conductivity in the non-regenerative infarcted VCS provides a molecular mechanism, distinct from scar-related re-entry and remodelling known to occur locally to the infarct/border zone or direct ischaemic damage, to explain conduction disease in MI patients. Moreover, by elucidating the cellular and molecular changes underpinning the response of the conduction system to MI across the regenerative window, these results identify the mechanisms by which normal cardiac conduction is re-established during heart regeneration, which can be extrapolated to adult stages.

Going forwards, it will be important to elucidate the interactions of Purkinje cells with other cardiac cell types more fully, to understand the cellular heterogeneity within the VCS as identified by scRNA-seq in time and space, to determine whether there is repopulation of the regenerating VCS and if so, what cell lineages drive this event, and to investigate the causal mechanisms driving the non-regenerative His/Purkinje pathological remodelling outside of the infarct area. Overall, the results presented here also afford several insights of value to understanding and treating conduction disease clinically. In the future, precise drug targeting of pathologically remodelled VCS cells to restore fast conductivity through the network may provide a promising avenue for CCS repair in MI patients.

8 Supplementary materials

Supplementary Table 1: The 30 most highly conserved genes of the cardiac conduction system cluster 13 identified through re-analysis of the Cui et al. dataset

Gene	Adjusted p-value	Annotations
Robo1	0.000e+00	Famous guidance receptor originally discovered in neurons
Cpne5	1.878e-252	Known CCS marker
Etv1	9.653e-220	Known CCS marker
Slit3	1.892e-214	Guidance ligand that binds the Robo receptor
Cacna2d2	4.694e-187	Known CCS marker
Slc6a12	1.192e-182	Na-Ca exchanger involved in kidney, liver, and neurotransmitter transport, and amino acid transport across the plasma membrane. Role in osmoregulation across basolateral epithelial membranes
Slit2	7.172e-180	Guidance ligand that binds the Robo receptor
Ntm	5.572e-144	Known CCS marker
Sema3a	2.964e-139	Known CCS marker
Stard10	5.083e-117	Phospholipid transfer protein; regulates insulin secretion/bile acid homeostasis
Tmem163	5.000e-114	Preferentially expressed in trabecular over compacted myocardium in the E10.5 heart. Synaptic vesicle membrane protein, Zn binding, expressed in brain especially in glutaminergic neurons
Cdh6	1.806e-108	Cadherin; Ca-dependent cell adhesion. High expression in brain, cerebellum, kidney. Highest heart expression in smooth muscle cells; differentially expressed in patients with dilated cardiomyopathy. Identified in the CCS ¹⁶⁹
b830012L1Rik	6.900e-95	
Fgf14	1.476e-94	Thought to be important in nervous system development; interacts with voltage gates sodium SCN8A
Fgf12	1.366e-92	Expressed in ventricle and modulates cardiac Nav1.5 (voltage-gated Na channel important for CCS)
Rgs6	3.334e-87	Known CCS marker
Grip1	6.520e-80	Important in dendrite development; positive regulation of neuron projection arborisation; vesicle transport at synapse; mis-splicing modulates kinesin association
Gja5	1.864e-75	Encodes CX40; known CCS marker
Meg3	1.454e-73	lncRNA implicated in CM apoptosis after MI
Vcan	5.532e-66	ECM component important in ventricular septal formation (known from SMC k/o), associated with CHD and adult cardiovascular diseases
Dapk2	7.153e-65	Regulates signalling to trigger autophagy / cell survival vs apoptosis; involved in pro-inflammatory responses regulated

by granulocytes		
Cdk14	1.143e-62	Cell cycle Wnt regulator during G2/M
Mpped2	3.803e-57	Metallo-phosphoesterase. May have role in the development of the nervous system; expressed in brain, heart kidney.
A930011G23Rik	1.912e-50	RIKEN cDNA; encodes long ncRNA with known expression in the nervous system
Fbn2	2.408e-46	Fibrillin-2. Extracellular Ca-binding microfibrils that occur in association with elastin or in elastin-free bundles. Mutations linked to congenital heart disease. Regulates endocardial morphogenesis
Magi2	3.430e-46	Scaffold molecule at synaptic junctions - assembles neurotransmitter receptors and cell adhesion proteins. Roles in neurite outgrowth, activin signalling, NGF signalling
Gabrg3	3.725e-46	GABA receptor subunit carrying the benzodiazepine binding site.
Sphkap	5.156e-46	Expressed mainly in neurons and heart muscle
2900055J20Rik	4.690e-45	RIKEN cDNA; encodes kctd16
Rabgap1l	2.463e-42	Involved in heart tissue intracellular membrane trafficking; involved in cell migration

Supplementary Table 2: Number of cells by condition in each cardiomyocyte cluster following re-analysis of the Cui et al. dataset

