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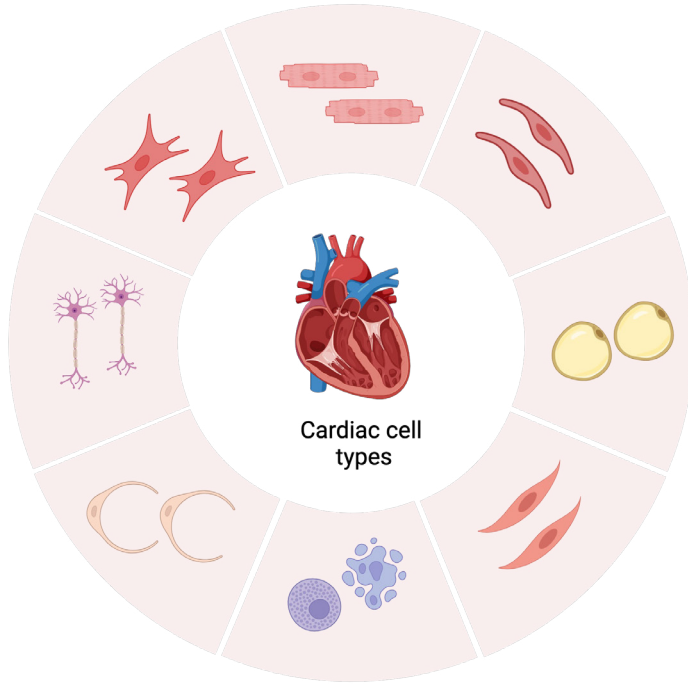
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Cellular individuality across the spectrum of heart diseases with implications for new therapeutic targets

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Cellular individuality across the spectrum of heart diseases with implications for new therapeutic targets

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Abstract:

Heart failure (HF) is a leading cause of death and disability globally. HF represents the common end-stage of most heart conditions, during which the heart is unable to generate sufficient output of blood for the metabolic demands of the body and intracardiac pressures increase. Different heart diseases display specific molecular pathophysiologies, but common pathways are also present that drive development and progression of HF. Specifically, the negative remodelling of the heart muscle that occurs gradually during prolonged load includes activation of a fetal gene expression program in heart muscle cells, immune cell activation, fibroblast activation, and increased fibrosis. However, both the molecular mechanisms of many underlying heart diseases and negative remodelling remain incompletely understood with limited therapeutic options beyond neurohormonal antagonists. The aims of this thesis were to (a) comprehensively investigate cell type-specific mechanisms involved in underlying heart diseases and negative remodeling in human hearts and (b) explore mechanisms linking the immunological mediator TSLP to HF mortality, as identified in a previous genetic study from the group.

In Paper I, we sought to develop a protocol for single cell isolation from frozen human hearts. However, a range of protocols was unable to isolate intact cells. Instead, we developed a protocol for isolation of single nuclei and show that nuclear transcriptomes are highly representative of the overall cellular and cytoplasmic transcriptome in human heart cells. By application of this protocol to human hearts and single nuclei RNA sequencing (snRNAseq) we developed a transcriptional atlas of the cell types and molecular profiles of the human heart. In Paper II, we greatly expanded this atlas to >100 human hearts with specific heart diseases and hearts without evidence of heart disease (controls). Compared to control hearts, the largest number of transcriptional differences were observed in dilated cardiomyopathy but most changes were also broadly shared with other conditions. In contrast, the largest number of unique transcriptional differences were seen in arrhythmogenic right ventricular cardiomyopathy. In Paper III, we find increased expression of TSLP in response to strain of cardiac fibroblasts. In addition, cardiac overexpression of TSLP resulted in increased expression of transforming growth factor β in myocardial mast cells, and tissue fibrosis. In Paper IV, we confirmed that the surface area of both cardiomyocytes and their nuclei were increased in HF patients, consistently with different underlying conditions, as compared to controls. Increased mechanical strain of iPSC-derived cardiomyocytes also resulted in increased cellular and nuclear size but these changes in nuclear size were not explained by changes in transcriptional activity as reflected by RNA content.

Collectively, this work shows the feasibility of dissecting the molecular pathophysiology of heart diseases from frozen single cardiac nuclei, highlights molecular signatures associated with specific heart muscle conditions, and implicates TSLP as a putative therapeutic target to prevent cardiac remodelling.

Key words: Heart failure, cardiac fibrosis, single nucleus RNA sequencing.

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*"If you want to shine like a sun,
first burn like a sun."
– Dr Abdul Kalam*

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Populärvetenskaplig sammanfattning

Hjärtsvikt är en av de vanligaste orsakerna till död och sjuklighet globalt, och utgör slutstadiet av de flesta hjärtsjukdomar. De vanligaste underliggande orsakerna är kranskärslssjukdom och högt blodtryck medan en mindre andel orsakas av primära hjärtmuskelsjukdomar (kardiomyopater). Förekomsten av hjärtsvikt har uppskattats till mer än 64 miljoner individer globalt och 200 000 i Sverige. Patienter med hjärtsvikt har en ökad dödlighet, ca hälften avlider inom 5 år, och nedsatt livskvalitet pga symptom som andfåddhet, ansträngningsintolerans, vätskeansamling med svullnad av ben och regelbundet behov av inneliggande sjukhusvård.

Vid hjärtsvikt är hjärtat oförmöget att upprätthålla tillräckligt utflöde av blod för kroppens behov. Trycket i hjärtat ökar, vilket resulterar i ökad belastning på hjärtmuskeln. Sådan ökad belastning resulterar över tid i kompensatoriska förändringar i hjärtmuskeln, såsom ökad storlek av hjärtmuskelceller och ökad bildning av ärrvävnad (fibros) som ökar hjärtmuskeln's stelhet, vilket kallas negativ remodellering. Sådana förändringar medför positiva effekter på kort sikt, men resulterar i progress av hjärtsvikt och ökad risk för död.

Det finns ingen bot för hjärtsvikt men flera behandlingar som minskar symptomen och förbättrar prognosen. De flesta sådana behandlingar riktas mot cirkulerande hormoner för att minska belastningen på hjärtmuskeln ytterst genom att minska blodtryck, blodvolym, hjärtfrekvens och liknande hemodynamiska effekter. Få behandlingar finns dock mot underliggande sjukdomar eller som direkt riktas mot negativ remodellering.

Hjärtmuskelcellerna (kardiomyocyterna) genererar den kraft som krävs för hjärtats pumparbete och är därför centrala för hjärtats funktion. Många andra celltyper är dock åtminstone lika vanliga i hjärtat och bidrar också till hjärtats funktion och tros ha en central roll i remodelleringsprocesser. Fibroblaster verkar genom att bilda den extracellulärmatrix som finns i hjärtat och ger strukturellt stöd. Endotelceller bildar det innersta lagret i blodkärl och hjärtrum och styr cirkulation och kärlgenomsläpplighet. Glatta muskelceller är ansvariga för blodkärlsreglering och att bibehålla elasticitet i blodkärlen. Skador mot hjärtat resulterar i död av hjärtmuskelceller och ökad aktivitet av fibroblaster som genererar ärrvävnad och frisätter ett stort antal signalämnen. Exakt vilken roll olika celltyper och deras molekylära profiler spelar för hjärtsjukdomar och remodellering är dock ofullständigt kartlagt, delvis pga svårigheter att få tag på intakt human hjärtvävnad.

Denna avhandling avsåg därför att undersöka strategier för att separera ut individuella hjärtceller så att hjärtvävnad kan studeras i detalj på singel-cell nivå, för molekylära analyser av hur enskilda celler och molekyler bidrar till specifika sjukdomar och remodellering.

I en första studie utvecklade vi protokoll för att isolera enskilda cellkärnor från humana hjärtan. Vi fann att frysande av celler, vilket är nödvändigt för preservation av hjärtprover till analys, resulterar i svårigheter att separera ut enskilda celler. Däremot cellkärnor var mycket mer robusta vid frysning och vi finner att det molekylära innehållet i cellkärnor i stort är representativt för hela celler. Vi använde därefter dessa metoder för att generera en karta över cellulära och molekylära komponenter i det humana hjärtat. I en andra studie använde vi dessa isoleringsmetoder för att studera flera specifika hjärtmuskelsjukdomar och fann ökat antal fibroblaster vid alla sjukdomarna. Många förändringar i genaktivitet var desamma vid olika sjukdomar, talande för en roll vid remodelering, medan andra förändringar var specifika för ett visst tillstånd. Det största antalet förändringar sågs vid arytmoden högerkammars kardiomyopati. I en tredje studie fokuserade vi på molekylen TSLP som identifierats som en möjlig bidragande faktor till remodelering i genetiska studier. Vi fann att TSLP frisätts vid belastning av hjärtceller och aktiverar fibroblaster till ökad fibros och hjärtstelhet. Slutligen, i studie fyra, fann vi att hjärtmuskelcellernas kärnor också ökar i storlek vid mekanisk belastning och är större i sviktande hjärtan än kontrollhjärtan.

Sammantaget ger studierna i denna avhandling inblick i cellulära identiteter vid hjärtmuskelsjukdomar, möjliga mekanismer för sjukdomsutveckling och remodelering, och nominerar nya potentiella terapeutiska mål inklusive TSLP som kan bidra till att förbättra prognosen vid hjärtsvikt.

Popular Science Summary

Heart failure (HF) is a leading cause of death and disability globally, representing the end-stage of most heart diseases. The most common underlying conditions are coronary artery disease and hypertension while a smaller subset of cases are due to primary diseases of the heart muscle (cardiomyopathies). The prevalence of HF has been estimated to more than 64 million individuals globally and 200 000 in Sweden. Patients with HF suffer from poor prognosis, with a five year mortality of approximately 50%, and reduced quality of life due to symptoms such as shortness of breath, exercise intolerance, fluid retention with swelling of the legs and frequent need for hospitalization.

During HF, the heart is unable to generate sufficient output of blood for the demands of the body. The pressure inside the heart increases, resulting in increased strain on the heart muscle. Such increases in strain act over time to result in compensatory changes in the heart muscle, such as increased size of heart muscle cells and increased deposition of scar tissue (fibrosis) which increases the stiffness of the heart muscle, referred to as negative remodelling. Such changes, although conferring positive effects in the short term, result in progression of HF and increased risk of mortality.

Although cure is not possible for HF, several therapies are available to relieve symptoms and improve prognosis. Most such therapies target circulating hormones to reduce strain on the heart muscle by ultimately reducing blood pressure, blood volume, heart rate and similar hemodynamic effects. However, therapy rarely targets the underlying conditions or directly the negative remodelling.

Although heart muscle cells (cardiomyocytes), which generate the force for cardiac pumping, are central for the function of the heart, many other cells types are at least equally abundant in the heart and also contribute importantly to cardiac function and are central for remodelling processes. Fibroblasts function by maintaining the extracellular matrix of the heart and provide structural support. Endothelial cells form the inner layer of blood vessels and heart chambers, regulating circulation and vessel permeability. Smooth muscle cells are responsible for vascular regulation and maintaining the elasticity of blood vessels. Injury to the heart results in death of heart muscle cells but increased activity of fibroblasts to generate scar tissue and release a range of signaling molecules. However, the precise contributions of specific cell types and their molecular profiles to heart diseases and remodelling remains incompletely understood, in part due to the difficulties of obtaining intact human heart tissue.

Therefore, this thesis aimed to investigate strategies to separate individual heart cells so that the tissue of the heart could be studied accurately at the single-cell level, for comprehensive molecular analysis of the contribution of such cells and molecules to specific diseases and remodelling.

In a first study, we developed a protocol to isolate single cell nuclei from human hearts. We find that freezing of cells, which is necessary for preservation of heart samples until analysis, results in difficulties in separating out single cells. However, cellular nuclei are much more robust to freezing and we find that the molecular contents of cellular nuclei are largely representative of whole cells. We then used these methods to generate a detailed atlas of the cellular and molecular constituents of the human heart. In a second study, we employed this isolation protocol to multiple heart muscle diseases, and we found increased number of fibroblasts in all the muscle diseases. Many changes in gene activity were broadly shared across diseases, consistent with a role in remodelling, while some changes were specific for a certain condition. The largest number of changes were seen in arrhythmogenic right ventricular cardiomyopathy. In the third study, we focused on the molecule thymic stromal lymphopoietin (TSLP), which was identified as a potential contributor to remodelling in genetic studies. We find that TSLP is released in response to cardiac cell strain and activates fibroblasts to increase fibrosis and cardiac stiffness. Finally, in study four, we found that cardiomyocyte nuclei increase in size when put under mechanical stress, and are larger in heart failure patients than in controls.

Together, the studies in this thesis provide insights into cellular identities across heart muscle diseases, potential mechanisms for disease onset and remodelling, and nominate new potential therapeutic targets including TSLP which may help improve outcomes in HF.

