UNIVERSITY OF CALIFORNIA SAN DIEGO

Heterogeneity of Natriuretic Peptide Expression in the Hearts of Mice and Humans using Single Cell Transcriptomics

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

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The thesis of Avinash Toomu is approved, and it is acceptable in quality and Form for publication on microfilm and electronically.

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT OF THE THESIS

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The atrial natriuretic (ANP) and B-type natriuretic peptides (BNP) are important clinical diagnostic markers that, in recent years, have also been leveraged in a therapeutic angle. The release of these peptides is commonly thought to be stimulated by myocardial stretch, whether that be due to a pressure or volume overload. These peptides have several effects in the body, but the main ones involve diuresis and natriuresis at the kidneys,

vasodilation, and an inhibition of the sympathetic nervous system. Bulk methods, such as quantitative PCR (qPCR) and bulk RNA-seq, have been able to identify the triggers for the transcription of the genes that encode for these peptides, *Nppa* and *Nppb*, in the setting of various cardiac injuries. Limitations of these methods involve not being able to identify who the producers of these peptides are in the injured myocardium, where they might be located, and how quickly the transcription of these genes begins in response to a stressor. Here, using single-cell RNA-seq, I analyze a combination of human and mouse cardiac tissue and identify a small subset of cardiomyocytes that seem to be responsible for professionally producing these peptides. These cardiomyocytes are located directly adjacent to the site of injury and are being robustly produced as soon as four hours following a cardiac injury.

INTRODUCTION

Atrial and B-type natriuretic peptides (ANP and BNP) are stored and released by the atria and ventricles of the heart, respectively. They are encoded by the paralogous genes *Nppa* and *Nppb* and are traditionally thought to be made in response to myocardial stretch. The transcription and translation of these genes takes place primarily in the cardiomyocytes of those respective chambers and when secreted into the bloodstream, these peptides have several effects. At the kidneys, they promote natriuresis and diuresis. In the blood vessels, they cause vasodilation. Furthermore, these peptides modulate the renin-angiotensin-aldosterone system (RAAS) to decrease water retention and inhibit the sympathetic nervous system.

B-type natriuretic peptide is commonly used in the hospital setting as a biomarker for heart failure. In the landmark Breathing Not Properly (BNP) Study, investigators found that the biomarker BNP was markedly elevated in those patients presenting to the emergency department with symptoms of dyspnea due to heart failure.[1] They found that values of BNP were higher in both settings of systolic and non-systolic left ventricular (LV) dysfunction. This study paved the way for BNP as it is now routinely measured in patients presenting to the emergency department with shortness of breath to determine whether they are suffering from cardiac-related issues. There have been several additional studies following this discovery that highlighted how BNP might be helpful in other settings along with the clinical utility of its counterpart protein, N-terminal pro-BNP (NTproBNP).[2]

Apart from having great diagnostic and prognostic value, these natriuretic peptides can be leveraged to improve outcomes for patients from a therapeutic lens. The PARADIGM-HF trial found that a novel medication, LCZ696 (brand name, Entresto), was better than using an

angiotensin-converting enzyme inhibitor (ACE-inhibitor) to treat patients with heart failure across several primary and secondary outcomes.[3] This medication combined two drugs, an angiotensin receptor blocker (ARB) and a neprilysin inhibitor. Of key interest is the second part, the neprilysin inhibitor. Neprilysin is the enzyme that degrades and clears BNP from the bloodstream; therefore, the rationale was that by inhibiting neprilysin, the endogenous BNP made by the heart would be able to exert its cardioprotective effects for a longer period. Today, Entresto is a commonly used drug to help treat heart failure, illustrating that BNP is not only a robust biomarker of heart failure but also a promising therapeutic target.

METHODS

In the lab, basic science investigators sought to reveal what stimulates the release of these peptides and where they might show up in an injured heart. These has been a particular focus in understanding where these peptides are made in the setting of a myocardial infarction. Using bulk methods, it was discovered that the *Nppa* and *Nppb* genes are transcriptionally upregulated in the cardiomyocytes adjacent to those cardiomyocytes in the area affected by a myocardial infarction.[4,5,6] However, there are several limitations to these methods. A main limitation of using bulk cell-input methods is that an unknown number of cells added in unknown ratios yield a gene expression profile that is unresolvable. My research sought to overcome this limitation by using single cell RNA sequencing, a method that individually barcodes and labels each cell's transcripts so that each cell is given an equal platform to have its transcripts read. By measuring the transcripts being produced by a given cell, I can infer what protein products are being produced.

A limitation of cardiomyocytes is that they do not survive the enzymatic digestion process to liberate single cells. Therefore, I developed a nuclei isolation protocol that I would

use in lieu of single cell isolation to acquire single nuclei. Following the release of single nuclei, I used the 10X Chromium Next Gem 3' workflow to generate single cell/nuclei transcriptome data. Data analysis was conducted using Seurat in R Studio. For generating spatial transcriptomics data, I used the 10X Visium workflow. To better understand what cells are making the ANP and BNP, in what amounts, and where in the tissue, I analyzed a collection of human and mouse cardiac tissue over several pathologies.