Cluster #	0	1	2	3	4	5	6	7	8	9	11	12	13	15	Total
P1MID1	197	312	514	249	90	210	173	73	42	40	31	44	32	9	2016
P2Sham	116	154	128	46	58	27	98	37	41	23	16	7	11	5	767
P1MID3	213	287	454	193	62	104	157	40	34	17	8	41	37	16	1663
P4Sham	333	513	150	126	198	59	198	227	150	103	140	6	37	3	2243
P8MID1	740	751	77	249	162	419	117	110	116	88	31	94	14	10	2978
P9Sham	1065	889	231	280	212	68	194	240	212	214	132	30	19	6	3792
P8MID3	834	320	108	126	231	206	78	154	157	178	9	81	17	9	2508
P11Sham	1259	698	82	173	178	97	154	208	192	159	114	16	19	4	3353

Supplementary table 3: The most highly conserved genes of each sub-cluster identified through CX40+ CD31- enriched single-cell RNA-sequencing

Cluster	Gene	Annotations
SMC.1	Col18a1	Multi-plexin collagen ECM protein
SMC.1	Lpp	SMC-enriched cell-cell adhesion, localises to focal adhesions
SMC.1	Cnn1	Calponin. Implicated in regulating SMC proliferation/contraction
SMC.1	Olfir1033	Olfactory receptor
SMC.1	Adarb1	Responsible for pre-mRNA editing of glutamate receptor subunit B/ K ⁺ voltage-gated channel; catalyses A-I RNA editing generally
SMC.1	Lmod1	Required for contractility of visceral SMCs, mediates nucleation of actin filaments
SMC.1	Igfbp7	Associated with cellular senescence, linked to abnormal cardiac structure/function in HF patients, stimulates cell adhesion
SMC.1	Crispld2	Promotes matrix assembly
SMC.1	Adamts9	Versican-degrading protease required for cardiac development and adult homeostasis. Expressed in non-myocardial cells of ventricles, VSMCs
SMC.1	Nr4a2	Transcriptional regulator. Protects cardiomyocytes vs MI, prolonged activation in myocytes causes dilated cardiomyopathy
SMC.1	Tagln	Calponin family actin-binding protein expressed in vascular and visceral SMC
SMC.1	Flna	In motile cells, remodelling, actin-binding
SMC.1	Cald1	Regulates smooth muscle and non-muscle contraction
SMC.1	Col4a2	Smooth muscle expression
SMC.1	Adamts1	Cleaves aggrecan, a cartilage proteoglycan. Angiogenic inhibitor activity. Expressed through myocardial tissue
SMC.1	Myh11	Smooth muscle myosin
SMC.1	Actg2	Actin gamma, smooth muscle. Also expressed in cardiomyocytes
SMC.1	Fgl2	Fibrinogen-like. Contributes to CM maturation and heart development
SMC.1	Col4a1	Type IV collagen alpha. Component of basement membrane
SMC.1	Mfge8	Downregulated in fibrosis, attenuates endothelial to mesenchymal transition through the smad3 pathway
Epi-VCS	Gsta3	Glutathione S-Transferase involved in oxidative stress response
Epi-VCS	Il18r1	Cytokine
Epi-VCS	Tfcp2l1	Transcription factor
Epi-VCS	Gpr39	Ghrelin receptor family
Epi-VCS	Fmod	ECM proteoglycan
Epi-VCS	Prg4	Boundary lubricant, elastic absorption at cartilage surfaces
Epi-VCS	Clic3	Chloride intracellular channel, implicated in atrial fibrillation
Epi-VCS	Lamc3	Laminin subunit
Epi-VCS	Ptgs1	Enzyme catalysing conversion of arachidonate to prostaglandin. Role in inflammatory response
Epi-VCS	Dpp4	Protects heart from acute myocardial ischaemia. Cell surface glycoprotein receptor involved in TCR-mediated T cell activation, ECM proteolysis