विज्ञान सारांश

हृदय अपयश हे जागतिक स्तरावर मृत्यू आणि अपंगत्वाचे एक प्रमुख कारण आहे, जे बहुतेक हृदयविकारांच्या शेवटच्या टप्प्याचे प्रतिनिधित्व करते. सर्वात सामान्य अंतर्निहित परिस्थिती म्हणजे कोरोनरी धमनी रोग आणि उच्च रक्तदाब, तर काही लहान उपसंच हृदयाच्या स्नायूंच्या प्राथमिक रोगांमुळे (कार्डिओमायोपथी) असतात. हृदय अपयश चा प्रसार जागतिक स्तरावर 64 दशलक्षाहून अधिक लोकांमध्ये आणि स्वीडनमध्ये 200,000 लोकांमध्ये असल्याचा अंदाज आहे. श्वास लागणे, व्यायाम असहिष्णुता, पाय सुजणे आणि वारंवार हॉस्पिटलायझेशनची गरज यांसारख्या लक्षणांमुळे एचएफ असलेल्या रुग्णांना खराब रोगनिदान, पाच वर्षांच्या मृत्यूचे प्रमाण आणि जीवनाची गुणवत्ता कमी होते.

हृदय अपयश दरम्यान, हृदय शरीराच्या मागणीसाठी पुरेशा प्रमाणात रक्त उत्पादन करण्यास असमर्थ असते. हृदयाच्या आतील दाब वाढतो, परिणामी हृदयाच्या स्नायूवर ताण वाढतो. हृदयाच्या स्नायूंच्या पेशींचा आकार वाढणे आणि स्कायर टिश्यू (फायब्रोसिस) वाढणे ज्यामुळे हृदयाच्या स्नायूंचा कडकपणा वाढतो, याला नकारात्मक रीमॉडेलिंग असे म्हणतात. असे बदल, जरी अल्पावधीत सकारात्मक परिणाम देतात, परिणामी हृदय अपयश ची प्रगती होते आणि मृत्यूचा धोका वाढतो.

हृदय अपयश साठी बरा होणे शक्य नसले तरी, लक्षणे दूर करण्यासाठी आणि रोगनिदान सुधारण्यासाठी अनेक थेरपी उपलब्ध आहेत. बहुतेक अशा उपचारपद्धती अंततः रक्तदाब, रक्ताचे प्रमाण, हृदय गती आणि तत्सम हेमोडायनामिक प्रभाव कमी करून हृदयाच्या स्नायूवरील ताण कमी करण्यासाठी परिसंचरण संप्रेरकांना लक्ष्य करतात. तथापि, थेरपी क्वचितच अंतर्निहित परिस्थिती किंवा थेट नकारात्मक रीमॉडेलिंगला लक्ष्य करते.

हृदयाच्या स्नायूंच्या पेशी (कार्डिओमायोसाइट्स), जे हृदयाच्या पंपिंगसाठी शक्ती निर्माण करतात, हृदयाच्या कार्यासाठी केंद्रस्थानी असले तरी, इतर अनेक पेशींचे प्रकार हृदयामध्ये कमीतकमी तितकेच विपुल असतात आणि हृदयाच्या कार्यामध्ये देखील महत्वपूर्ण योगदान देतात आणि पुनर्निर्मित प्रक्रियेसाठी केंद्रस्थानी असतात. . फायब्रोब्लास्ट्स हृदयाच्या बाह्य मॅट्रिक्सची देखभाल करून आणि संरचनात्मक समर्थन प्रदान करून कार्य करतात. एंडोथेलियल पेशी रक्तवाहिन्या आणि हृदयाच्या कक्षांचा आतील थर तयार करतात, रक्ताभिसरण आणि रक्तवाहिन्यांची पारगम्यता नियंत्रित करतात. गुळगुळीत स्नायू पेशी संवहनी नियमन आणि रक्तवाहिन्यांची लवचिकता राखण्यासाठी जबाबदार असतात. हृदयाला झालेल्या दुखापतीमुळे हृदयाच्या स्नायूंच्या पेशींचा मृत्यू होतो परंतु फायब्रोब्लास्ट्सची क्रिया वाढल्याने डाग टिश्यू तयार होतात आणि सिग्नलिंग रेणूंची श्रेणी सोडते. तथापि, हृदयविकार आणि रीमॉडेलिंगमध्ये विशिष्ट पेशींच्या प्रकारांचे आणि त्यांच्या

आण्विक प्रोफाइलचे अचूक योगदान अपूर्णपणे समजले नाही, काही अंशी अखंड मानवी हृदयाच्या ऊती मिळविण्याच्या अडचणींमुळे.

म्हणूनच, या प्रबंधाचे उद्दिष्ट वैयक्तिक हृदयाच्या पेशी विभक्त करण्याच्या धोरणांची तपासणी करणे होते जेणेकरून हृदयाच्या ऊतींचा एकल-सेल स्तरावर अचूकपणे अभ्यास केला जाऊ शकतो, विशिष्ट रोग आणि पुनर्निर्मितीमध्ये अशा पेशी आणि रेणूंच्या योगदानाचे सर्वसमावेशक आण्विक विश्लेषण.

पहिल्या अभ्यासात, आम्ही मानवी हृदयातून सिंगल सेल न्यूक्ली वेगळे करण्यासाठी एक प्रोटोकॉल विकसित केला. विश्लेषण होईपर्यंत हृदयाचे नमुने जतन करण्यासाठी आवश्यक असलेल्या पेशी गोठवल्यामुळे एकल पेशी वेगळे करण्यात अडचणी येतात असे आम्हाला आढळून आले आहे. तथापि, सेल्युलर न्यूक्लीय हे गोठवण्यापेक्षा जास्त मजबूत असतात आणि आम्हाला आढळले की सेल्युलर न्यूक्लीची आण्विक सामग्री मोठ्या प्रमाणात संपूर्ण पेशींचे प्रतिनिधी आहेत. त्यानंतर आम्ही मानवी हृदयाच्या सेल्युलर आणि आण्विक घटकांचे तपशीलवार अँटलस तयार करण्यासाठी या पद्धती वापरल्या. दुसऱ्या अभ्यासात, आम्ही हा आयसोलेशन प्रोटोकॉल अनेक हृदयाच्या स्नायूंच्या रोगांसाठी वापरला आणि आम्हाला सर्व स्नायूंच्या आजारांमध्ये फायब्रोब्लास्ट्सची संख्या वाढलेली आढळली. जनुकांच्या क्रियाकलापातील अनेक बदल हे सर्व रोगांमध्ये व्यापकपणे सामायिक केले गेले होते, रीमॉडेलिंगच्या भूमिकेशी सुसंगत, तर काही बदल एका विशिष्ट स्थितीसाठी विशिष्ट होते. एरिथमोजेनिक उजव्या वेंट्रिक्युलर कार्डिओमायोपॅथीमध्ये सर्वात जास्त बदल दिसून आले. तिसऱ्या अभ्यासात, आम्ही थायमिक स्ट्रोमल लिम्फोपोएटिन (TSLP) रेणूवर लक्ष केंद्रित केले, जे अनुवांशिक अभ्यासांमध्ये पुनर्निर्मितीसाठी संभाव्य योगदानकर्ता म्हणून ओळखले गेले. आम्हाला आढळले की TSLP कार्डियाक सेल स्ट्रेनला प्रतिसाद म्हणून सोडले जाते आणि फायब्रोसिस आणि हृदयाचा कडकपणा वाढवण्यासाठी फायब्रोब्लास्ट सक्रिय करते. शेवटी, चौथ्या अभ्यासात, आम्हाला असे आढळून आले की, यांत्रिक तणावाखाली असताना कार्डिओमायोसाइट न्यूक्लीचा आकार वाढतो आणि हार्ट फेल्युअर रूग्णांमध्ये नियंत्रणापेक्षा जास्त असते.

एकत्रितपणे, या प्रबंधातील अभ्यास हृदयाच्या स्नायूंच्या रोगांमधील सेल्युलर ओळख, रोगाची सुरुवात आणि रीमॉडेलिंगसाठी संभाव्य यंत्रणा, आणि TSLP सह नवीन संभाव्य उपचारात्मक लक्ष्ये नामांकित करतात जे हृदय अपयश मध्ये परिणाम सुधारण्यास मदत करू शकतात.

List of papers

Paper I

Neha Pimpalwar, Tomasz Czuba, Maya Landenhed Smith, Johan Nilsson, Olof Gidlöf, J. Gustav Smith. Methods for isolation and transcriptional profiling of individual cells from the human heart. **Heliyon** 2020; 6 (12): e05810.

Paper II

Olof Gidlöf, **Neha Pimpalwar**, Tomasz Czuba, Joakim Sandstedt, Jakob Lundgren, Entela Bollano, Selvi Celik, Erik Linnér, Sebastian Albinsson, Göran Dellgren, Jan Borén, Anders Jeppsson, Johan Nilsson, Pradeep Natarajan, Patrick T. Ellinor, Tuuli Lappalainen, Kristina Vukusic, Malin Levin, J. Gustav Smith. Single nucleus profiling of heart muscle diseases.

Manuscript

Paper III

Neha Pimpalwar, Selvi Celik, Mardjaneh Karbalaeei Sadegh, Tomasz Czuba, Olof Gidlöf, J. Gustav Smith. Analysis of genetic variant associated with heart failure mortality implicates thymic stromal lymphopoietin as mediator of strain-induced myocardial fibroblast-mast cell crosstalk and fibrosis. **FASEB J** 2024 ; 38 (4): e23510.

Paper IV

Neha Pimpalwar, Kristina Vukusic, J. Gustav Smith, Olof Gidlöf. Effects of heart failure and mechanical strain on nuclear size in human cardiomyocytes.

Manuscript

Selected abbreviations

AAV	Adeno-associated virus
ChIP.	Chromatin immunoprecipitation
CVD	Cardiovascular diseases
DCM	Dilated cardiomyopathy
eGFP	Enhanced green fluorescent protein
EMSA	Electrophoretic mobility shift assay
HCF	Primary human cardiac fibroblasts
HCM	Hypertrophic cardiomyopathy
HF	Heart failure
HFrEF	Heart failure with reduced ejection fraction
HFmrEF	Heart failure with mildly reduced ejection fraction
HFpEF	Heart failure with preserved ejection fraction
ICM	Ischemic cardiomyopathy
iPS-cells	Induced pluripotent stem
NHLH1	Nescient helix-loop helix 1
qRT-PCR	Quantitative real time-polymerase chain reaction
SNP	Single nucleotide polymorphism
snRNA-Seq	Single nucleus RNA sequencing
TSLP	Thymic stromal lymphopoietin

Introduction

The heart

The heart is a muscular organ located centrally within the chest behind the sternum. The main function of the heart is to serve as a pump, maintaining circulation of blood and providing all tissues throughout the body with oxygen and nutrients. The heart is a four-chambered structure consisting of two upper smaller chambers, referred to as atria, and two lower chambers referred to as ventricles [4]. The superior and inferior vena cava transport deoxygenated blood from the body to the right atrium, whereas the pulmonary veins transport oxygenated blood from the lungs to the left atrium. The right ventricle receives blood deoxygenated blood from the right atrium and generates pressure to drive blood to the lungs through the pulmonary artery. Conversely, the left ventricle receives oxygenated blood from the left atrium and generates pressure sufficient to transport blood to the rest of the body through the aorta [1]. To ensure unidirectional flow of blood through the heart, the heart has four valves that prevent reverse flow between chambers: two atrioventricular valves prevent backflow from ventricles into atria: the tricuspid valve on the right side and the mitral valve on the left side. The aortic valve prevents backflow from the aorta into the left ventricle and the pulmonary valve prevents backflow from the pulmonary artery to the right ventricle. The heart itself is supplied with blood from arterial branches from the aorta - the coronary arteries.

The heart consists of three layers: The innermost layer is called the endocardium, lines the chambers and valves and is similar in structure, function and embryological origin to the endothelial lining of blood vessels. The middle layer is the thick muscular myocardium, consisting of heart muscle cells (cardiomyocytes), blood vessels and connective tissue, and is responsible for the contraction of the heart. The outermost layer is the epicardium which is a protective layer composed of connective tissue and epithelium [2]. The epicardium borders to the fluid-filled pericardial cavity which allows the heart to move without friction during contraction (**Figure 1**).

The heart undergoes a sequence of changes in pressure and volume known as a cardiac cycle that lasts one whole beat. The sinoatrial node (SA), located in the right atrium, first generates an electrical signal that is transmitted through the atria by the flux of ions across the cell membrane. This signal causes the coordinated contraction of the atrial myocardium and ejection of blood from atria to ventricles after an initial filling phase. The signal then travels from the atria through the atrioventricular (AV) node

and a specialized conduction system to the ventricles, prompting contraction of the ventricular myocardium and ejection of blood into the arteries. After contraction and ejection of blood into arteries, a phase of the cardiac cycle referred to as systole, the chambers relax and filling with blood begins, a phase referred to as diastole. Diastole starts when the pulmonary and aortic valves close and finishes when the tricuspid and mitral valves close. Systole begins when the tricuspid and mitral valves close and ends with the closing of the pulmonary and aortic valves [3] [4].

Impulse generation in the pacemaker cells of the SA node and conduction through the AV node is regulated by the autonomic nervous system, regulating the heart rate as the cardiac cycle continuously repeats. A constant blood flow that is matched to tissue demands throughout the circulatory system is assured by the coordinated cardiac cycle [5, 6].