RESULTS

I first looked in a piece of cardiac tissue collected from a patient suffering from nonischemic dilated cardiomyopathy at the time of their heart transplant (Figure 1.1). Collecting and analyzing the single nuclei of this sample revealed several cell types over 5,489 single nuclei (Figure 1.2). Subsetting and reclustering the 2,454 cardiomyocytes revealed a population of cardiomyocytes that were distinct in their production of their *Nppa* and *Nppb* genes (Figure 1.3 and 2). This suggests that only a small subset of cardiomyocytes is responsible for producing ANP and BNP. Though it is unknown where in the heart these cells might be located, it is interesting that only a small subset of cells produces these peptides in the end-stage heart failure setting.

To investigate whether this subset of cells naturally exists in any given heart, I next analyzed cardiac tissue collected from a patient with eosinophilic myocarditis also at the time of their transplant (Figure 3.1). Single nuclei RNA sequencing experiments yielded 3,149 single nuclei of which 758 nuclei were from cardiomyocytes (Figure 3.2 and 3.3). Similarly, subsetting and reclustering these nuclei showed that none of these cardiomyocytes professionally produced ANP and BNP (Figure 4). This further suggests and supports the hypothesis that stretch related injury leads to ANP and BNP production and that a pathology

not involving this stretch-related stress would not stimulate cardiomyocytes to produce ANP and BNP. Furthermore, these cells that produce these peptides are not present at baseline; they are specially activated in certain settings but not all.

To understand whether this small population of ANP and BNP producing cardiomyocytes exists following a myocardial infarction, I moved to a mouse model where I can access cardiac tissue and be able to modulate time and severity parameters of the infarct. I first looked at the cardiac tissue of a mouse three days after inducing a myocardial infarction by performing a permanent ligation of the left anterior descending artery (Figure 5.1). Single nuclei RNA sequencing yielded 4,147 single nuclei with several cell types being represented (Figure 5.2). Subsetting and analyzing the 426 cardiomyocytes here reveals a population, roughly 30%, that are professionally producing ANP and BNP (Figure 5.3 and Figure 6). This indicates that myocardial infarction also activates the production of ANP and BNP in cardiomyocytes in a similar way to the dilated cardiomyopathy setting. Where these cells are in the heart remains to be seen; however, of particular interest is whether these cells localize around a particular region of interest or are randomly scattered through the myocardium.

Given that at three days post-myocardial infarction, the landscape within the heart has radically evolved with the arrival of an immune infiltrate, I was interested in an earlier timepoint. To this end, I collected the cardiac tissue from a mouse just four hours after inducing a myocardial infarction by similar methods as outlined above (Figure 7.1). Single nuclei RNA sequencing analysis revealed 9,611 single nuclei (Figure 7.2). Of these, 1,032 nuclei were cardiomyocyte nuclei (Figure 7.3). Subsetting and reclustering these cells revealed a population of cardiomyocytes that were once again producing ANP and BNP. This data supports the theory that the activation of these special cardiomyocytes is not dependent

on an immune infiltration, remodeling, or fibrosis, given that all of these events take place at a much longer time course than by four hours.

To make sure that this special subset of cardiomyocytes was not present at baseline in mouse hearts, I similarly analyzed a healthy mouse's heart and found that this subset of cardiomyocytes vanished (Figures 8, 9, and 10). This underscores the fact this special subset of cardiomyocytes is only present after myocardial infarction but not before and that only a subset of these cells professionally produces ANP and BNP. However, this does not yet suggest where these cells might be in the infarcted heart.

By performing 10X Visium on a mouse heart following a myocardial infarction (3 days post), I was able to generate spatial transcriptomics data to highlight where these cells might be (Figure 11.1). Prior single cell data illustrates that there are three distinct subtypes of cardiomyocytes present following a myocardial infarction – two of which only really emerge following the injury (Figure 11.2). Prior to examining the single cell data on the tissue, a slice is taken for H&E staining (Figure 11.3). A crude outline is drawn around the area of the tissue that was subjected to the infarct. Using a collection of genes, a score can be assigned to each region of the tissue – the remote zone, the border zone, and the ischemic zone (Figure 11.4). Looking at some example genes that characteristically show up and separate one zone from another, we can see that the *Nppa* and *Nppb* genes are distinctly located directly adjacent to the ischemic zone that can be identified by some canonical immune markers (*Arg1, Cd68*) (Figure 11.5). This data suggests that the cardiomyocytes that produce ANP and BNP following a myocardial infarction do so only at the border of the injury where they are somehow able to sense the injury. These cells are clearly able to be recruited without the need for remodeling or fibrosis to occur.

CONCLUSION

In all, these data suggest that there is only a small subset of cardiomyocytes that are responsibly for producing ANP and BNP. These cells start making these peptides as early as four hours after an injury. They are not dependent on long term cardiac remodeling to be activated. In the case of myocardial infarction, they are in the border zone next to the infarct zone. Further research needs to be conducted to understand whether it might be a loss of neighboring cell that might activate these cells. Perhaps a different mouse model that can induce a global stressor would help elucidate what triggers the activation of ANP and BNP production. A trans-aortic constriction mouse model could potentially work to this end. Nonetheless, these data offer novel insights into how the widely studied ANP and BNP are produced in such a unique and specialized way.