Epi-VCS	Dhrs9	Involved in MI response, CM immune response
Epi-VCS	Lrp2	Endocytic receptor, mediates endocytosis of high-density lipoproteins. Roles in cardiovascular development, CHD gene
Epi-VCS	Cybrd1	Cytochrome b reductase expressed in smooth muscle, fibroblasts
Epi-VCS	Wt1os	WT1 TF, opposite strand, lncRNA
Epi-VCS	Wt1	TF in epicardium, endothelium
Epi-VCS	Lrrn4	Role in hippocampal long-term memory, linked to dilated cardiomyopathy, expressed in ventricular cardiomyocytes
Epi-VCS	Pard6b	Par6 cell polarity (epithelial)
Epi-VCS	Nkain4	Epicardial sodium/potassium transporting ATPase-interactor
Epi-VCS	Tuft1	Involved in adaptation to hypoxia?
Epi-VCS	Cgn	Tight junction assembly; cadherin binding
SMC.2	Carmn	SMC
SMC.2	Olf558	Olfactory receptor
SMC.2	Mob2	Actin cytoskeleton organisation
SMC.2	Slc38a11	Amino acid TM transport
SMC.2	Acta2	Actin, smooth muscle
SMC.2	Sorbs2	Disrupts sarcomeric integrity of cardiomyocytes, variants found in patients with arrhythmogenic cardiomyopathy
SMC.2	Wtip	Wilms Tumour interacting protein. Knockdown causes hypertrophy, expression in SMC and fibroblast too
SMC.2	Kcnab1	Potassium voltage-gated channel. Deletion leads to electrical changes and hypertrophy - long QRS and QT, increased action potential duration
SMC.2	Dgkb	Linked to atrial fibrillation
SMC.2	Ccdc3	Negative regulator of TNF α -pro-inflammatory response in endothelial cells
SMC.2	Lmod1	Required for contractility of visceral SMCs, mediates nucleation of actin filaments
SMC.2	Dstn	SMC link
SMC.2	Rplp1e	Cell adhesion protein
SMC.2	Map3k7cl	Myocyte enriched
SMC.2	Nrip2	Involved in proteolysis, in notch pathway?
SMC.2	Rgs7bp	Regulates GPCR signalling, in myocytes
SMC.2	Tpm2	Isoform of tropomyosin, in SMC and cardiomyocytes, and non-muscle cells
SMC.2	Olf78	Olfactory receptor
SMC.2	Mustn1	Pan musculoskeletal marker
SMC.2	Gucy1a1	Catalyses conversion GTP to 3,5 cGMP. GO: cardiac conduction
FbVCS.1	Cthrc1	May be a negative regulator of collagen matrix deposition. Needed in cardiac fibroblasts after MI - absence leads to ventricular rupture
FbVCS.1	Mfap4	ECM protein involved in cell adhesion/intercellular interaction. Regulates stress-induced cardiac remodelling; non-myocyte derived ²⁶⁹ . Role in elastic fibre formation/maintenance?
FbVCS.1	Dpt	ECM protein. Role in dilated cardiomyopathy. Expressed in cardiac fibroblasts
FbVCS.1	Col15a1	Basement membrane collagen. Stabilises muscle cells and micro-vessels
FbVCS.1	Pdgfra	Fibroblast cell surface tyrosine kinase receptor. Promotes or inhibits

		cell proliferation and migration depending on context
FbVCS.1	Lum	ECM proteoglycan lumican. Increased expression in failing hearts. Mainly fibroblast expression
FbVCS.1	Serpinf1	Secreted protein that inhibits angiogenesis; neurotrophic factor in retinoblastoma cells inducing neuronal differentiation
FbVCS.1	Gpm6b	Membrane glycoprotein. Role in neuronal development, maintenance of actin cytoskeleton
FbVCS.1	Mmp2	Famous cardiac matrix metalloproteinase. Activated by oxidative stress.
FbVCS.1	Htra3	Regulates heart failure by maintaining identity of quiescent fibroblasts by degrading TGFb ²⁷⁰
FbVCS.1	Lpar1	Expressed in cardiac fibroblasts. Genetic ablation attenuates myocardial fibrosis in HCM mice. Actin cytoskeleton organisation, differentiation, proliferation
FbVCS.1	Cygb	Cytoglobin. Protection during oxidative stress, cardiac progenitor cell survival
FbVCS.1	Mfap5	ECM protein promoting attachment of cells to microfibrils via integrin. Regulates growth factors
FbVCS.1	Kcnt2	Potassium Na-activated channel
FbVCS.1	Gxylt2	Glycosyltransferase that elongates glucose bound to EGF repeats
FbVCS.1	Cd34	Adhesion molecule with roles in attachment of stem cells to bone marrow ECM
FbVCS.1	Fndc1	Fibronectin; positive regulation of cardiomyocyte apoptotic process; upstream of cellular response to hypoxia
FbVCS.1	Tnxb	ECM glycoprotein. Anti-adhesive, functions in matrix maturation during wound healing, accelerates collagen fibril formation
FbVCS.1	Matn2	Matrillin. Involved in formation of filamentous networks of tissues; needed for muscle and nerve regeneration; matrix assembly ²⁷¹
FbVCS.1	Ace	Angiotensin converting enzyme. Polymorphism associated with cardiac conduction defects and electrical remodelling. Different levels in MI, cardiomyopathy, left ventricular hypertrophy ²⁷²
SMC.3	Rgs5	Regulator of G protein signalling. Protects vs hypertrophy and fibrosis in pressure overload
SMC.3	Chrm3	Muscarinic acetyl choline receptor, controls SMC contraction
SMC.3	Kcnab1	Potassium voltage-gated channel. Deletion leads to electrical changes and hypertrophy - long QRS and QT, increased action potential duration
SMC.3	Reln	Secreted ECM protein; roles in neuronal migration, neuronal development. Lymphoangiocrine signal that improves heart repair after neonatal MI ²⁷³
SMC.3	Rasd1	Activator of G protein signalling. modulates hormone secretion in volume-overloaded heart
SMC.3	Dgkb	Linked to atrial fibrillation
SMC.3	Cabp1	Regulates CaV L-type Ca channels. calcium binding protein, regulates gating of voltage-gated Ca channels
SMC.3	Ntrk2	Neurotrophin receptor expressed in epicardium and blood vessels. Roles in development and maturation of nervous system. Mediates communication between neurons and glia.
SMC.3	Prkg1	SMC contraction, neurons, cerebellar Purkinje expression. Candidate for heart failure
SMC.3	Gucy1a1	Catalyses conversion GTP to 3,5 cGMP. GO: cardiac conduction
SMC.3	Mef2c	Important to cardiac development
SMC.3	Mylk	Myosin light chain kinase - SMC contraction, roles in striated muscle