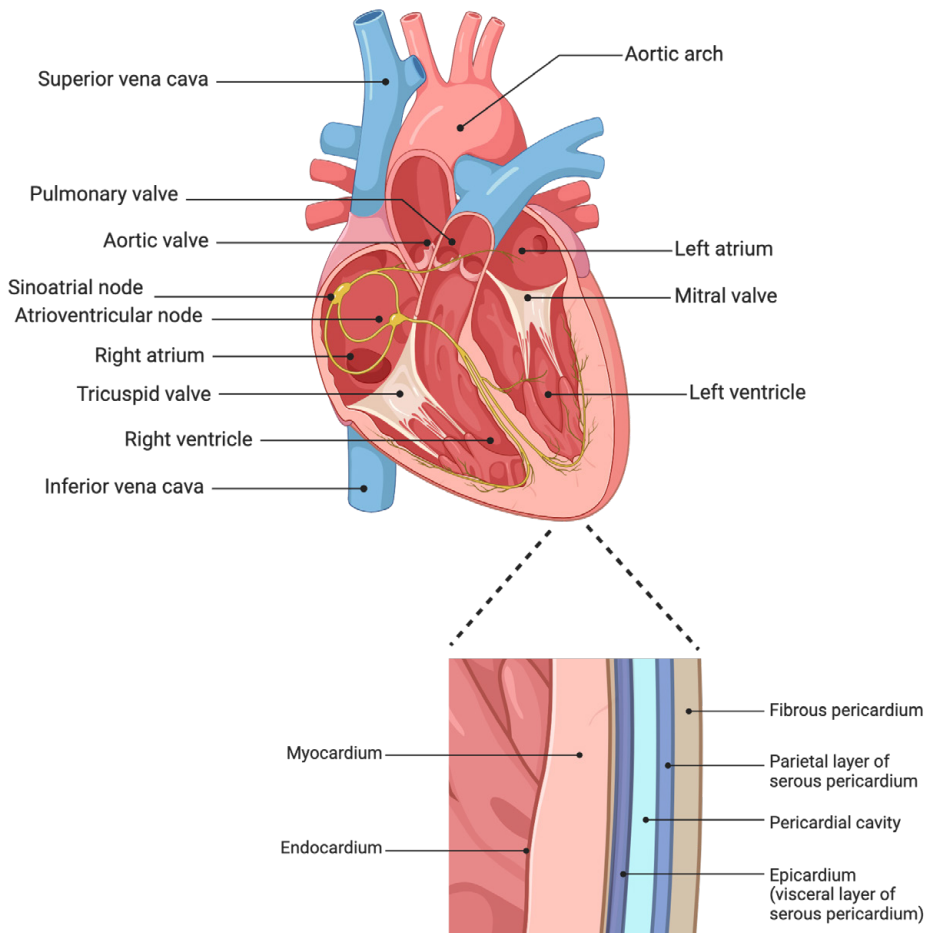


Figure 1. Human heart anatomy.

Cellular heterogeneity in the human heart

The heart is often viewed as a muscular organ, and although the large cardiomyocytes make up a substantial proportion of the heart volume, other cell types are equally or more common in the three layers that make up the heart. The most prominent cell types have been shown in histological studies to be endothelial cells, fibroblasts, cardiomyocytes, and smooth muscle cells [7, 8]. In addition, the heart is known to contain populations of adipocytes, pericytes, neurons, and immune cells (**Figure 2**) [9, 10].

The abundance, molecular profiles, and spatial arrangement of cardiac cell types as well as their surrounding matrix determine the properties of the heart, including its stiffness, contractility, and conductive properties [11]. Importantly, the distribution of distinct cell types in the heart may also determine how it responds to injury, strain and other pathological processes as different cell types respond differently. In addition, understanding the distribution of different cell types in the heart is essential for understanding its development [12].

The implications of cellular heterogeneity in the heart are thus significant both for understanding cardiac physiology, development, pathologies, and potential therapeutic strategies [9].

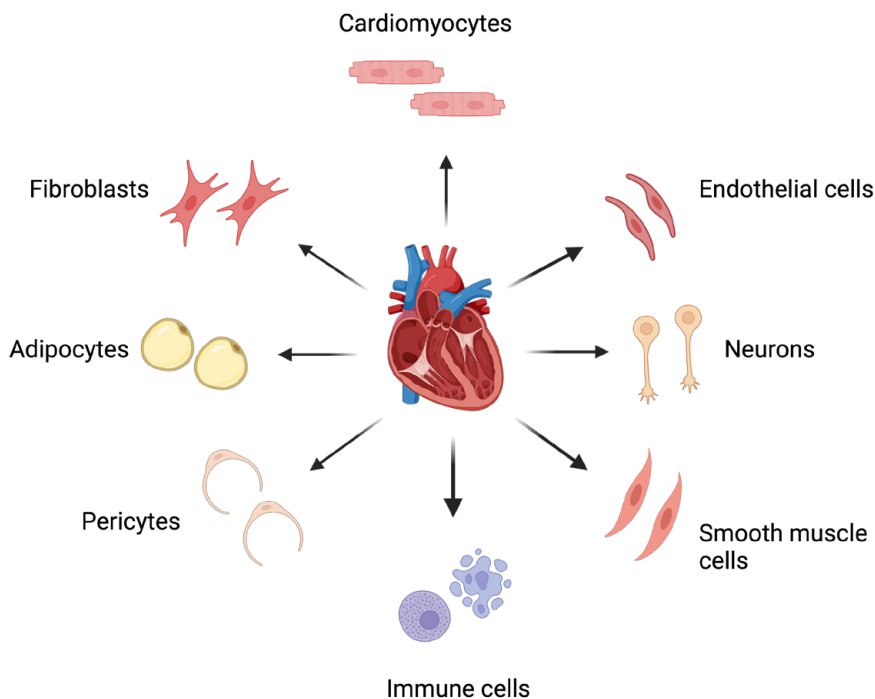


Figure 2. Schematic representation of cardiac cell types.

Cardiomyocytes

Cardiomyocytes are a type of specialized muscle cells in the heart which generate contractile force and active relaxation upon electrochemical excitation. These cells comprise around 30% of cells in the human heart [7], connecting to each other through membrane structures referred to as intercalated discs, in which gap junctions allow flow of ions between cardiomyocytes, forming a network throughout the heart referred to as a functional syncytium.

Cardiomyocytes have a tubular shape with a central nucleus, although a subset have two or more nuclei, and a plasma membrane known as the sarcolemma [13]. They are densely packed with mitochondria for energy generation and myofibrils, rod-like organelles within the cell responsible for the active contraction and relaxation of cardiomyocytes. In close proximity with both the myofibrils and the sarcolemma is also a specialized type of smooth endoplasmic reticulum that stores calcium ions and plays a central role in activation and regulation of myofibril function.

Cardiomyocyte membrane potential is precisely regulated by a spatially defined network of ion channels and exchangers that regulate the flux of sodium, potassium, and calcium ions across the sarcolemma and sarcoplasmic reticulum. The electrical activity across the sarcolemma controls Ca^{2+} release from the sarcoplasmic reticulum which in turn activates myofibril contractility [14].

Myofibrils are made up of repeated sarcomeres, the basic contractile units of muscle cells [15]. The sarcomere consists of two types of protein filaments: thick and thin filaments, mainly consisting of larger numbers myosin and actin molecules respectively (**Figure 3**). Upon activation by increased cytosolic calcium ions, myosin in the thick filaments climb across the thin filaments, resulting in shortening of the sarcomere and myofibril (contraction). As calcium is pumped back into the sarcoplasmic reticulum and cytosolic concentrations decrease, in an energy-consuming active relaxation process, myosin releases its grip to actin and the sarcomere returns to its extended shape, due to the spring-like titin protein and elastic components in the extracellular matrix [2, 16].

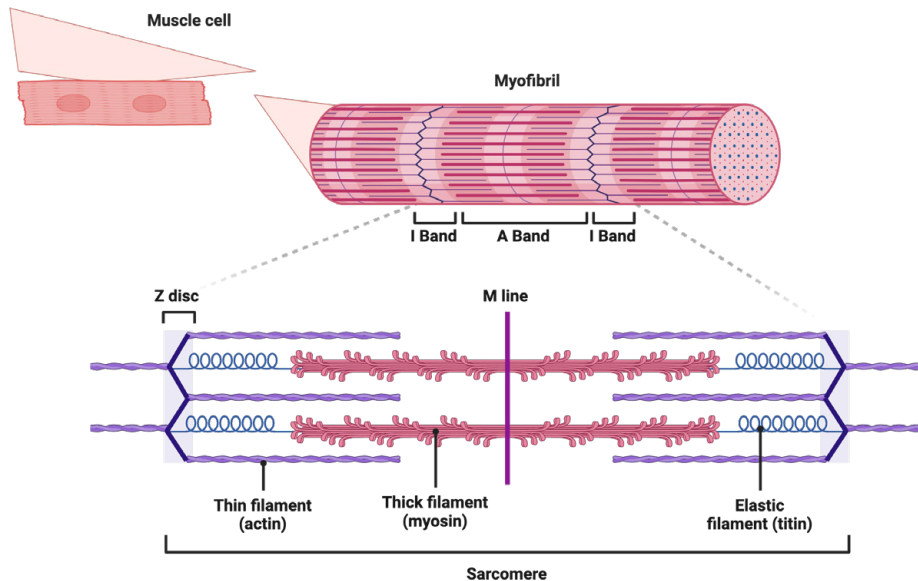


Figure 3. Structure of myofibrils and sarcomeres in cardiomyocytes.

Fibroblasts

Fibroblasts are the most common cells in connective tissue and can be found in most tissues [17]. Fibroblasts are more abundant than cardiomyocytes in the human heart [18]. However, fibroblast populations have been shown to be heterogeneous, representing fibroblast precursors, tissue-specialized fibroblasts and disease-activated fibroblast states [19]. Fibroblasts can be activated by several stimuli, including transforming growth factor beta and strain, and differentiate into myofibroblasts, also referred to as “activated fibroblasts”, with increased production of extracellular matrix components such as collagens which increase the structural integrity of the surrounding tissue [20]. A hallmark of late stages of cardiac injury, such as after myocardial infarction, or with increased strain during heart failure, is myocardial fibrosis deposited by myofibroblasts [21].

Cardiac fibroblasts are thought to play an important role in heart disease, especially in cardiac remodeling during pathological conditions including myocardial infarction and pathological strain. Excessive cardiac fibrosis can compromise heart function by multiple mechanisms, including increased restriction to filling and by altering electrophysiological characteristics resulting in increased risk both for bradycardia and ventricular reentry circuits that provide substrate for tachyarrhythmias [22, 23] [24]. In addition to their central role in remodelling of the extracellular matrix, fibroblasts are involved in cell-cell communication with myocytes and immune cells, and other fibroblasts, secreting both cytokines and growth factors [25].

Single-cell RNA sequencing to assess RNA heterogeneity

Single-cell RNA sequencing (scRNAseq) is a developing high-throughput method for examining the cellular heterogeneity of tissues by global RNA profiling of individual cells and clustering of cells into cell types and subpopulations. scRNA-seq has allowed researchers to identify and classify cell types within tissues with greater resolution, revealing previously unknown cell subtypes and their transcriptional profiles [26] [27].

The application of this powerful tool to dissect cellular heterogeneity has provided initial important insights for understanding the molecular complexity of tissues and organs. In the last several years, there has been notable progress in the evaluation of cellular heterogeneity at single-cell resolution in several organs[19]. As we set out with this thesis project, no protocols were available and no single cell study of the human heart was available. Although both atlas studies of the normal human heart [28] [9] and a few specific heart conditions have recently been published, resulting mainly in important molecular perspectives on fibroblast activation in the heart [29], snRNAseq applications to human heart conditions remain underexplored. However, important insights and novel cell types have been identified in the kidney, lung and brain fields as summarized in the section below.

Findings from single-cell profiling across human organs

Kidney

scRNA-seq has to date proved particularly useful in the kidney field, helping to advance the understanding of kidney immunology and disease. In particular, scRNA-seq has been used to investigate immunological contributions to a range of kidney illnesses, including lupus nephritis, diabetic kidney disease, and IgA nephropathy [30]. The method has been applied to thoroughly examine immune cells in blood, secondary lymphoid tissues, kidney biopsy, and urine samples, resulting in the identification of novel immune cell types, gene regulation, and signalling pathways related to kidney conditions [30] providing several opportunities for novel therapies..The technique has also facilitated the discovery of novel specialized cell types, for example a uroepithelial cell expressing TNNT1, a marker typical of skeletal muscle fibers. This cell type is proposed to play a role in the stretching functions of the bladder and urinary tract [19]. scRNA-seq has also revealed the sensitivity of renal lymphatic endothelial cells to acute kidney damage [31].

Finally, scRNA-seq data applied to kidneys of human donors has revealed the cellular architecture of the normal human kidney, demonstrated 10 clusters of normal human renal cells [32] and over multiple nephron segments [33].

Lung

scRNA-seq has been utilized to investigate numerous features of the lung, including lung cancer and pulmonary fibrosis. A study used scRNA-seq to profile differentially expressed genes (DEGs) at single-cell resolution in human non-small cell lung cancer (NSCLC) epithelial cells. This led to the identification of candidate genes and biomarkers for early-stage lung cancer [34]. This method has also been used to generate a single-cell atlas of pulmonary fibrosis, demonstrating heterogeneity across alveolar macrophages and epithelial cells and providing insights into disease pathobiology [35]. A complete cell atlas of the human lung, containing the gene expression patterns and structural locations of 58 cell groups, was generated utilizing droplet- and plate-based scRNA-seq. The technique has facilitated discovery of a new rare, cell type, the ionocyte [36]. Ionocytes are primarily responsible for the function of cystic fibrosis transmembrane conductance regulator (CFTR) within conductive epithelium. [37]. In addition, the technique has identified aerocytes, which are unique cells involved in pulmonary gas exchange [38].