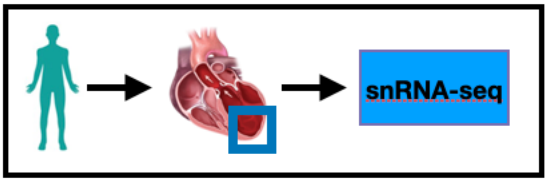

Figure 1.1: Schematic of human nonischemic cardiomyopathy cardiac tissue collection

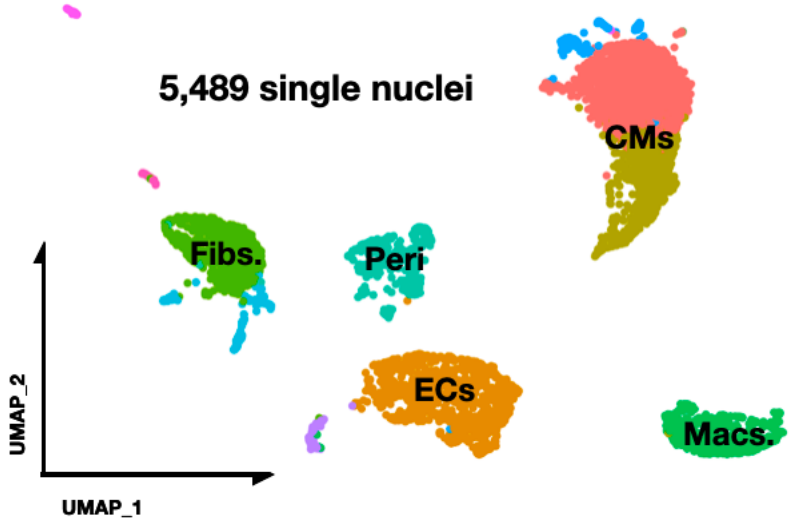

Figure 1.2: UMAP plot of single nuclei collected with the major cell types labelled

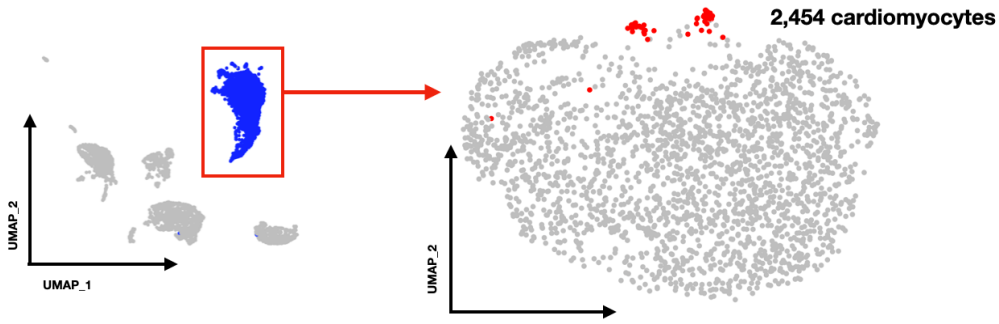

Figure 1.3: Subsetting cardiomyocytes and displaying UMAP plots

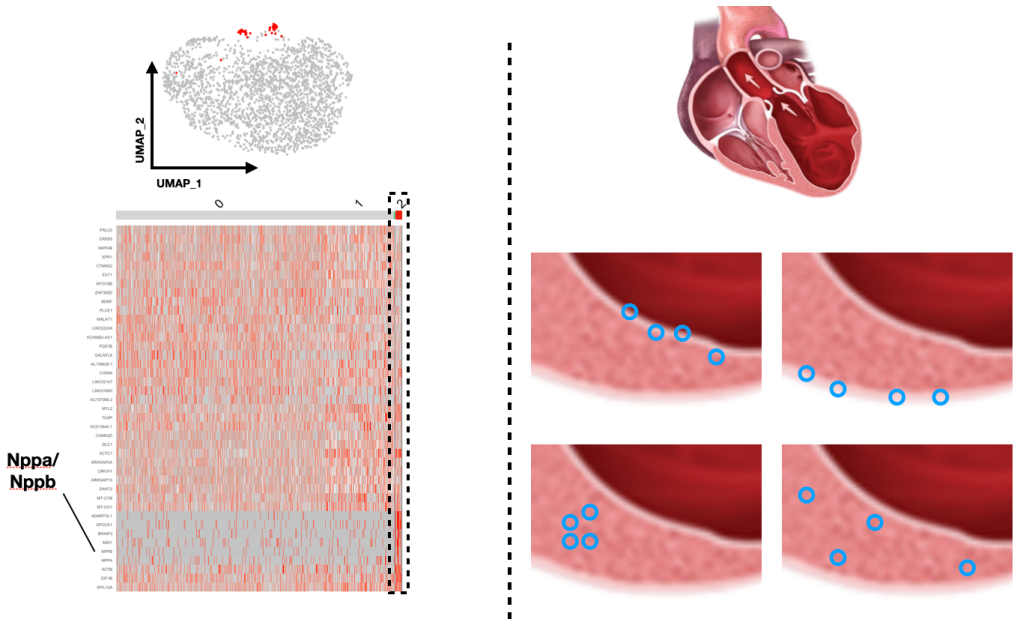

Figure 2: Heatmap of subsetted cardiomyocytes and depiction of predicted cell locations

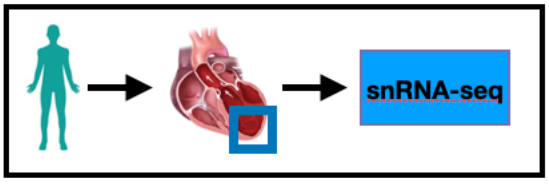

Figure 3.1: Schematic of human eosinophilic myocarditis cardiac tissue collection