SMC.3	Crispld2	Promotes matrix assembly
SMC.3	S1pr3	GPCR, key regulator of cardiac rhythm, expressed in AVN and His/Purkinje ²⁷⁴ . Also regulates vascular endothelial cell function?
SMC.3	Eli2	Elongation factor
SMC.3	Cd93	Cell surface glycoprotein, intercellular adhesion
SMC.3	Arih1	Links to aortic and cardiovascular diseases
SMC.3	Adamts9	Versican-degrading protease required for cardiac development and adult homeostasis. Expressed in non-myocardial cells of ventricles, VSMCs
SMC.3	Sparcl1	RV-specific biomarker for maladaptive right ventricle function, ECM binding
SMC.3	Gm13889	Pericyte, SMC expression?
FbVCS.2	Svep1	Linked to coronary artery disease
FbVCS.2	Dpep1	Dipeptidase but also adhesion receptor for neutrophil recruitment in inflamed lung/liver
FbVCS.2	Abca8a	Catalyses ATP import of organic anions, role in sphingomyelin production, role in regulating cholesterol levels
FbVCS.2	Gpm6b	Membrane glycoprotein. Roles in neuronal development, maintenance of actin cytoskeleton
FbVCS.2	Eya2	Transcription coactivator, inhibits development of cardiac hypertrophy
FbVCS.2	Qpct	Glutaminy peptide cyclotransferase
FbVCS.2	Ramp2	Acts as receptor for adrenomedullin. Expressed in myocytes and non-myocytes, levels increased in failing CM
FbVCS.2	Lpar1	Expressed in cardiac fibroblasts. Genetic ablation attenuates myocardial fibrosis in HCM mice. Roles in actin cytoskeleton organisation, differentiation, proliferation
FbVCS.2	Pdgfra	Fibroblast cell surface tyrosine kinase receptor. Promotes or inhibits cell proliferation and migration depending on context
FbVCS.2	Srpx2	Roles in angiogenesis, cellular migration, adhesion, synapse formation
FbVCS.2	Tcf21	Pro-epicardial, fibroblast marker
FbVCS.2	Robo2	Roundabout guidance receptor 2. Known roles in CM, conserved cluster 13 snRNA-seq marker identified in Supplementary Table 2
FbVCS.2	Col15a1	Basement membrane collagen. Stabilises muscle cells and micro-vessels in heart
FbVCS.2	Negr1	Neuronal growth regulator promoting regenerative axon sprouting; roles in cell-adhesion
FbVCS.2	Mmp2	Famous cardiac matrix metalloproteinase. Activated by oxidative stress
FbVCS.2	Plekhg1	Regulates small GTPase mediated signal transduction, endothelial, oligodendrocyte, B cell enriched
FbVCS.2	Cygb	Protective function in oxidative stress, promotes cardiac progenitor cell survival
FbVCS.2	Dpt	ECM protein. Role in dilated cardiomyopathy. Expressed in cardiac fibroblasts
FbVCS.2	Prelp	In connective ECM
FbVCS.2	Fndc1	Fibronectin; positive regulation of cardiomyocyte apoptotic process; upstream of cellular response to hypoxia
SMC.4	Myl9	Smooth muscle and non-muscle expression
SMC.4	Myl6	Smooth muscle and non-muscle expression
SMC.4	Mustn1	Pan musculoskeletal marker
SMC.4	Tpm2	Isoform of tropomyosin, in SMC and cardiomyocytes, and non-muscle cells