Brain

scRNAseq has been applied to generate an atlas of cell type heterogeneity in the human brain. In addition, scRNAseq has been applied to both map cell-type-specific therapeutic responses in Parkinson's disease [34, 35], Huntington's disease to identify gene expression changes in different cell types and subtypes [36], multiple sclerosis and brain tumours and the tumour microenvironment. scRNAseq uncovered specialized cortical neurons in specific brain areas, a wide range of neurons in the midbrain and hindbrain, and varying astrocyte populations across regions. Additionally, distinct types of oligodendrocyte precursors were identified, each specific to different regions of the brain [39]. The technique has also allowed detection of hundreds of different cell statuses during brain development, including early neuroepithelium in the developing mouse [40]. Furthermore, diverse mouse medial ganglionic eminence (MGE) cell types were identified that include proliferating progenitor cells in the ventricular region of the MGE [41].

Heart failure

Heart failure is a clinical syndrome with typical symptoms and clinical findings that presents when the heart is unable to provide sufficient output of blood to meet the demands of the body. This syndrome is a leading cause of death and disability globally, estimated to affect about 64 million people worldwide [42], with high burden in developed countries and increasing prevalence in emerging countries [43].

Heart failure is characterized by high intracardiac pressures and decreased cardiac output at rest or during stress. Typical symptoms include dyspnea and exercise intolerance, which has a multifaceted pathophysiology that includes pulmonary congestion and impaired perfusion of skeletal muscle. Other symptoms are related to fluid accumulation in the legs and abdomen, resulting in peripheral edema and ascites. [44]

Heart failure is most commonly due to dysfunction of the left ventricle but also in a smaller subset due to predominant dysfunction of the right ventricle [45]. However, left ventricular dysfunction frequently progresses to dysfunction of both ventricles through several mechanisms, importantly including pulmonary hypertension resulting from congestion of blood in the pulmonary circulation.

Left ventricular heart failure is also frequently classified based on whether the ejection fraction, a measure of contractility and cardiac emptying, is reduced (HFrEF), mildly reduced (HFmrEF) or preserved (HFpEF) [46]. HFrEF and HFmrEF is often thought to result from limitations in the number or contractile function of cardiomyocytes, such as after a myocardial infarction or with a genetic cardiomyopathy which impairs sarcomere function. HFpEF on the other hand is thought to often represent impaired relaxation and increased stiffness related to hypertrophy and fibrosis resulting in impaired filling [47]. Hypertension and diabetes are important risk factors for HFpEF.

Therapy for HFrEF targets the underlying condition as well as the neurohormonal pathways that result from limitations in output and high filling pressures that drive disease progress. Such neurohormonal therapies include betablockers, angiotensin receptor blockers, angiotensin converting enzyme inhibitors, neprilysin inhibitors, and mineralocorticoid receptor antagonists. For HFpEF, SGLT2 inhibitors was recently the first therapy to show evidence of benefit in phase III clinical trials, and this new group of therapies also provides benefit in HFrEF, potentially acting through a combination of positive effects on blood pressure, glucose concentration, fluid volume and others. Diuretics are also used to reduce fluid overload and symptom burden. Surgical or endovascular alternatives are available for a subset of patients and include heart transplantation, ventricular assist devices, coronary revascularization, implanted cardioverter-defibrillators, and pacemakers. However, even with this broad range of therapies, morbidity and mortality remains high in heart failure [48].

Heart muscle diseases

Most cardiac diseases result in some degree of myocardial damage and may ultimately result in heart failure. A large proportion of cases occurs in patients with coronary artery disease resulting in myocardial ischemia or infarction or with

hypertension, resulting in increased strain on cardiomyocytes. Other conditions, including diabetes mellitus, valvular heart disease, congenital heart disease, or infections explain a smaller proportion of cases. A subset of cases also occur in the absence of underlying systemic disease, termed primary diseases of the heart muscle - cardiomyopathies [49] which includes contributions from genetic factors and systemic factors such as amyloidosis and cardiac inflammation that may not result in manifestations from other organs.

Coronary artery disease

Diseases in the coronary artery that limit blood supply to the heart is, together with hypertension, the most common cause of heart failure. Such limitations in blood supply result in ischemia and potentially infarction. The most common cause of limitations in blood supply is buildup of atherosclerotic plaque in the branching points of coronary arteries, with lipid accumulation, followed by inflammatory processes, smooth muscle cell and fibrous matrix growth, and calcification [50]. Increased pressure as well as the contributions of macrophages and other inflammatory cells may damage the plaque wall, resulting in plaque rupture, intravascular thrombosis and marked vessel occlusion ultimately resulting in myocardial infarction [50, 52].

Treatment of coronary artery disease is based on management of risk factors by a combination of lifestyle changes and medications. In addition, improved myocardial perfusion may be obtained by revascularization, that is the widening of an atherosclerotic vessel by percutaneous coronary intervention or shunting of blood around the plaque by open heart surgery involving bypass grafting [53, 54].

Cardiomyopathy

A cardiomyopathy is defined by the European Society of Cardiology as “a myocardial disorder in which the heart muscle is structurally and functionally abnormal, in the absence of coronary artery disease (CAD), hypertension, valvular disease, and congenital heart disease (CHD) sufficient to cause the observed myocardial abnormality” [55]. Cardiomyopathies are further subgrouped based on morphological findings upon imaging of the heart, for which the two major groups are dilated and hypertrophic cardiomyopathy.

Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is the most common cause of early-onset severe heart failure which requires heart transplantation. Dilated cardiomyopathy (DCM) is a progressive cardiac condition, characterised by an increase in internal chamber size and weakness of the heart chambers, particularly the left ventricle, resulting in

limitations in cardiac emptying and output. Continued ventricular dilation and contractile decline results in gradually worsening heart failure, and may be followed by cardiac fibrosis, conduction system abnormalities, ventricular arrhythmias, and thromboembolism [56]. The most common identifiable causes of DCM are viral or idiopathic myocarditis and genetic variants that truncate the titin gene [55].

Hypertrophic cardiomyopathy

HCM is characterized by increased cardiomyocyte volumes and wall thickness, and often fibrosis, resulting in impaired filling, myocardial ischemia, and risk for ventricular arrhythmias. Symptoms are largely the same as for other causes of heart failure. [57, 58]. A majority of patients with HCM are carriers of mutations in sarcomere genes, particularly including myosin heavy chain and myosin-binding protein C genes.

Cardiac remodeling

Cardiac remodelling is a term that encompasses a set of molecular, cellular, and interstitial alterations of the heart that appear clinically as changes in the size, mass, shape, and function of the heart following injury [59]. Several pathophysiological stimuli, including injury, pressure and volume overload, trigger the remodeling cascade (**Figure 4**), a process that initially confers protection to the heart as a compensatory mechanism but in the longer-term results in adverse clinical outcomes.

The onset and course of remodeling is influenced both by cardiomyocytes and other cell types. In cardiomyocytes, remodelling is associated with activation of a fetal expression profile which includes class switches in sarcomeric proteins and ventricular expression of atrial natriuretic peptide. Energy metabolism changes towards increasing glycolysis and increased oxidative stress contribute to activate hypertrophic, inflammatory and profibrotic signalling pathways [60]. Gradual cardiomyocyte loss has been widely characterized to occur by necrosis, necroptosis, apoptosis, or autophagy. In parallel, subclinical myocardial immune system activation and inflammation may persist after heart injury or be triggered by other contributing factors and result in increased cytokine production, contributing to fibroblast proliferation and metalloproteinase activity [61]. Fibrosis occurs through fibroblast proliferation and extracellular matrix (ECM) reconfiguration as described above and may impact both relaxation and contractility [59, 61-63]. This loss of cardiomyocytes, excess fibrosis, and reduced contractile strength further drives remodelling.

The initial clinical manifestations of remodeling include morphological changes such as increased intracardiac cavity diameter, ventricular mass and wall thickness

by echocardiography, increased natriuretic peptides in plasma, and signs of fibrosis by magnetic resonance imaging [64]. Progressive remodelling drives deterioration of both cardiac contractile function and filling, resulting in onset and progression of heart failure, worsening symptoms, and risk of both bradycardias, tachyarrhythmias and mortality.

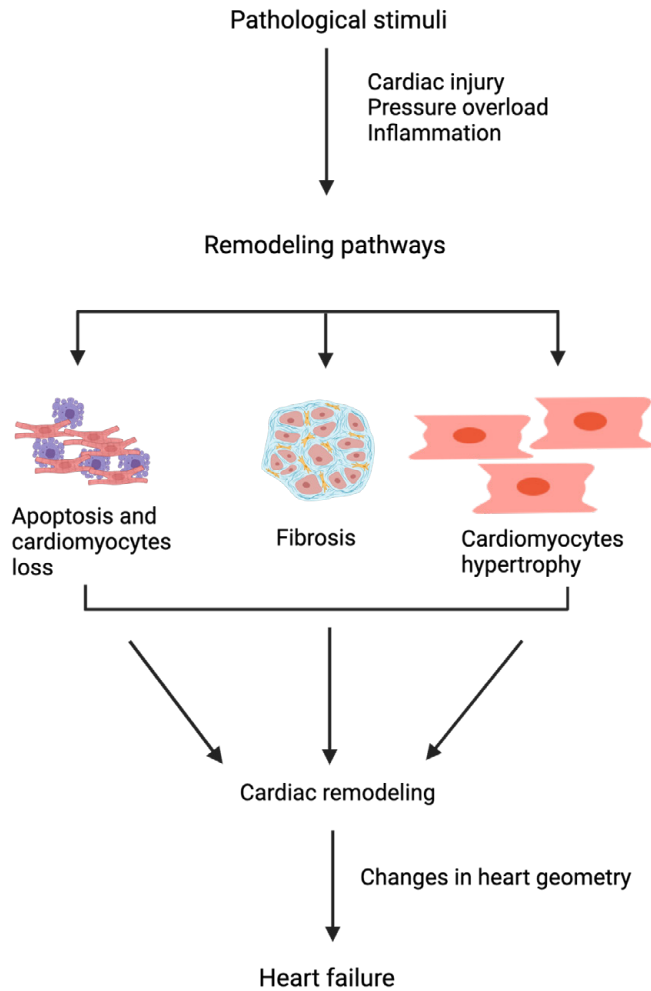


Figure 4. Schematic representation of cardiac remodeling.

Cardiac Fibrosis

Excessive myocardial fibrosis is well established to confer increased risk of morbidity and death [65]. Essentially all myocardial disease processes, including hypertensive heart disease, diabetic heart disease, and idiopathic dilated cardiomyopathy [66, 67], involve deposition of extracellular matrix (ECM) proteins by cardiac fibroblasts (CFs) which influences risk for development of cardiac dysfunction, heart failure and arrhythmia [65, 68, 69]. Fibrosis mechanisms have been particularly well studied in the context of myocardial infarction in particular, which may lead to extensive death of cardiomyocytes, focal tissue damage and an inflammatory response with upregulation of proinflammatory cytokines and profibrotic factors. As regenerative capacity in the postnatal human heart is limited, such damage results instead in tissue scarring, with activation of cardiac fibroblasts, proliferation, transition to a myofibroblast phenotype [70] and replacement of dead myocardium by a collagen-based scar, particularly including collagen type I [61, 71]. The transition of tissue fibroblasts to myofibroblast is the key cellular event that drives the fibrotic response (**Figure 5**) [71]. Transforming growth factor beta (TGF- β) is a key driver of fibroblast activation and proliferation in the heart following injury or stress [65, 71]. However, many of the molecular details remain incompletely understood.

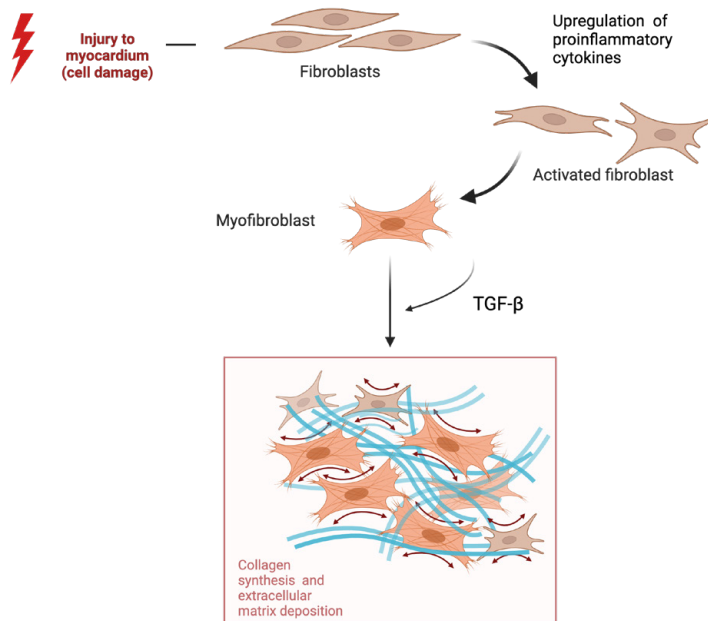


Figure 5. Schematic representation of cardiac fibrosis.