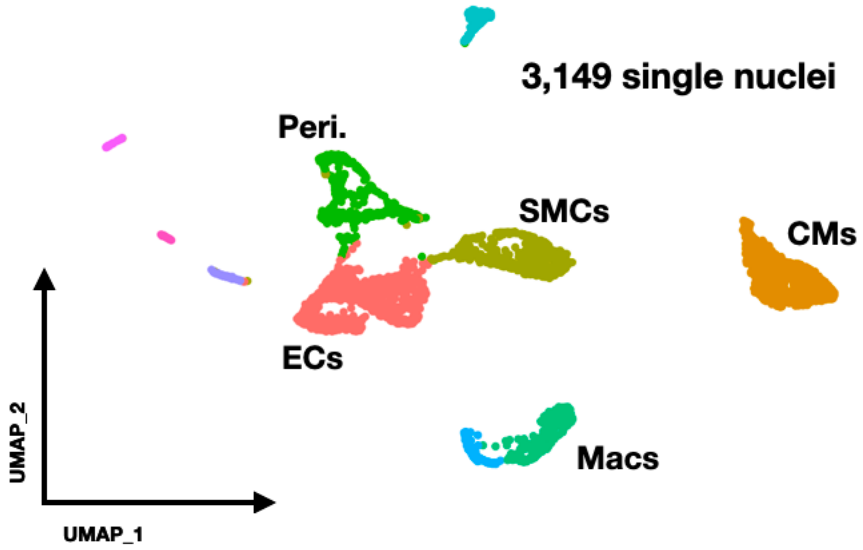

Figure 3.2: UMAP plot of single nuclei collected with the major cell types labelled

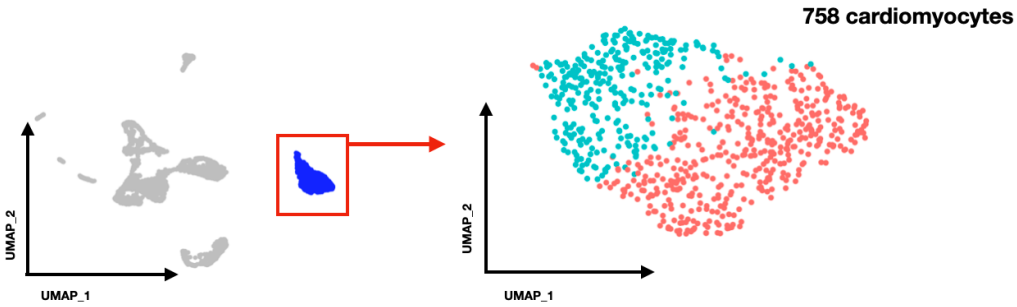

Figure 3.3: Subsetting cardiomyocytes and displaying UMAP plots

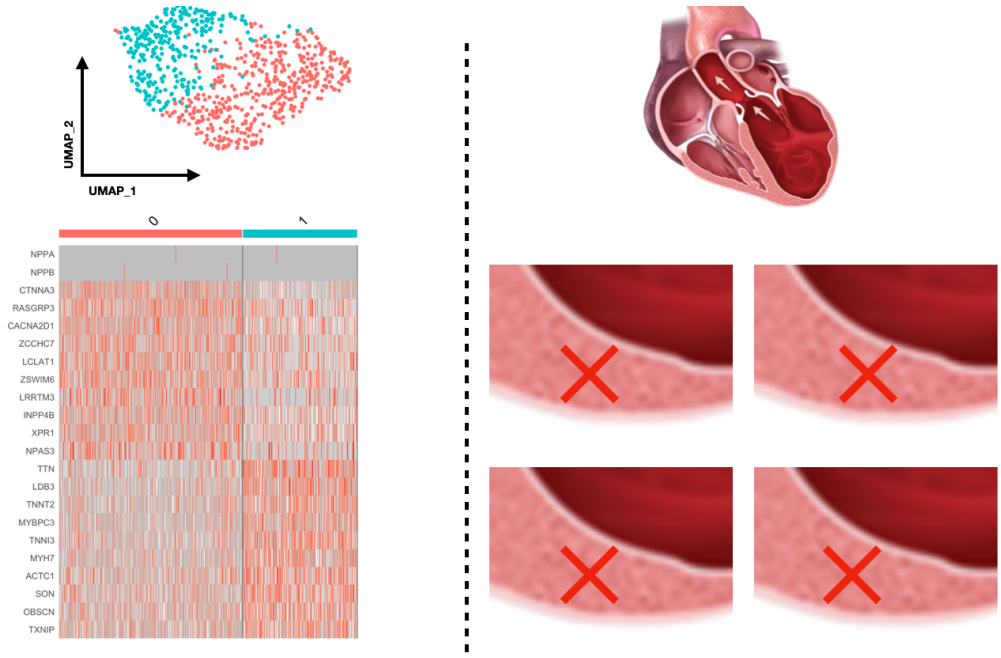

Figure 4: Heatmap of subsetted cardiomyocytes and depiction of predicted cell locations

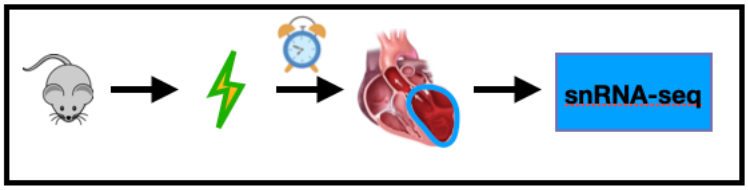

Figure 5.1: Schematic of mouse cardiac tissue collection three days following myocardial infarction