SMC.4	Crip1	Immune-related protein; intracellular zinc transport
SMC.4	Tagln	Calponin family actin-binding protein expressed in vascular and visceral SMC
SMC.4	Tinagl1	Secreted glycoprotein, upregulated in cardiac ECM in porcine ischaemia/reperfusion model
SMC.4	Acta2	Actin, smooth muscle
SMC.4	Gm13889	Pericyte, smooth muscle cell expression?
SMC.4	Ppp1r14a	Cellular stress response
SMC.4	Cavin3	Involved in caveola formation
SMC.4	Csrp1	Cardiac mesoderm and endoderm expression, associated with endocardial fibroelastosis, role in neuronal development
SMC.4	Csrp2	Vascular expression, roles in development of vascular system
SMC.4	Ckb	Associated with subendocardial MI. Central role in energy transduction – catalyses transfer of a phosphate from ATP and phosphagens
SMC.4	Sparcl1	RV-specific biomarker for maladaptive right ventricle function, ECM binding
SMC.4	Actg2	Actin
SMC.4	Dstn	SMC link
SMC.4	Ppp1r15a	Cellular stress response
SMC.4	Junb	TF involved in regulating gene activity after primary growth factor response. Decreased level in MI hearts in mice
SMC.4	Ptp4a3	Protein tyrosine phosphatase, stimulates G1 to S progression in mitosis, potential role in cardiac hypertrophy by inhibiting intracellular calcium mobilisation in response to angiotensin II
CmVCS.1	Ankrd23	Titin binding, transcriptional regulator
CmVCS.1	Cox5b	Mitochondrial electron transport - known LV enrichment
CmVCS.1	Tmem182	Negative regulator of myogenesis and skeletal muscle regeneration
CmVCS.1	Fhl2	ECM assembly? Negative regulation of calcineurin/NFAT in CM
CmVCS.1	Acadl	Mitochondrial role, associated with cardiac hypertrophy
CmVCS.1	Tuba4a	Tubulin - major microtubule constituent
CmVCS.1	Des	Desmin. Linked to ventricular arrhythmia, helps maintain sarcomere structure, cardiac conduction GO
CmVCS.1	Ptma	Provides permissive environment for CM proliferation, linked to regeneration in post-infarct hearts
CmVCS.1	Ndufa10	Mitochondrial electron transport chain
CmVCS.1	Tnni1	Troponin I - striated muscle
CmVCS.1	Tnnt2	Cardiac troponin T
CmVCS.1	Mpc2	Mitochondrial pyruvate carrier
CmVCS.1	Coq8a	Mitochondrial protein
CmVCS.1	Esrrg	Linked to heart failure, dilated cardiomyopathy, fibrosis
CmVCS.1	Atp5c1	Mitochondrial ATP synthase overexpressed in LV
CmVCS.1	Vim	Intermediate filament protein. Expressed in fibroblasts
CmVCS.1	Neb1	Cardiac z-disk protein, variants linked to arrhythmia
CmVCS.1	Crat	Metabolic roles
CmVCS.1	Ak1	Energy metabolism
CmVCS.1	Ndufa8	Electron transfer from NADH to respiratory chain
Immune	Ptpn18	ERBB2 / cytokine signalling. PTP protein

Immune	Inpp5d	Terminating signal transduction from activating immune/hematopoietic cell receptor systems
Immune	St8sia4	Required for synthesis of an NCAM1 modulator
Immune	Cxcr4	Chemokine receptor; ventricular septum formation; arrhythmia links
Immune	Ptprc	PTP protein
Immune	Rgs1	GTPase activator, calmodulin binding
Immune	Ncf2	Neutrophil cytosolic factor
Immune	E330020D12Rik	
Immune	Fcgr2b	Phagocytosis of immune complexes
Immune	Fcgr3	Receptor for Fc fragment of IgG
Immune	Fcer1g	Adapter protein, transduces activation signals from immunoreceptors
Immune	Arhgap30	Rho GTPase activator of RAC1 and RHOA; small GTPase signal transduction
Immune	Cd48	GPI-anchored cell surface glycoprotein, can regulate immune function/activation
Immune	Cd84	Membrane glycoprotein, role in regulating innate and adaptive immune response
Immune	Ifi207	Interferon-induced
Immune	Mrc1	Mannose receptor, mediates endocytosis of glycoproteins by macrophages
Immune	Arhgap15	Rho GTPase activator, overexpression leads to increase in actin stress fibres and cell contraction
Immune	Spi1	Pioneer TF, controls hematopoietic cell fate
Immune	Lmo2	Regulates RBC development or acts with LDB1 to maintain erythroid precursors in immature state
Immune	Gatm	Involved in response to heart failure by elevating local creatine synthesis
FbVCS.3	Bmx	Endocardial signal transduction
FbVCS.3	Ahsg	Serum glycoprotein
FbVCS.3	Tmem132c	Enriched in neurons and oligodendrocytes
FbVCS.3	Nrxn1	Electrophysiologic
FbVCS.3	Sall3	TF; knockdown accelerates cardiac differentiation
FbVCS.3	Rtl4	Cognitive brain function
FbVCS.3	Car3	Associated with MI
FbVCS.3	Ccl19	Inflammatory
FbVCS.3	Kirrel3	Synaptic cell adhesion molecule
FbVCS.3	Enpp6	High in developing oligodendrocytes. Choline-specific phosphodiesterase
FbVCS.3	Prss35	Enriched in muller glia cells of retina. Serine protease
FbVCS.3	Ptprb	Cell adhesion, neurite growth, neuronal differentiation, endothelial. PTP protein. Connects to contactin, tenascin c, sodium channels
FbVCS.3	Ptx3	Pentraxin. In myocardium, endothelial, SMC etc., plasma levels rise after MI
FbVCS.3	Wnt16	Involved in neuronal signalling, enriched in B cells
FbVCS.3	Rspo3	Expressed in CM, also in fibroblasts, SMC, endo, neurons
FbVCS.3	Bmper	EMC protein. Expressed in endo. Roles in cardiac development
FbVCS.3	Msx1	transcriptional repressor. Function with T-box to suppress Cx43 and working myocardial genes