Genetic contribution to cardiac remodeling and outcomes

Although it has long been clear that heart failure patients have very different propensity for accelerated myocardial remodelling, the mechanisms for this remain unclear as does any genetic contributions to cardiac remodelling. However, our group has shown that patients with a family history of poor prognosis in heart failure also have a poor prognosis when developing heart failure [72]. In an effort to better understand mechanisms underlying familial propensity to remodelling and poor outcomes, our group previously conducted a genome-wide association study of prognosis in heart failure patients, in broad international collaboration [73]. In such studies, single nucleotide polymorphisms (SNPs, i.e. single-base pair substitutions in the DNA sequence that occur in a large proportion of a population that represent the most common form of genetic variation in populations) across the genome are related to differences in phenotype. This study identified a SNP on chromosome 5q22 (rs9885413) associated with increased mortality in heart failure [73]. It was further reported that the SNP confers a change to the regulatory motif of an enhancer region upstream of the thymic stromal lymphopoietin gene (TSLP), which is predicted to bind the transcription factor nescient helix-loop helix 1 (NHLH1) (**Figure 6**). Any role for TSLP, NHLH1, or other genes at the locus in the heart have remained unclear.

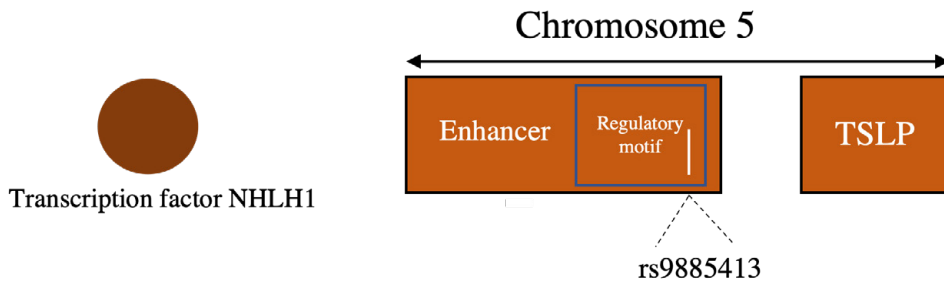


Figure 6. Schematic representation of the location of the SNP rs9885413 on chromosome 5.

Key Methods

Cell culture

In paper I, III and IV we used human cardiac fibroblasts, cardiomyocytes derived from induced pluripotent stem cells (iPS-cells) and human cardiac microvascular endothelial cells. The cells were cultured in Fibroblast Growth Medium, iCell Cardiomyocyte Plating or Maintenance Medium and endothelial basal medium respectively.

Overexpression of *Tslp* in mice

In paper III an AAV9 viral vector was used to induce transgenic expression of *Tslp* under the control of the *Tcf21* promoter [74]. The transcription factor Tcf21 is specific for the cardiac fibroblast cell lineage. Tcf21 is expressed in epicardial progenitor cells, which give birth to cardiac fibroblasts, and its expression persists in adult fibroblasts [75]. Furthermore, Tcf21 expression was shown to be considerably higher in resident fibroblasts throughout the fibrotic phase [76, 77]. Therefore, we used the *Tcf21* promoter to create a cardiac fibroblast-selective AAV9 vector. A P2A self-cleaving peptide linker was used to attach an enhanced GFP (eGFP) reporter gene downstream of *Tslp*. A vector expressing solely eGFP under the *Tcf21* promoter was created as a control. Oligonucleotides corresponding to the genomic region 500 base pairs upstream of the Tcf21 transcription start site, the *Tslp* open reading frame (NCBI Reference Sequence: NM_021367.2), the self-cleaving peptide P2A sequence, eGFP (GenBank ID: MH458079.1), Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE), simian virus 40, and poly (A) were synthesised and cloned into the plasmid containing the inverted terminal repeats (ITRs) from AAV9. Plasmid AAV9-Tslp-eGFP and the control plasmid AAV9-eGFP were delivered into HEK293T cells, collected, purified, and quantified as previously published [78].

Animal procedure

Female C57BL/6 mice, aged 8–10 weeks, were utilized for all the animal experiments. The mice received intravenous injections of AAV9-Tslp-eGFP or AAV9-eGFP (at 10^9 genomic copies/animal diluted in PBS) through the tail vein. Following injection, the mice were isolated for 72 hours before collecting heart, kidneys, lungs, spleen, liver, and whole blood samples for subsequent analysis. All animal procedures conducted in Lund were sanctioned by the local ethics committee for animal research.

Human heart tissue

In Study I human cardiac tissue samples were obtained from the free wall of the left ventricle of four orthotopic heart transplant recipients; three of the recipients with dilated cardiomyopathy (DCM), and one with ischemic cardiomyopathy. Biopsies were sectioned, placed in RNA-later, frozen at -80°C , kept for 12 months, and only thawed for cell isolation. All participants provided written informed permission, and the study was approved by the local ethics committee approved of the study. In Study II heart biopsies were obtained from explanted hearts at Swedish transplantation centres in Gothenburg and Lund as part of the ongoing SwedeHeartSeq research program. Control samples from unused donor hearts without heart disease were collected in Gothenburg. The cardiac tissue was preserved either snap-frozen or stored in RNALater and frozen at -80°C . Clinical data were extracted from electronic health records, and heart failure phenotypes were determined following ESC guidelines by specialized cardiologists. The study was ethically approved, and all participants provided written consent.

Strain of cells

We mechanically strained cells to replicate the biomechanical stress characteristic of cardiac failure in cell culture. The cells were seeded on silicon chambers with flexible membranes. The cells were subsequently subjected to a 48-hour uniaxial strain in a single direction at a frequency of 60 cycles per minute, 10% elongation, or a distance of 2.0 mm (Figure 4). After that, cells were removed to extract RNA.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) is the gold standard technique for studying protein-DNA interactions. It aims to determine if proteins, such as transcription factors or other DNA binding sites, are associated with particular genomic areas. In Study III we used ChIP in order to ascertain the binding of transcription factor NHLH1 to the enhancer region flanking the risk allele rs9885413 by co-precipitating the DNA and protein complex from human cardiac fibroblasts. Cells were fixed and crosslinked, then subjected to enzymatic digestion for fragmentation of chromatin. Then the chromatin was pulled down with antibodies against Histone H3, NHLH1 or non-specific IgG. The protein:DNA:antibody complex was captured in a Protein A filter spin column for washing and the chromatin sample was recovered by reverse cross linking. The DNA was then quantified with qPCR.

Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assay (EMSA) is used to detect and quantify the interaction between protein and DNA. As nucleic acid-protein complexes move slower than free nucleic acids through the gel during electrophoresis, the difference in band location indicates whether the protein of interest has bound to the potential target DNA [79]

We used EMSA in Study IV to determine whether NHLH1 binding is affected by the rs9885413 risk allele in HCF. The oligonucleotides were designed based on the region spanning the SNP. Two probes were designed: one carrying the wild type G allele and the other the T risk allele. HCF nuclear protein extract was incubated with the DNA oligonucleotides. Compared to the wild-type G, binding to the T allele was significantly stronger.

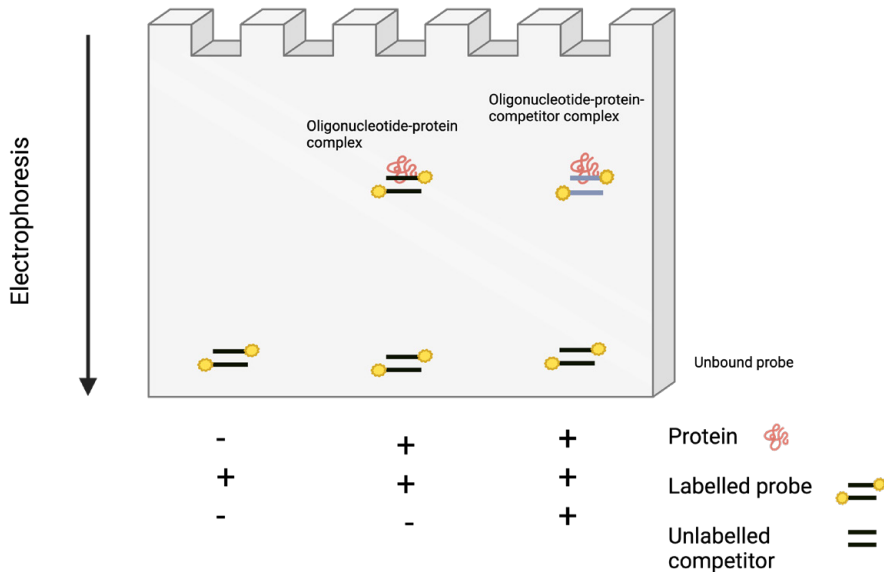


Figure 7. Schematic representation of electrophoretic mobility shift assay/ gel shift assay. Nuclear protein extract is mixed with fluorescently labeled DNA probes corresponding to the DNA region of interest. When a protein **binds to the** labelled probe, the probe is retarded during electrophoresis and shifted up. An excess of unlabeled competitor probe reduced the intensity of the band.

Single nuclei isolation

This technique is used to study gene expression patterns at the level of individual cells, providing insights into cellular heterogeneity and function. By analyzing nuclei instead of whole cells, snRNA-seq helps minimize issues associated with cell dissociation and stress-induced transcriptional changes. It is also essential for studying tissues where single intact cells are difficult to isolate, *e.g.* from the brain and heart. The technique involves multiple steps.

In Study I we used the extraction of single nuclei from tissue based on sucrose gradient centrifugation. Briefly, tissue is first homogenised in lysis buffer using a rotor-stator homogenizer followed by repeated strokes with a dounce homogenizer. The homogenate is filtered through a series of cell strainers of different sizes. Next, the tissue suspension is layered with sucrose buffer and centrifuged to remove debris [80]. The nuclear pellet is suspended in a buffer and stained with the desired antibody or nuclear stain for analysis by flow cytometry. In Study I, the isolated nuclei were sorted into cardiomyocyte and non-cardiomyocyte population for further downstream processing based on the cardiomyocyte nuclear marker PCM1. In Study II we implemented a sucrose free nuclei isolation protocol. Briefly, tissue

was first homogenised in NP40 lysis buffer using a rotor-stator homogenizer followed by 8 strokes with a dounce homogenizer. The homogenate was filtered through a 70 μm cell strainer. The nuclei suspension was then centrifuged and suspended in a buffer with the nuclear dye 7AAD. The nuclear suspension was FACS-sorted to remove doublets and debris.

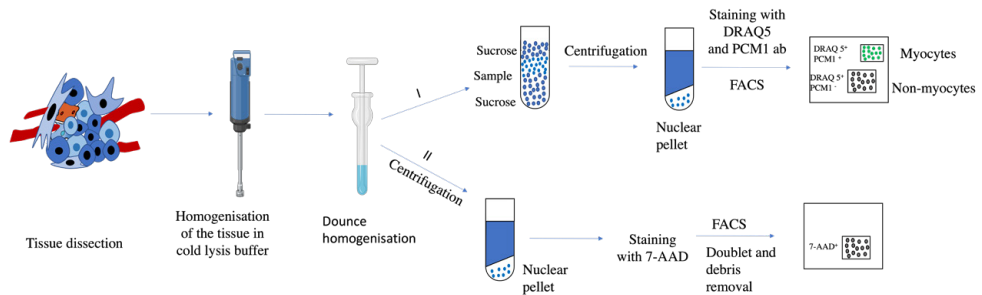


Figure 8. Single nuclei isolation experimental workflow in Study I and II. Frozen tissue was dissected, homogenized, washed, filtered and either I) layered with sucrose, centrifuged and stained with DRAQ5 and PCM1 antibody or II) directly centrifuged and stained with 7AAD.

Library preparation for droplet-based single-cell RNA sequencing

In droplet-based single cell RNA-sequencing, the first step of library preparation involves loading of the nuclear suspension in a microfluidic controller (Chromium, 10x Genomics). The instrument utilises microfluidics to produce Gel Beads-in-emulsion (GEMs), that contain sequencing primers, a unique molecular identifier (UMI) and a reverse transcriptase master mix. Upon loading of the sample, single nuclei are captured and lysed within a GEM. Incubation of the GEMs produces barcoded, full-length cDNA from the mRNA of the captured cell or nucleus. In Study I and II, single nuclei cDNA libraries were produced with the 3' Gene expression chemistry (10x Genomics) and sequenced on a NovaSeq 6000 system. Raw base calls were imported into CellRanger software (see below) for quality control and mapping of reads to the human reference genome.