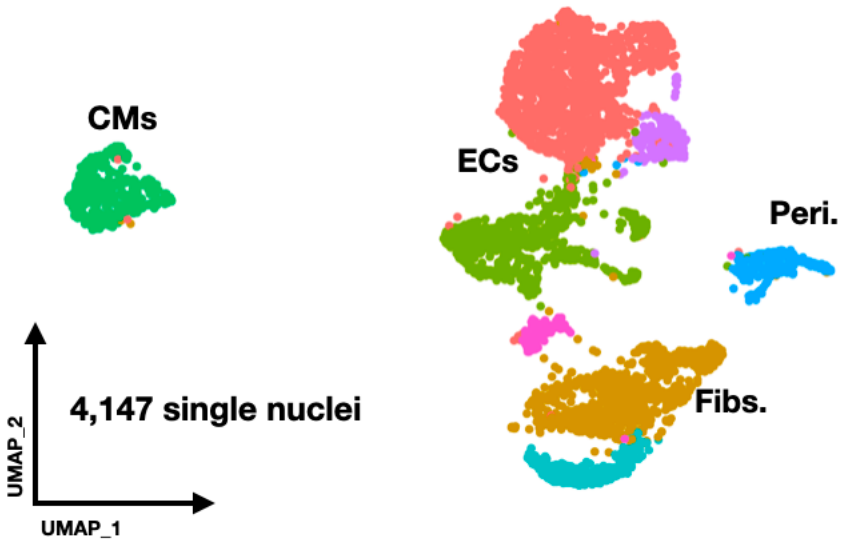

Figure 5.2: UMAP plot of single nuclei collected with the major cell types labelled

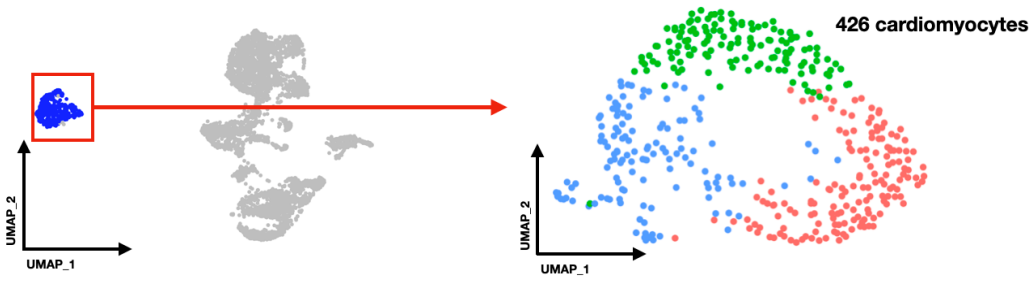

Figure 5.3: Subsetting cardiomyocytes and displaying UMAP plots

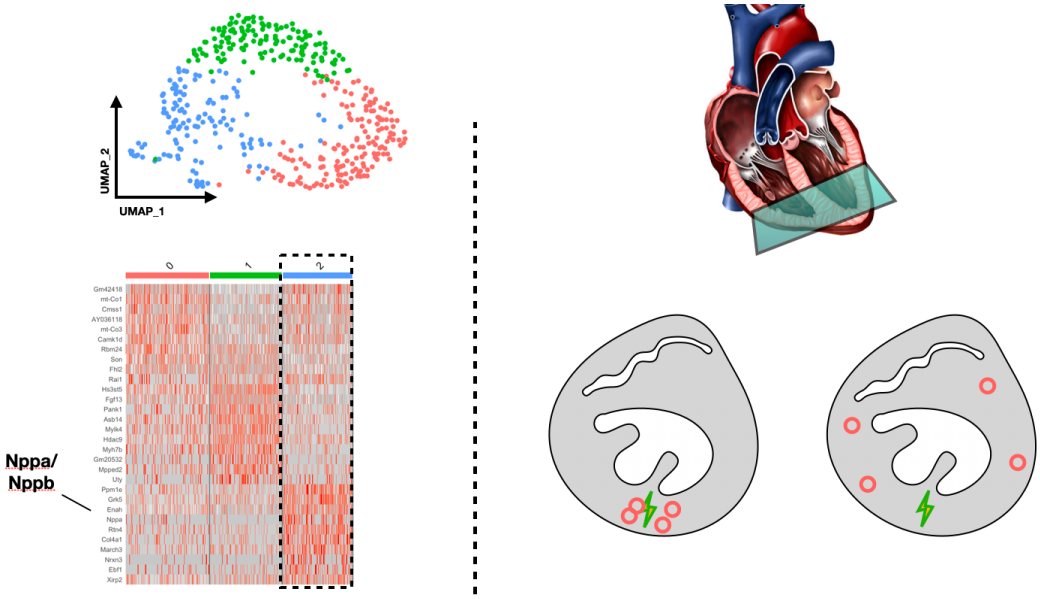

Figure 6: Heatmap of subsetted cardiomyocytes and depiction of predicted cell locations

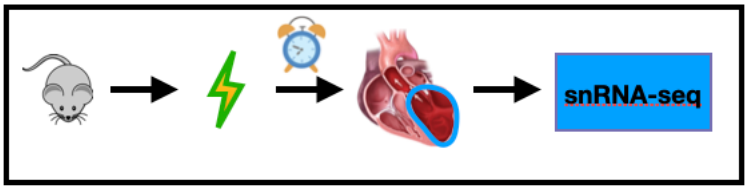

Figure 7.1: Schematic of mouse cardiac tissue collection four hours following myocardial infarction