FbVCS.3	Wif1	Wnt inhibitory factor
FbVCS.3	Erbb4	Roles in CM proliferation
FbVCS.3	Unc5c	Netrin receptor, axon guidance
prolif.3	Pbk	MAPKK. Only active in mitosis, protective role in MI, linked to heart failure
prolif.3	Depdc1a	Cell cycle gene that regulates mitotic progression
prolif.3	Sgo1	Interacts with HCN4 to stabilise cardiac pacing. Mutant has reduced heart rate, reduced cardiac function. General: role in chromosome cohesion during mitosis
prolif.3	Ccna2	Cyclin A2 - can induce cardiac regeneration in small-animal MI models through cytokinesis ^{275,276}
prolif.3	Kn1	Kinetochore scaffold. Role in kinetochore-MT attachment and chromosome segregation
prolif.3	Neil3	DNA repair gene. Regulates cardiac fibroblast proliferation preventing myocardial rupture after MI ²⁷⁷
prolif.3	Cep55	Centrosomal protein with role in mitotic exit and cytokinesis
prolif.3	Tpx2	Microtubule nucleation factor, spindle assembly factor
prolif.3	Ndc80	Component of kinetochore complex. Required for proper chromosome segregation and spindle checkpoint activity
prolif.3	Aurkb	Aurora kinase family. Chromosomal passenger complex member - key mitosis regulator. Regulates alignment / segregation of chromosomes in mitosis
prolif.3	Ska1	Spindle and kinetochore associated complex. Microtubule binding complex essential for proper chromosome segregation
prolif.3	Dlgap5	Potential cell cycle regulator. Key regulator of adherens junctions' integrity. Mitotic phosphoprotein
prolif.3	Kif4	Kinasein. May be involved in maintaining chromosome integrity in mitosis, roles in metaphase-anaphase transition
prolif.3	Hmmr	Hyaluronan mediated motility receptor. Cardiac regeneration inhibited after Hmmr knockdown. Required for epicardial EMT during heart regeneration ²⁷⁸
prolif.3	Kif20b	Kinesin. Motor protein required for completion of cytokinesis. Regulates neuronal polarisation
prolif.3	Anln	Anillin - actin binding protein with roles in cytokinesis, cell growth, migration
prolif.3	Ckap2l	Mitotic spindle protein
prolif.3	Spc25	Component of kinetochore complex, associated with proliferation. Potential roles in spindle checkpoint and kinetochore-microtubule interaction
prolif.3	Ncapg	Subunit of condensin, stabilising chromosomes in mitosis and meiosis
prolif.3	Racgap1	Rac GTPase activating protein. Roles in cytokinesis, cell growth, differentiation. Proliferation associated
prolif.2.FbVCS	Mxd3	Transcriptional repressor. Disruption leads to proliferation
prolif.2.FbVCS	Efcab11	Calcium ion binding
prolif.2.FbVCS	Pbk	MAPKK. Only active in mitosis. Protective role in MI. linked to heart failure
prolif.2.FbVCS	Melk	Serine/threonine protein kinase. Roles in cell cycle regulation in mitosis, stem cell self-renewal, needed in neural progenitors
prolif.2.FbVCS	Ncapg	Subunit of condensin, stabilising chromosomes in mitosis and meiosis
prolif.2.FbVCS	Kif15	Kinesin-like motor protein - mitotic spindle assembly
prolif.2.FbVCS	Bub1	Mitotic checkpoint serine/threonine kinase. Role in myocardial proliferation ²⁷⁹
prolif.2.FbVCS	Kn1	Kinetochore scaffold. Role in kinetochore-MT attachment and chromosome segregation