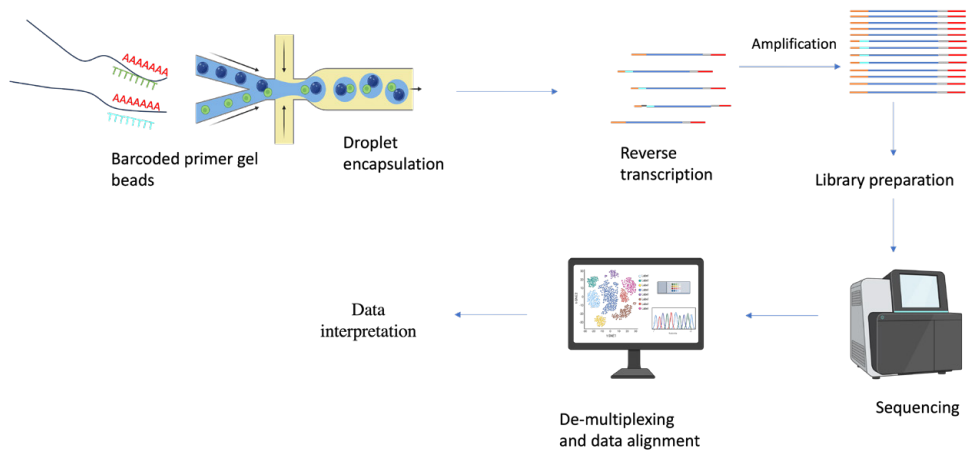


Figure 9. Single cell RNAseq sample processing. Schematic representation of a typical workflow for an scRNA-seq experiment. The cell or nuclear suspension is placed into a microfluidic chip, and cells are separated and partitioned into gel beads-in-emulsion (GEMs) droplets that include barcoded gel beads and reverse transcription (RT) reagents. After cell or nuclear lysis, reverse transcription takes place. The pooled cDNA is amplified in bulk, fragmented by enzymatic fragmentation, and libraries are formed. The sequencing libraries are sequenced, and the results are analysed by alignment and demultiplexing, before being interpreted.

Bioinformatic processing of snRNA-Seq data

In Study I and II, Seurat (see below) was used for further processing and analysis of single nucleus RNA-sequencing data. Molecular counts were normalised for variations in sequencing depth across nuclei, and the variance was stabilised using regularised negative binomial regression. Dimensionality reduction was carried out using principal component analysis. Two-dimensional projections based on the top 50 major components were generated using the UMAP (Uniform Manifold Approximation and Projection for Dimension Reduction) approach [81]. The UMAP projection's shared nearest neighbor network was used for graph-based clustering of nuclei. The expression of traditional cell-type informative markers often employed in immunohistochemical research was used for annotation of cell clusters.

Cell ranger

Cell Ranger is a suite of analytic pipelines developed by 10x Genomics that use Chromium Next GEM single-cell data to align reads, construct feature-barcode matrices, cluster, and perform secondary analysis. It includes five pipelines for the 3' Single Cell Gene Expression and 5' Immune Profiling Solutions [82].

Seurat

Seurat, a comprehensive and versatile R software toolbox for examining and understanding scRNA-seq data, was developed by the Satija Lab at the New York Genome Centre. Seurat can handle data from many scRNA-seq systems and is compatible with a variety of file types. Some of the important aspects of Seurat are Quality control and pre-processing of single-cell data, cell type grouping and identification, multiple gene identification, and dimensional reduction. Seurat provides a variety of tools for viewing single-cell data, such as feature plots, violin plots, and heat maps. t-SNE and Uniform manifold projection approximation (UMAP) plots are popular methods for displaying high-dimensional data in two or three dimensions [83].

Aims and Results

Paper I

Aim

This paper aimed to develop a framework for analysing frozen human heart tissue at the single-cell level, providing a comprehensive view of the human heart's transcriptional activity beyond the limitations of bulk tissue analysis.

Results

Single nuclei isolation from the frozen human heart.

We sought to evaluate the previously reported single nuclei RNA sequencing (sn-Seq) analysis methodology on frozen human cardiac tissue. Left ventricular tissue from a surgically excised (explanted) heart was utilized and PCM1 was used as a marker to distinguish cardiomyocyte nuclei from nuclei originating from other cell types. Two distinct populations of DRAQ5⁺/PCM1⁺ and DRAQ5⁺/PCM1⁻ were detected with flow cytometry (Figure 10 A, D). The purity of the isolated nuclei was checked by qPCR using markers *PCMI*, *TNN3* for cardiomyocytes and *VIM* for fibroblasts, respectively. As expected, PCM1⁺ nuclei were enriched in *TNN3* and PCM1⁻ nuclei were enriched in *VIM* (Figure 10 E, G).

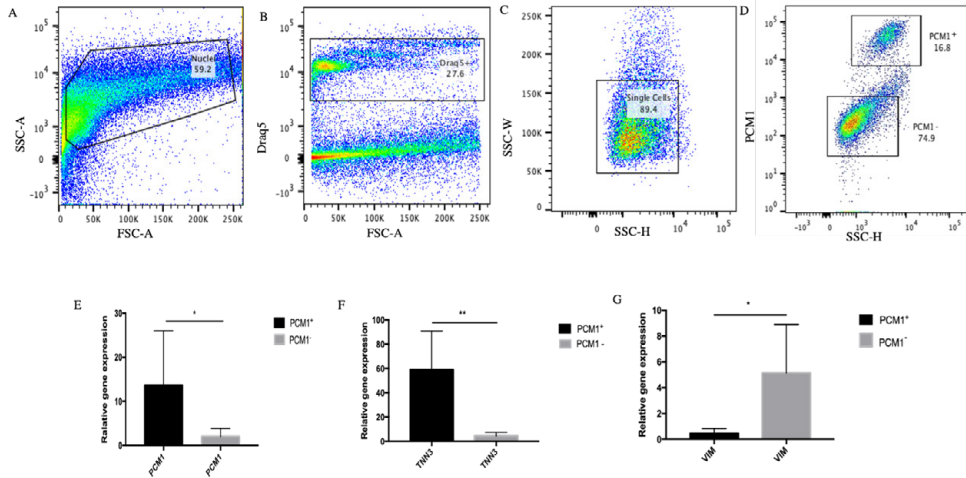


Figure 10. Assessment of nuclei isolation protocol from frozen human heart tissue. (A, D) Flow cytometry analysis of nuclei, DRAQ5⁺ nuclei identified and gated. (E, F, G) Isolated PCMI⁺ nuclei were enriched in cardiomyocytes marker *PCMI* and *TNN3*, where PCMI⁻ nuclei fraction was enriched in *VIM*. Data is presented as mean + SD from three independent experiments.

Single-cell transcriptomic analysis of human heart

Next, we generated single-cell transcriptome profiles from a patient with dilated cardiomyopathy. A total of 4390 PCMI⁺ and 4070 PCMI⁻ nuclei were analysed, clustered and used to generate Uniform Manifold Approximation and Projection (UMAP) projections. We identified nine cell clusters, including cardiomyocytes, fibroblasts, endothelial cells, vascular smooth muscle cells, macrophages, lymphocytes, and neurons. Cell type annotations were made using gold-standard marker genes and Gene Ontology pathway analyses. Further analysis of cardiomyocyte clusters revealed one cluster characterized by high natriuretic peptide expression. TCF21, a tissue-resident fibroblast marker, was widely expressed in the fibroblast cluster (Figure 11).

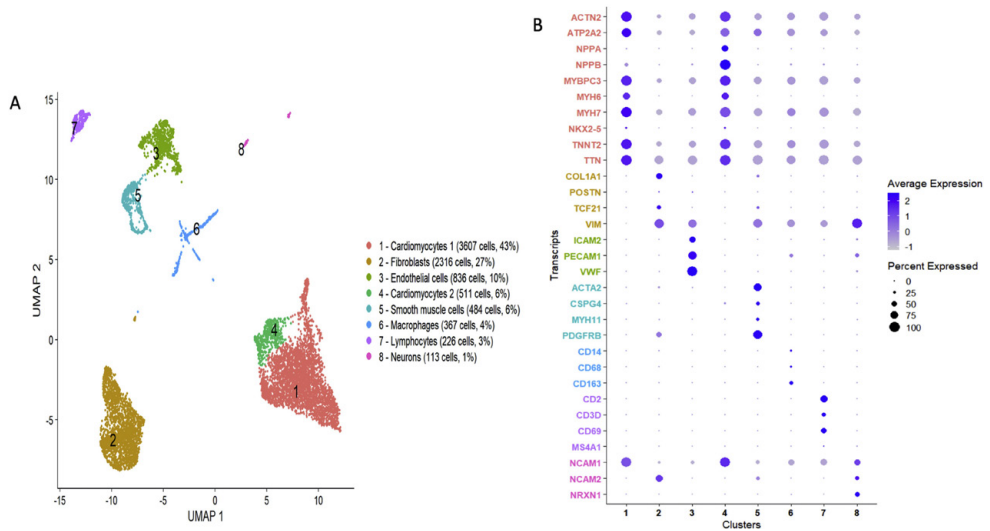


Figure 11. Single nuclei analysis of the human heart. (A) Uniform Manifold Approximation and Projection (UMAP) graphic was generated from 8,460 individual nuclei from a human heart with 4,390 chosen from PCM1⁺ and 4,070 from PCM1⁻ nuclear fractions using fluorescence-activated cell sorting. Panel B displays a dot plot of gene expression across clusters of certain known marker genes that are representative of the main cell types found in the human heart. The colours red, yellow, green, cyan, blue, purple, and pink represent cardiomyocytes, fibroblasts, endothelial cells, vascular smooth muscle cells, pericytes, macrophages, lymphocytes and neurons, respectively.

Paper II

Aim

The aim of this paper was to apply single nuclei RNA sequencing on a larger cohort of heart failure patients with various etiologies to improve our understanding of heart disease pathophysiology.

Results

A single nucleus transcriptional atlas of healthy and diseased human hearts

We performed droplet-based RNA sequencing of single nuclei from the hearts of 103 individuals, including 96 heart failure patients and 7 unused donor hearts without heart disease. After sample and nuclear level quality control, doublet removal and sample integration; principle component analysis and shared nearest neighbour clustering of 398 917 nuclei from 102 individuals were used to identify distinct cell populations in the human heart. We identified 14 clusters representing 10 distinct cell types (Figure 12 A). Endothelial cells were the largest cell cluster, followed by cardiomyocytes and fibroblasts (Figure 12 B). Transcriptionally diverse

subgroups were identified, with markers unique to Cardiomyocytes II suggesting they represent cardiomyocytes subjected to hemodynamic strain.

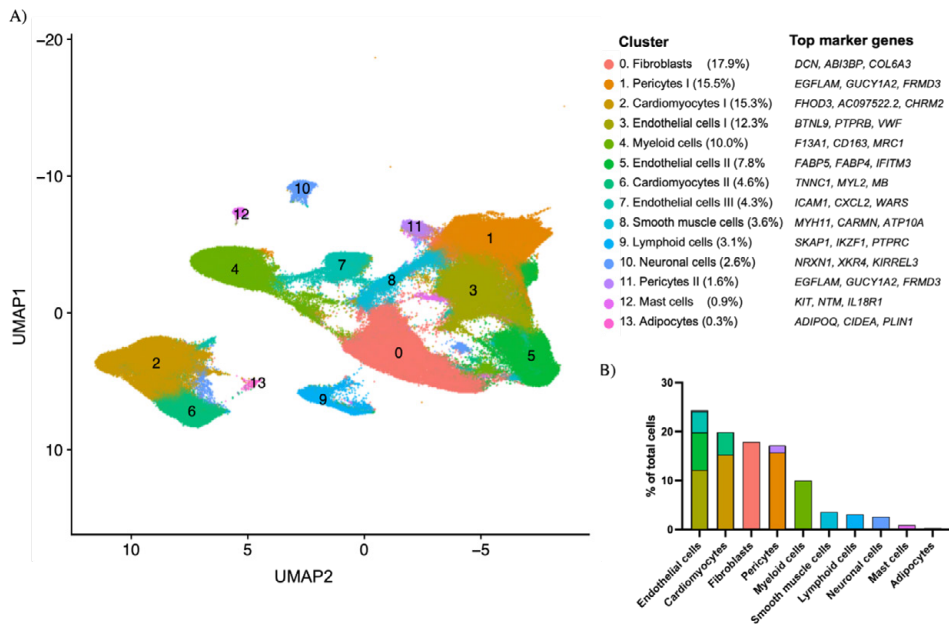


Figure 12. Cellular composition of the adult human heart. (A) Uniform Manifold Approximation and Projection (UMAP) graphic generated from 398 917 individual cardiac nuclei (B) Cell composition across all clusters and cell types

Differential gene expression in heart muscle diseases

We sought to investigate the effects of heart failure on cardiac cell composition and gene expression. Consistent with earlier research (single-nucleus profiling of human dilated and hypertrophic cardiomyopathy) heart failure patients had an extended fibroblast cluster, substantial decreases in pericyte and endothelial cell clusters, and neuronal cell reduction across 10 major heart failure etiologies (Figure 13A). Next, differential gene expression analysis comparing heart failure with control samples was carried out using a pseudobulk approach. Myeloid cells, cardiomyocytes I and fibroblast cell clusters contained the most differentially expressed genes (Figure 13 B). Natriuretic peptide gene (*NPPA*) and Periostin (*POSTN*) were highly upregulated in cardiomyocyte cluster II and fibroblast clusters, respectively. The next step was to identify transcriptional effects related to individual heart failure etiologies compared to control hearts. Here, DCM was found to have the highest number of DE genes (Figure 13 C).