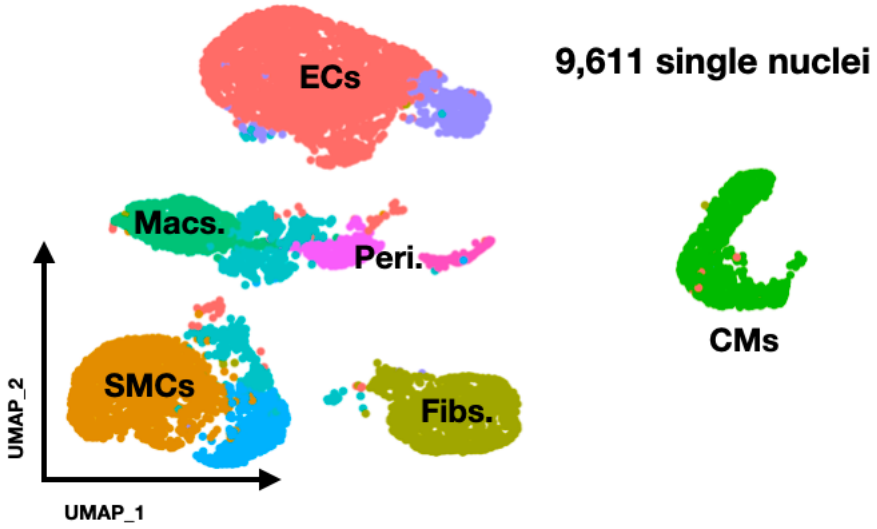

Figure 7.2: UMAP plot of single nuclei collected with the major cell types labelled

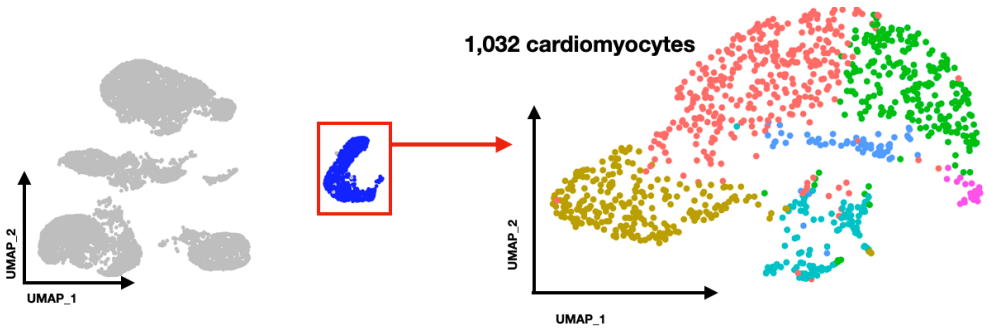

Figure 7.3: Subsetting cardiomyocytes and displaying UMAP plots

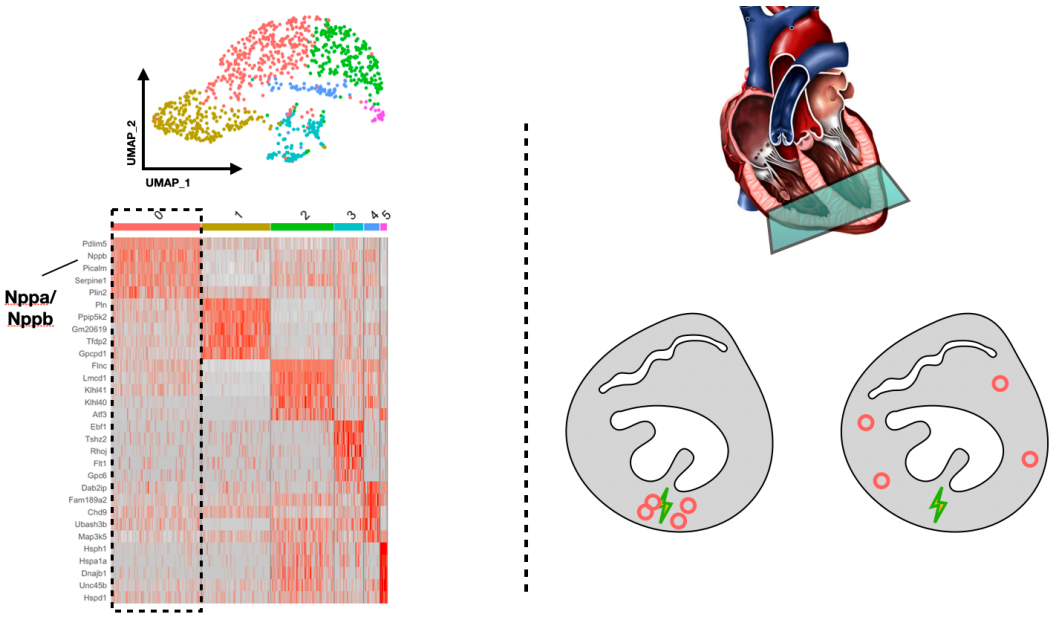

Figure 8: Heatmap of subsetted cardiomyocytes and depiction of predicted cell locations