prolif.2.FbVCS	Hist1h2af	Involved in heterochromatin assembly and negative regulation of cell population proliferation?
prolif.2.FbVCS	Fbxo5	Regulator of APC activity during cell cycle
prolif.2.FbVCS	Esco2	Role in S phase of mitosis
prolif.2.FbVCS	Ndc80	Component of kinetochore complex. Required for proper chromosome segregation and spindle checkpoint activity
prolif.2.FbVCS	Nuf2	Kinetochore component (spindle assembly checkpoint, microtubule-kinetochore interaction)
prolif.2.FbVCS	Hist1h2ab	Histone protein
prolif.2.FbVCS	Neil3	DNA repair gene. Regulates cardiac fibroblast proliferation preventing myocardial rupture after MI ²⁷⁷
prolif.2.FbVCS	Pimreg	PICALM interacting mitotic regulator. Foetal-specific cell cycle promoter in cardiomyocytes ²⁸⁰ . Involved in cell division, proliferation marker.
prolif.2.FbVCS	Ska1	Spindle and kinetochore associated complex. Microtubule binding complex essential for proper chromosome segregation
prolif.2.FbVCS	Asf1b	Histone chaperone. Replication-dependent chromatin assembly
prolif.2.FbVCS	Ttk	Mitotic kinase that controls progression through mitosis. In MI datasets. Also, an angiogenic modulator
prolif.2.FbVCS	Cep55	Centrosome protein with role in mitotic exit and cytokinesis
CmVCS.2	Rpm20	
CmVCS.2	Rpl19	
CmVCS.2	mt-Co3	
CmVCS.2	Rps29	
CmVCS.2	Rpl6	
CmVCS.2	mt-Atp6	
CmVCS.2	Rpl37	
CmVCS.2	Rpl41	
CmVCS.2	Rpl38	
CmVCS.2	Rps24	
CmVCS.2	mt-Co2	
CmVCS.2	mt-Cytb	
CmVCS.2	Rplp1	
CmVCS.2	Rpl24	
CmVCS.2	Rpl35a	
CmVCS.2	Rps9	
CmVCS.2	Rps8	
CmVCS.2	Rpl36	
CmVCS.2	mt-Nd4	
CmVCS.2	Tpt1	
SMC.5	Grp	Gastrin releasing peptide
SMC.5	Cdh18	Cadherin expressed in CNS, involved in synaptic adhesion, cell-cell adhesion
SMC.5	Prdm6	Smooth muscle and endothelial roles
SMC.5	Ccn3	Involved in ventricular septum development
SMC.5	Ntrk3	Expressed in developing CCS but not adult CCS
SMC.5	Mtus2	MT associated scaffold protein.

SMC.5	Fbln5	ECM fibulin. Promotes endothelial adhesion
SMC.5	Erbb4	Roles in CM proliferation
SMC.5	Pcdh7	Protocadherin. Cell-cell adhesion. Roles in neuronal signalling
SMC.5	Slc24a4	Na/Ca exchanger
SMC.5	Eln	Connective tissue, fibroblasts, extracellular fibres, SMC
SMC.5	Rgs7	GTPase regulation. Modulates potassium channels; role in synaptic vesicles?
SMC.5	Ncam1	Cell-cell adhesion, identified Purkinje marker ¹⁶⁹
SMC.5	Mecom	Endothelial
SMC.5	Sgcd	Sarcoglycan complex, expressed in CM, dilated cardiomyopathy and conduction defects
SMC.5	Alcam	Cell-cell adhesion, identified Purkinje marker ¹⁶⁹
SMC.5	Plcb1	Intracellular signal transduction. Neurons, oligodendrocyte precursors, astrocytes
SMC.5	Nav2	Neuron navigator gene family. Cellular growth/migration. Roles in neuronal development
SMC.5	Lgr6	Stem-cell marker
SMC.5	Itga8	Integrin subunit in neurons, SMCs, oligodendrocyte precursors, microglia
CmVCS.3	Gja5	Connexin-40 - fast-conductance connexin and famous Purkinje marker
CmVCS.3	Sbk2	MAPKK
CmVCS.3	Slc16a12	TM transporter of monocarboxylic acid
CmVCS.3	Lmntd1	Nuclear lamina
CmVCS.3	Fgf12	Genetic association with ventricular tachycardia
CmVCS.3	Cacna2d2	Known Purkinje marker. Voltage-dependent calcium channel
CmVCS.3	Pcp4	Regulates Purkinje cell excitability and rhythmicity ²⁸¹
CmVCS.3	Tmem163	Enriched in neurons
CmVCS.3	Myl1	Myosin in striated muscle
CmVCS.3	Mybphl	Implicated in arrhythmia
CmVCS.3	Kcnj3	Potassium inwardly-rectifying channel
CmVCS.3	Gm31659	
CmVCS.3	Gm15543	
CmVCS.3	Scn5a	Purkinje marker
CmVCS.3	Chrm2	Muscarinic Ach receptor linked to DCM
CmVCS.3	Fam78a	
CmVCS.3	Ank1	Ankyrins previously linked to arrhythmia
CmVCS.3	Kcnj5	Potassium inwardly-rectifying channel linked to cardiac conduction
CmVCS.3	Hspb3	Inhibitor of actin polymerisation
CmVCS.3	Lrrc3b	Inhibits cell growth and replication?
prolif.1.CmVC S	Esco2	Role in S phase of mitosis
prolif.1.CmVC S	Ccna2	Cyclin A2 - can induce cardiac regen in small-animal MI models through cytokinesis ^{275,276}
prolif.1.CmVC S	Kn11	Kinetochore scaffold. Role in kinetochore-MT attachment and chromosome segregation
prolif.1.CmVC S	Hmmr	Hyaluronan mediated motility receptor, cardiac regeneration inhibited after Hmmr knockdown, required for epicardial EMT during heart