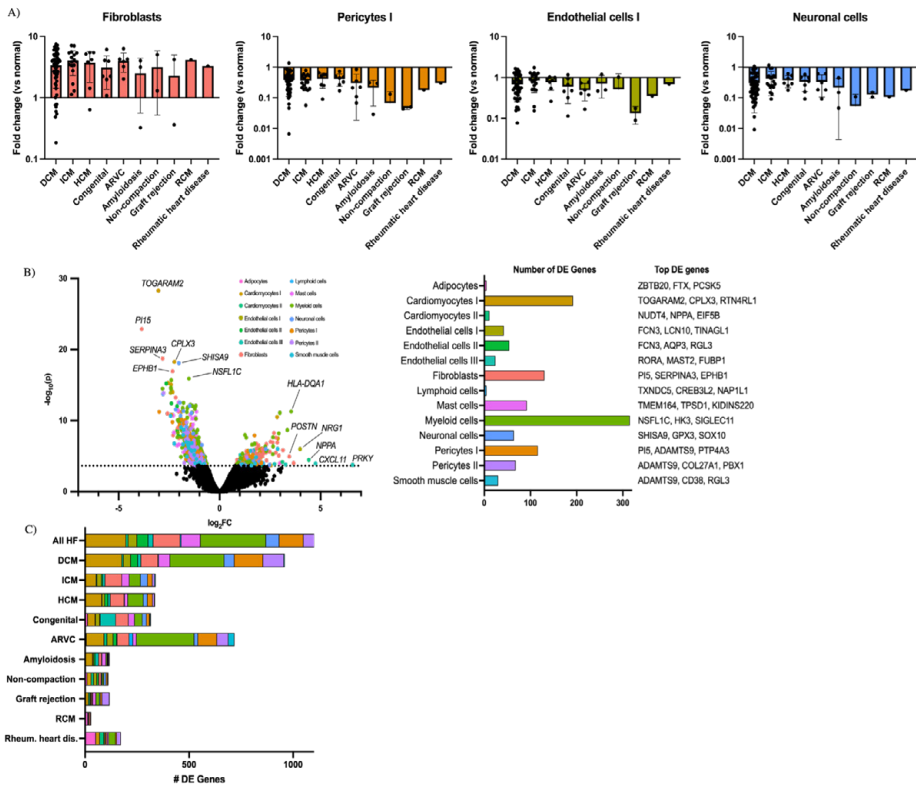


Figure 13. Transcriptional profile of differential gene expression of heart muscle diseases. (A) Comparison of fibroblasts, pericytes I, endothelial cells I and neuronal cell cluster sizes across HF etiologies. (B) Left, volcano plot of differentially expressed genes comparing all heart failure patients with controls. Right, comparison of the number of differentially expressed genes per cluster (C) Number of differentially expressed genes per cluster and etiology.

Paper III

Aim

This study aimed to (1) explore the impact of the SNP rs9885413 on TSLP expression in cardiac fibroblasts and (2) to study the role of cardiac TSLP as a potential therapeutic target in cardiac remodeling and fibrosis.

Results

NHLH1 differentially binds to the enhancer region spanning rs9885413 in human cardiac fibroblasts

We wanted to demonstrate the binding of NHLH1 to the enhancer region through chromatin immunoprecipitation. The result showed significant enrichment of NHLH1 at the rs9885413-containing region compared to the negative control antibody and compared to a region in the *GAPDH* promoter lacking a NHLH1 motif, indicating the specific interaction of NHLH1 to the regulatory motif in human cardiac fibroblasts (HCF) (Figure 14A). We further assessed whether NHLH1 binding is affected by the risk allele using EMSA, where nuclear protein binding to the risk allele (T, Fig. 14C, lane 5) was significantly higher than for the wild-type allele (G, Fig. 14D, lane 2, Fig. 14C). The presence of an excess amount of unlabelled probe lowered the intensity of the band, indicating the specificity of the interaction with the nuclear extract (Figure 14B, lane 6). We concluded that NHLH1 binds to the rs9885413 motif in HCF, with the risk allele increasing the affinity of the enhancer motif.

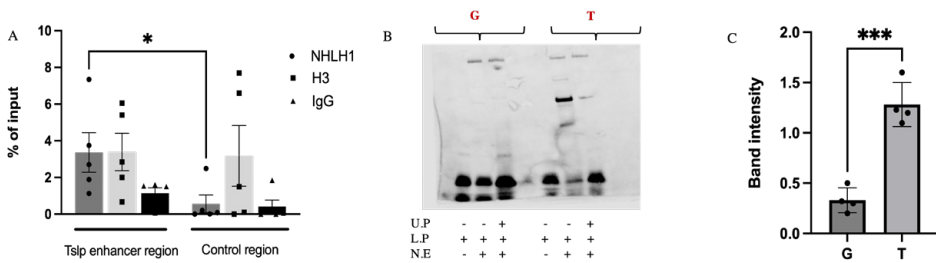


Figure 14. Differential binding of NHLH1 to the enhancer region flanking rs9885413 in human cardiac fibroblasts. (A) ChIP of NHLH1 in HCF, chromatin from HCF was pulled down using NHLH1 antibody, a histone 3 positive control antibody, and a non-specific negative control IgG antibody. DNA from the specific related region and an unrelated segment within the *GAPDH* promoter region was quantified using qPCR and presented as a percentage of the input DNA n=3. (B) EMSA of HCF nuclear extract, stronger binding of risk allele T to the HCF nuclear extract can be seen (lane 5) compared to the wild type allele G (lane 2). (C) Quantification of band intensities n=4. Statistical differences were assessed using t-tests *p < .05, ***p < .01. Data are shown as mean \pm SEM, from 4 independent experiments.

NHLH1 regulates TSLP expression in cardiac fibroblasts

We aim to investigate the effect of cyclic mechanical strain on the expression of *NHLH1* and *TSLP*. To this end 48 hours of cyclic mechanical strain was applied on HCF. A 4-fold increase in *NHLH1* expression and a 10-fold increase in *TSLP* expression was observed compared to non-stretched control cells (Figure 15 A, B). Further, we wanted to determine if the effect of stretch-induced *TSLP* expression is dependent on *NHLH1* in HCF during pathophysiological biomechanical conditions. To this end, HCF was transfected with *NHLH1* siRNA and subjected to 10% cyclic mechanical strain for 48 hours. The expression of stretch-induced *TSLP* was

significantly decreased (Figure 15 C). Likewise, stretch-induced expression of markers of fibroblast activation *COL1A1* and *FNI* was also reduced compared to negative control siRNA (Figure 15 D, E). A similar trend was observed with stretch-induced expression of the myofibroblast marker *POSTN* (Figure 15 F). These results indicate that the NHLH1/TSLP signalling pathway plays a significant role in the development of cardiac fibrosis triggered by mechanical stress.

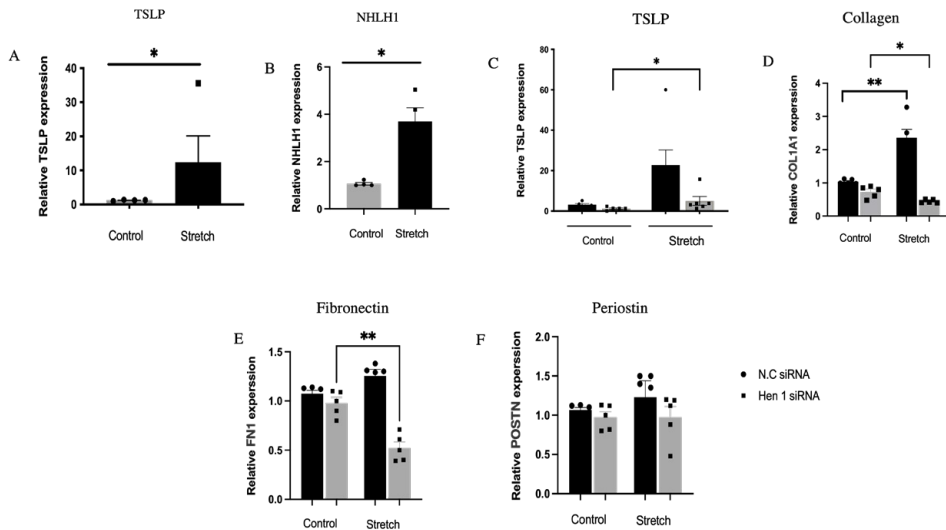


Figure 15. Knockdown of NHLH1 affects TSLP expression in stretched human cardiac fibroblasts. (A, B) *TSLP* and *NHLH1* gene expression after 48 hours of cyclic mechanical strain, analysed by qPCR and normalized to *ACTB*. (C, F) Gene expression of *TSLP*, Collagen, Fibronectin and Periostin in HCF after transfected with *NHLH1* siRNA or negative control siRNA and subjected to 48 hours of cyclic mechanical strain. The data was assessed by qPCR normalized to *ACTB* and shown relative to the average of their respective negative control siRNA sample. Statistical differences were assessed with a t-test * $p < .05$.

Adeno-associated virus (AAV9) mediated overexpression of TSLP in mice

Next, the effect of *Tslp* overexpression was investigated in vivo. Transgenic expression of *Tslp* in cardiac fibroblasts was achieved by injection of a AAV9 vector where *Tslp* had been inserted downstream of the *Tcf21* promoter. A control vector containing eGFP downstream of the *Tcf21* promoter was used as a negative control. C57BL/6 mice were injected with *Tslp* and control virus and a significant increase in *Tslp* expression after 3 days of viral injections was observed (Figure 16A). A trend towards increased *Tslp* protein expression was also detected at day 5 (Figure 16B). Further, the role of *Tslp* overexpression was detected in the context of fibrosis and remodeling, where the evaluation of collagen deposition revealed increased fibrosis in mice injected with the *Tslp* virus (Figure 16C, D). We conclude that *Tslp* stimulates cardiac collagen synthesis and may contribute to fibrosis in heart failure.

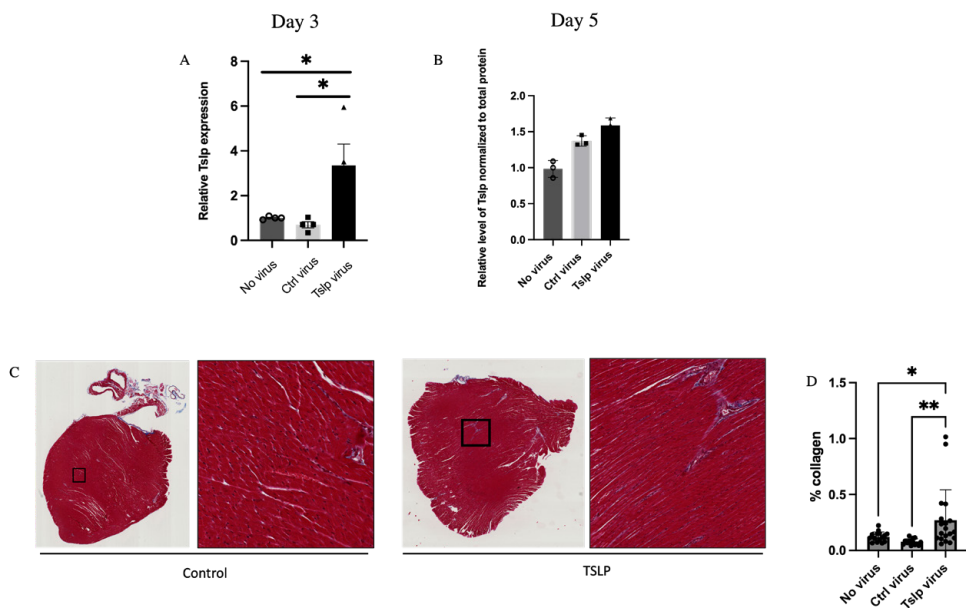


Figure 16. *Tslp* overexpression drives collagen deposition. (A) Gene expression analysis of cardiac *Tslp* at day 3 after injection with AAV9-*Tslp*-eGFPtcf21, AAV9-eGFPtcf21 and no virus (n= 3 per group). (B) Quantification of Western blot data. (C) Masson trichrome staining of tissue sections from mice hearts after 5 days of injections. (D) Quantification of collagen in Masson's trichrome stained sections.

Mast cell stimulation

Single nuclei RNA sequencing data generated in Study II revealed that both the *TSLP* receptor subunits (*i.e.* *IL7R* and *CRLF2*) were expressed in mast cells. Mast cells have previously been shown to release TGF- β , a profibrotic factor [68] in response to TSLP [84] [85]. We considered the possibility that cardiac mast cells release TGF- β in response to TSLP and investigated it in a human mast cell line. A significant TGF- β upregulation was found after 3 hours of stimulation with recombinant TSLP (Figure 17A, B). To further explore this finding *in vivo*, cardiac mast cells were stained with toluidine blue and c-KIT antibody. Mice injected with *Tslp* virus showed a significant increase in toluidine blue+ cells (Figure 17C, E). The result indicated that overexpression of TSLP in fibroblasts leads to the activation of cardiac mast cells, prompting the production of TGF- β .