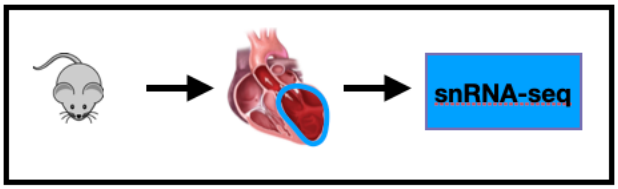

Figure 9.1: Schematic of mouse cardiac tissue collection at steady state

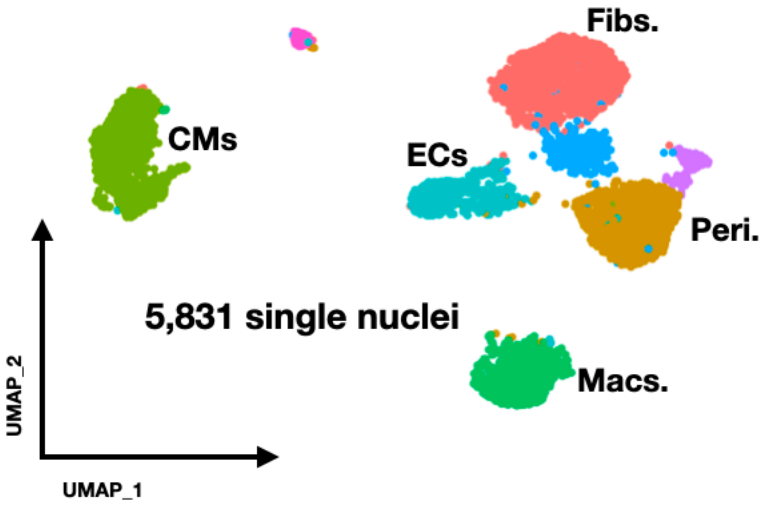

Figure 9.2: UMAP plot of single nuclei collected with the major cell types labelled

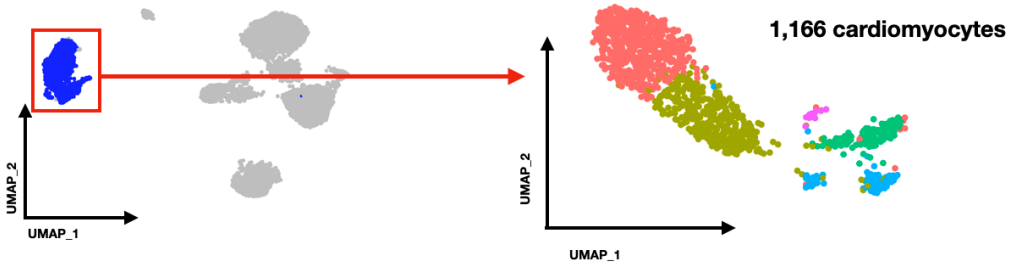

Figure 9.3: Subsetting cardiomyocytes and displaying UMAP plots

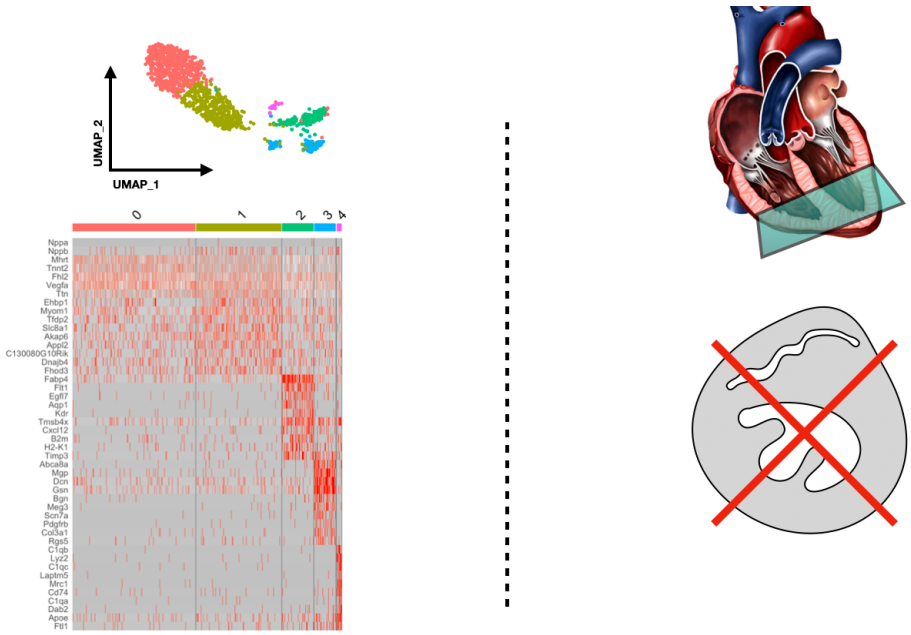

Figure 10: Heatmap of subsetted cardiomyocytes and depiction of predicted cell locations

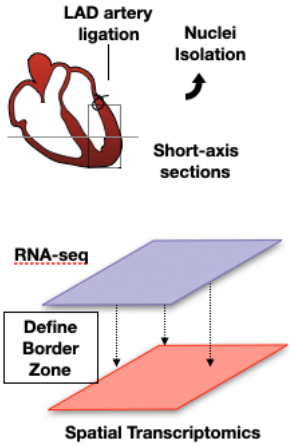

Figure 11.1: Schematic of mouse cardiac tissue collection three days following myocardial infarction for Visium