		regeneration ²⁷⁸
prolif.1.CmVC S	Kif11	Kinesin. Roles in chromosome positioning, centrosome separation, bipolar spindle formation in mitosis
prolif.1.CmVC S	Depdc1a	Cell cycle gene that regulates mitotic progression
prolif.1.CmVC S	Ckap2l	Mitotic spindle protein
prolif.1.CmVC S	Iqgap3	Enables calmodulin binding activity/ myosin light chain binding activity; regulates actin cytoskeleton. Signal transduction roles in binucleated CM ²⁸²
prolif.1.CmVC S	Bub1	Mitotic checkpoint serine/threonine kinase. Role in myocardial proliferation ²⁷⁹
prolif.1.CmVC S	Spc25	Component of kinetochore complex, associated with proliferation
prolif.1.CmVC S	Rrm2	Ribonucleotide reductase. Synthesis regulated in cell-cycle dependent way. Can improve CM proliferation after MI reperfusion through Hippo/YAP ²⁸³
prolif.1.CmVC S	Nuf2	Kinetochore component (spindle assembly checkpoint, microtubule-kinetochore interaction)
prolif.1.CmVC S	Cdca8	Cell division cycle associated 8; identified in Cui et al cardiomyocyte dataset ¹
prolif.1.CmVC S	Kif4	Kinesin. May be involved in maintaining chromosome integrity in mitosis, metaphase-anaphase transition etc.
prolif.1.CmVC S	Sgo1	Interacts with HCN4 to stabilise cardiac pacing. Mutant has reduced heart rate, reduced cardiac function. General: role in chromosome cohesion during mitosis preventing premature cohesin dissociation
prolif.1.CmVC S	Ncapg	Subunit of condensin, stabilising chromosomes in mitosis and meiosis
prolif.1.CmVC S	Racgap1	Rac GTPase activating protein. Roles in cytokinesis, cell growth, differentiation. Proliferation associated
prolif.1.CmVC S	Shcbp1	SHC binding and spindle associated 1. Roles in regulation of neural precursor cell proliferation / FGFR signalling
prolif.1.CmVC S	Arhgap11a	Rho GTPase activating protein. Involved in cell-cycle arrest/apoptosis after DNA damage
prolif.1.CmVC S	Aspm	Mitotic spindle regulation, preferential role in neurogenesis
SMC.6	Vtn	Ventricle enriched adhesive glycoprotein. Tissue remodelling roles
SMC.6	Chl1	Neural cell adhesion molecule L1 like
SMC.6	Klk8	Myocardial expression, link to cardiac hypertrophy
SMC.6	Fabp7	Lipid binding, needed for radial glial fibres in brain development
SMC.6	Colec11	C-type lectin
SMC.6	Nostrin	Controls muscarinic M3 receptor localisation. Mediates neurotransmission. Adipose tissue ECM organisation
SMC.6	Ednrb	Endocardium and endothelium. Dispensable for CCS development
SMC.6	Kcnj8	Potassium inward-rectifying channel. Linked to arrhythmia. In heart muscle CM and SMC
SMC.6	Egfl8	Schwann cell enriched
SMC.6	Lin7a	Scaffold important for synaptic function, linked to heart failure
SMC.6	Adgrf5	Adhesion GPCR
SMC.6	Plp1	Predominant component of myelin in central nervous system
SMC.6	Nrxn1	Linked to neurodevelopmental disease. Neurexin, forms complexes at CNS synapses
SMC.6	Cdh19	Cadherin. CM muscle contraction, expressed in brain white matter too

SMC.6	Adgrl3	Adhesion GPCR linked to ion transport in brain
SMC.6	Ets1	TF expressed in cardiac neural crest and heart mesoderm of embryo
SMC.6	Dach1	Endothelial
SMC.6	Higd1b	Hypoxia inducible gene. Localises to cell membrane, involved in angiogenesis, high expression in brain
SMC.6	Adap2	Known to be upregulated after MI and suggested as a diagnostic marker
SMC.6	Gng2	Mutation linked to familial sinus node and AV conduction dysfunction

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