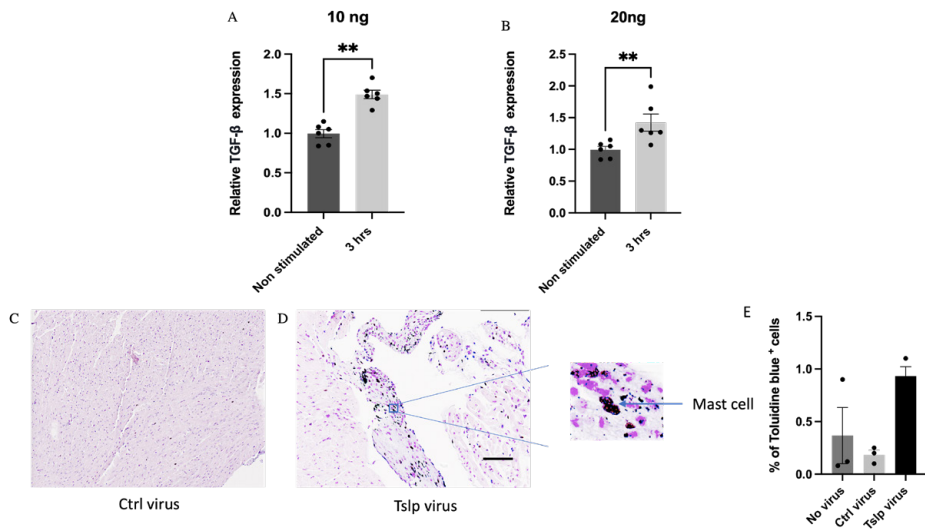


Figure 17. TSLP triggered TGF- β production in mast cells. (A, B) TGF- β expression in mast cells after being stimulated by 10 and 20 ng of recombinant TSLP respectively for 3 hours. (C, D) Toluidine blue staining of cardiac sections from mice at day 5, scale bar 100 μ m (E) Percentage of total toluidine blue⁺ cells.

Paper IV

Aim

This study aimed to compare cardiomyocyte nuclear size in individuals with and without heart failure, and to assess whether cardiomyocyte strain affects nuclear size.

Results

Cardiomyocyte nuclei and cells are larger in failing hearts

The first step was to compare cardiomyocyte cell and nuclear size between heart failure patients and controls. We performed immunofluorescence staining of cardiac tissue sections using an antibody to cardiomyocyte marker Troponin T, cell membrane dye WGA and nuclear dye DRAQ7. High content screening and automated quantification of cell and nuclear size revealed cardiomyocyte size was larger in heart failure patients compared to controls, in line with the expected heart failure-related cardiomyocyte hypertrophy and remodeling. Interestingly, cardiomyocyte nuclei were also found to be larger in heart failure patient's samples than controls (Figure 18 A, B).

A

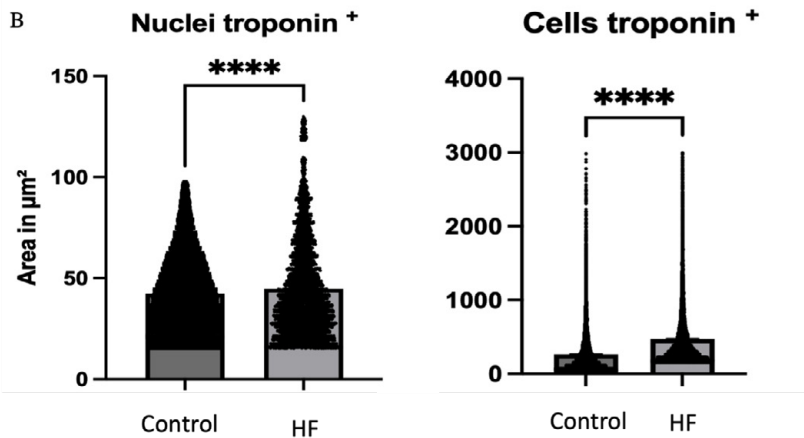
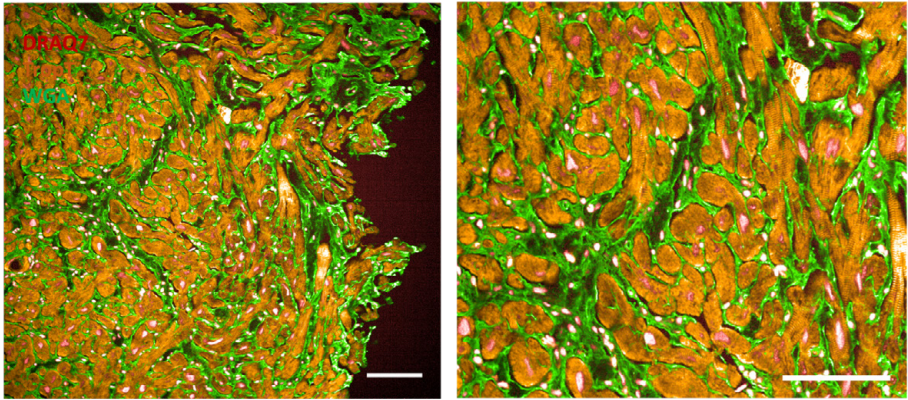


Figure 18. Cardiomyocytes nuclei and cells are larger in failing hearts. (A) Troponin T staining of paraffin tissue sections, Troponin T (orange), membrane staining with WGA (green), and counter-staining with DRAQ 7 (Red). Scale bar: 50 μm . (B) A comparison of the nuclear and cellular regions of cardiomyocytes in control and patient samples. The analysis was performed in the Operetta CLS high-content imaging system. Statistical differences were assessed using Paired t-tests **** $P < 0.0001$ data is presented as mean \pm SEM.

Cyclic mechanical strain increases cardiomyocyte nuclei size

We sought to determine if mechanical strain could cause cardiomyocyte nuclear hypertrophy. iPS-derived cardiomyocytes were subjected to cyclic mechanical strain. *NPPA* gene expression was significantly increased after 48 hours, confirming that the assay produced physiologically meaningful levels of mechanical strain (Figure 19 A, B). Further, the size of cardiomyocyte nuclei was assessed and a substantial increase in nuclear size was observed (Figure 19 C). This result suggests that mechanical strain could be the driver of nuclear hypertrophy.

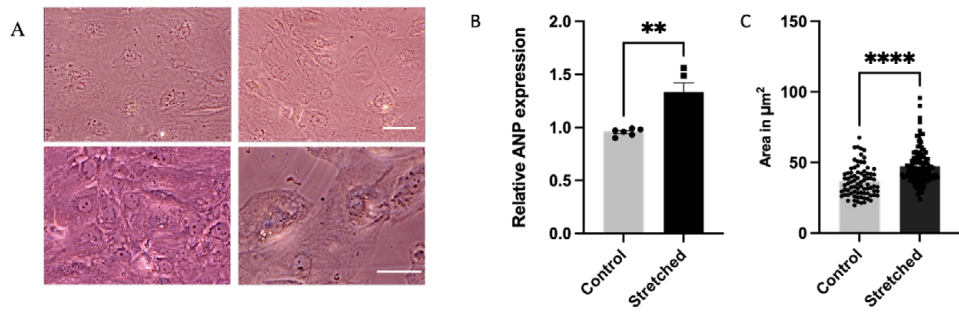


Figure 19. Cyclic mechanical strain increases the nuclear size. (A) iPS-derived cardiomyocytes were subjected to cyclic mechanical strain for 48 hours, representative images were taken in the brightfield, scale bars 50 and 100 μm respectively. (B) qPCR quantification of ANP gene expression in iPS-derived cardiomyocytes after 48 hours of strain. (C) Manual analysis of the nuclear area of stretched and non-stretched controls. Statistical differences were assessed using unpaired t-test **P < 0.01, ****P < 0.0001 data is presented as mean \pm SEM.

Discussion

Paper I

In this study, we developed a protocol for robust isolation of single nuclei from human hearts for individual transcriptional profiling, which was not available when we first set out on this project. Using frozen heart tissue, we first applied enzymatic digestion protocols but found limited cell yield and quality. Instead, we developed a protocol for nuclear dissociation from frozen tissue.

Importantly, we found that nuclear transcriptomes are strongly associated with cytosolic and whole-cell transcriptomes in human heart cells, supporting that our single nucleus sequencing protocol provides representative information on the overall cellular transcriptome. We note that our findings are concordant with similar work from nerve tissues which found correlation between transcriptomes of individual nerve cells and nuclei [86]. In addition, we have also shown that PCM1-gating can efficiently be used to separate cardiomyocytes from non-cardiomyocytes. Using PCM1⁺ based sorting, we confirm that the transcriptome of bulk heart tissue is most strongly associated with the cardiomyocyte enriched PCM1⁺ cell subset.

We then applied our snRNA-Seq sequencing protocol to generate a cell atlas of the human heart, from patients with DCM and ischemic cardiomyopathy (ICM), which had not previously been published at the time. By application of cluster analysis, we identified eight different cell clusters, and confirmed that fibroblasts, cardiomyocytes, and endothelial cells are the major cell types in human hearts, followed by smooth muscle cells, pericytes, macrophages, lymphocytes, neurons, and adipocytes, consistent with published work based on histology [18]. Our findings were also consistent with a subsequently published atlas of the human heart from the Human Cell Atlas project [9]. Clusters were identified using existing cell type markers and pathway-based grouping with global transcriptional patterns.

Interestingly, we noted two cardiomyocyte clusters. We believe that the smaller group of cardiomyocytes, marked by natriuretic peptide expression, might represent nuclei from cardiomyocytes that have been particularly exposed to strain or injury and are therefore particularly active in remodelling processes. This cluster was again observed in our larger study of independent hearts in Paper II but further study is required before any mechanistic inferences can be drawn.

Paper II

In this study, we sequenced a large number of human hearts with ten different phenotypes and control hearts without evidence of heart failure. We identified 14 cell clusters representing 10 distinct cell types among which endothelial cells, cardiomyocytes and fibroblasts were again the most common cell types, consistent with our previous study. Furthermore, as mentioned we again observed two cardiomyocyte clusters of which the smaller cluster expressed a transcriptional profile consistent with increased strain and fetal gene expression program remodelling. We further observed a notable increase in the number of fibroblasts and a decrease in vascular cell populations in failing hearts [29] consistently across all etiologies, highlighting significant cellular changes in heart failure. Further, we detected multiple differentially expressed genes across all ten etiologies. The highest unique differentially expressed genes were found in arrhythmogenic right ventricular cardiomyopathy, a condition not previously studied in single cell studies. The validity of our findings will need to be confirmed in additional samples using independent methods, ideally with spatial resolution to allow understanding of the tissue context of these findings.

Paper III

In this study, we explored mechanisms for a SNP previously linked to heart failure mortality. Our findings highlight *TSLP* as the likely gene underlying the association of the SNP with HF mortality. The SNP was located in an enhancer motif upstream of the *TSLP* gene and conferred increased binding of the transcription factor *NHLH1* and increased *TSLP* expression in human heart. Furthermore, we detected increased expression of *TSLP* in primary human cardiac fibroblasts in response to mechanical strain, recapitulating conditions in heart failure with high strain. A significant decrease in *TSLP* expression was observed both at rest and, more markedly, after strain, when *NHLH1* was suppressed indicating that *TSLP* is likely regulated by *NHLH1* in human cardiac fibroblasts. In addition, overexpression of *TSLP* in cardiac fibroblasts, by use of a *TFC21*-promoter vector, resulted in increased fibrosis and collagen deposition. In our snRNAseq dataset from study II, we found that the *TSLP* receptor was present in cardiac mast cells and observed that *TSLP* administration to mast cells resulted in increased TGF- β expression, suggestive that strain-induced crosstalk between fibroblasts and mast cells in the human heart may contribute to tissue fibrosis. Mast cells have also previously been described to release TGF- β [68].

It has been previously shown in other conditions that *TSLP* is released in parallel with IL-25 and IL-33 [87], and that IL-33 is released from cardiac fibroblasts in

response to increased strain during heart failure [88]. Our findings suggest that combined targeting of IL-33 and TSLP may confer benefits to prevent cardiac remodelling. Additional experiments to evaluate this therapeutic strategy in animal models of heart failure are warranted.

Paper IV

The main observation of this study was that in addition to larger cell size, nuclear size is also larger in failing hearts as compared to non-failing hearts. This observation was consistent across multiple etiologies which highlights the robustness of the observed phenomena across a range of heart failure phenotypes. We also observed that increased strain results in increased nuclear size, suggesting a role of mechanoresponsive pathways.

We argued that the larger size of cardiomyocyte nuclei in failing hearts may indicate increased transcriptional activity, but in our cardiomyocyte strain experiment we observed reduced total RNA abundance in both nuclei and cytoplasm, which does not support a role of broadly increased transcription. Further experiments are therefore needed to understand the mechanisms underlying these findings, which could potentially include factors such as DNA abundance, open chromatin, or altered nuclear-cytoplasmic transport. Furthermore, future studies will need to explore whether strain-induced changes in DNA replication and polyploidization in iPS-CMs as used here could contribute to increased nuclear size and contribute to our findings.

Conclusions and perspectives

The results of this work establish the feasibility of dissecting the molecular pathophysiology of heart diseases by RNA sequencing of frozen single cardiac nuclei. Furthermore, we sought to apply such methods at scale to >100 human hearts and identified molecular signatures associated with specific heart muscle conditions, which need to be confirmed in independent cohorts and expanded for improved precision. Finally, our work implicates TSLP as a putative therapeutic target to prevent cardiac remodelling which will need to be studied further in experimental HF models.

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