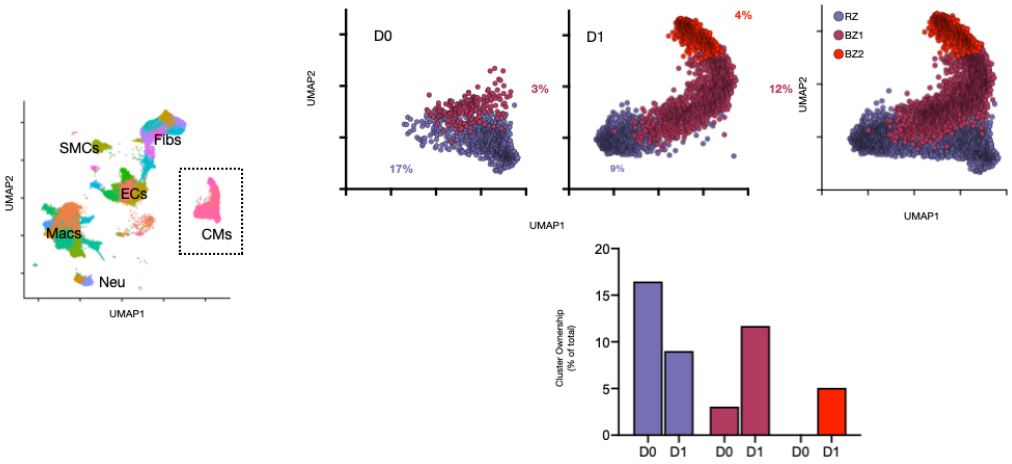

Figure 11.2: UMAP plot of single nuclei collected with the major cell types labelled and bar plot of cluster ownership vs day

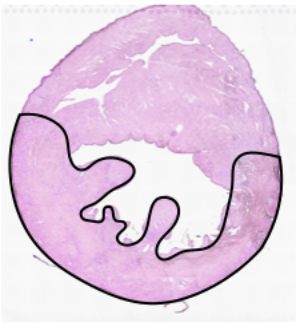

Figure 11.3: H&E staining of short axis section with infarct area highlighted in black

Figure 11.4: Remote zone (RZ), border zone (BZ), and infarct zone (IZ) identified by characteristic genes by Visium

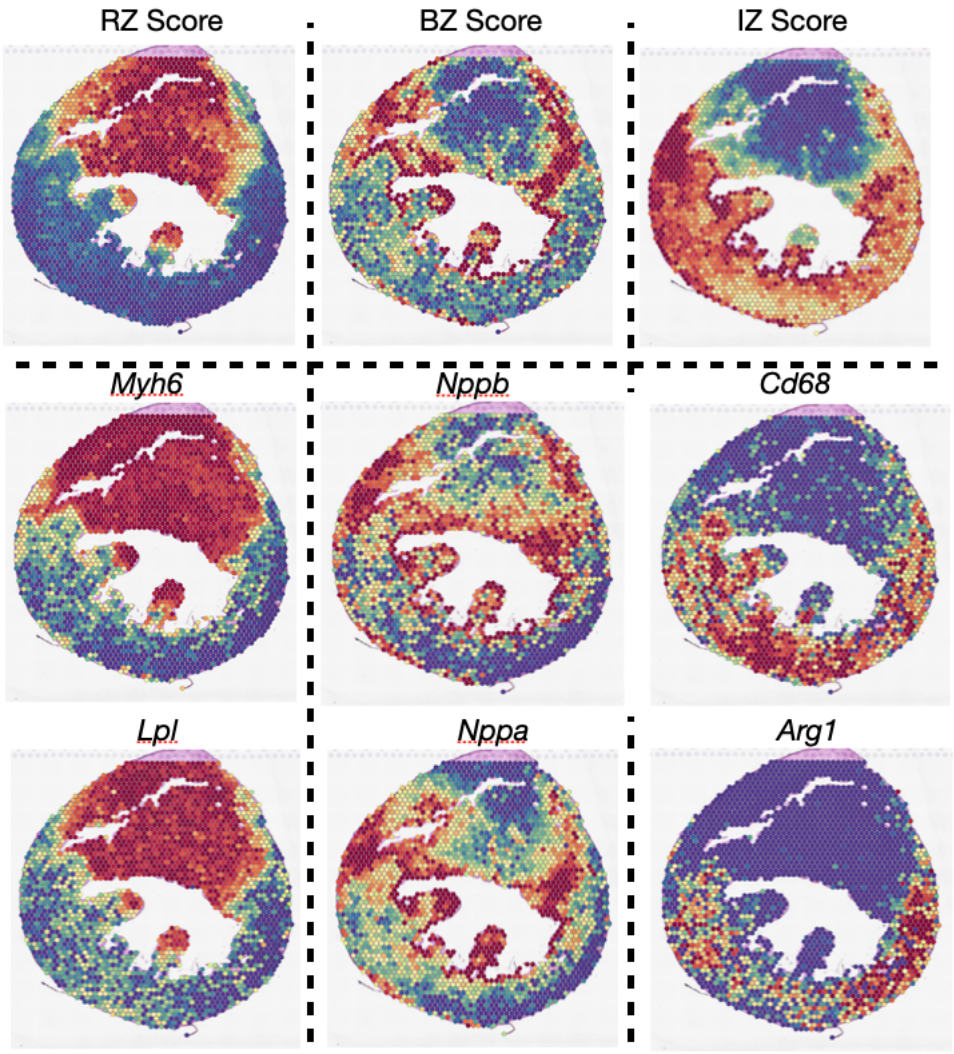

Figure 11.5: Remote zone, border zone and infarct zone displayed with two example characteristic genes per region

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