From Department of Medicine, Huddinge
Karolinska Institutet, Stockholm, Sweden

Focus on fibroblasts: Development, plasticity, and therapeutic challenges in the cardiac fibroblast lineage

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Stockholm 2020

Published by Karolinska Institutet.
Printed by E-Print AB 2020

IBSN 978-91-7831-714-1

Cover: Confocal image of primary human cardiac fibroblasts in culture.

Focus on Fibroblasts:
Development, plasticity, and therapeutic challenges in the cardiac fibroblast lineage

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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This thesis is dedicated to my family
for their sacrifice and encouragement,
for their love, affection and support
through time and distance.

ABSTRACT

Cardiac fibroblasts (CFs) are cells of mesenchymal origin and represent a heterogeneous population in the mammalian heart. They secrete extracellular matrix (ECM) molecules providing structural support during heart development and homeostasis. In response to injury, CFs are activated and undergo phenotypic conversion to myofibroblasts (myoFBs). MyoFBs, in turn, produced high levels of ECM proteins which can lead to cardiac fibrosis, thereby contributing to tissue stiffness, reduced contractility, and eventually heart failure (HF).
Currently, there are no effective treatments for HF. However, a better understanding of CF biology and the mechanisms of cardiac pathology may lead to novel therapeutic strategies for treatment of heart disease.

The overall aim of this thesis was further our understanding of CFs, with particular emphasis on their embryonic development, their plasticity, and the therapeutic challenges and opportunities presented by CFs.

In study I, we defined the transcriptional profiles of cardiac cells of mesenchymal origin during embryonic development in the mouse heart. The results suggested a new model for cell fate acquisition and differentiation within the mesenchymal lineage of the heart.
In study II, we developed a novel approach for the reprogramming of mouse CFs into an immature, scalable cardiac progenitor cells (CPCs) that could be differentiated into other cardiac cell types that may be more beneficial for cardiac function.
In study III, we established cell culture conditions that supported extracellular deposition of mature collagen from primary human CFs. This method was then used to develop an *in vitro* cardiac fibrosis assay that allows high-throughput screening to identify compounds with anti-fibrotic activity.
In study IV, we presented a new methodology for cell encapsulation, where human cells were encapsulated in microparticles made of new polymer materials and a new synthesis method that enables the delivery of secreted molecules for therapeutic use.

In summary, the work presented in this thesis contributes to a better understanding of CFs at the cellular and molecular level, and points to how they can be targeted for therapeutic purposes for treatment of heart diseases.

LIST OF SCIENTIFIC PAPERS

I. Stefanos Leptidis*, Aldo Moggio*, **Giorgia Palano,** Husain Ahammad Talukdar, Emil M. Hansson. Deconstructing mesenchymal cell lineages in the mouse heart using single cell RNA sequencing.
Manuscript

II. Jason S. L. Yu, **Giorgia Palano**, Cindy Lim, Aldo Moggio, Lauren Drowley, Alley T Plowright, Mohammad Bohlooly-Y, Barry S. Rosen, Emil M. Hansson, Qing-Dong Wang, Kosuke Yusa. CRISPR-KO screen identifies Dmap1 as a regulator of chemically-induced reprogramming and differentiation of cardiac progenitors. *Stem Cells 2019,* 37:958–972.

III. **Giorgia Palano***, Märta Jansson*, Anna Backmark, Sofia Martinsson, Kjell Hultenby, Alan Sabirsh, Kenneth Granberg, Karin Jennbacken, Peter Åkerblad, Erik Müllers, Emil M. Hansson. A high-content, *in vitro* cardiac fibrosis assay for high-throughput, phenotypic identification of compounds with anti-fibrotic activity.
Manuscript submitted JMCC in December 2019.

IV. Xiamo Chianty Zhou, Tommy Haraldsson, Salvatore Nania, Federico Ribet, **Giorgia Palano**, Rainer Heuchel, Matthias Löhr, and Wouter van der Wijngaart. Human cell encapsulation in gel microbeads with cosynthesized concentric nanoporous solid shells. *Advanced Functional Material 2018*, 1707129.

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LIST OF ABBREVIATIONS

α-SMA	alpha-smooth muscle actin
CCN2	connective tissue growth factor 2
CD90/Thy1	cluster of differentiation 90/thymus cell antigen 1
cDNA	complementary DNA
CF	cardiac fibroblast
CPC	cardiac progenitor cell
Cnn1	calponin 1
CM	cardiomyocyte
CRISPR	clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
DDR2	discoidin domain receptor 2
Dmap1	DNA methyltransferase 1-associated protein 1
Ebi3	Epstein-barr virus induced 3
EC	endothelial cell
ELISA	enzyme-linked immunosorbent assay
EGFP	enhanced green fluorescent protein
EndMT	endothelial-mesenchymal transition
EMT	epithelial-mesenchymal transition
ECM	extracellular matrix
FSP1	fibroblast specific protein 1
Gli1	glioma-associated protein 1
gRNA	guide RNA
HCF	human cardiac fibroblast
HF	heart failure
HRP	horseradish peroxidase
HTS	high-throughput screening
IL-10	interleukin-10
KO	knockout
LOX	lysyl oxidase
MAPK	mitogen-activated protein kinase

MyoD	myoblast determination protein 1
myoFB	myofibroblast
MMP	matrix metalloproteinase
mRNA	messenger RNA
miRNA	microRNA
MS	mass spectrometry
NG2	neuronal-glial antigen 2
Nkx2.5	NK-2 homeobox 5
OSTE	off-stoichiometry thiol-ene
PDGFRα	platelet-derived growth factor receptor alpha
PDGFRβ	platelet-derived growth factor receptor beta
PEGDA	polyethylene glycol diacrylate
PEO	proepicardial organ
Riok2	RIO kinase 2
scRNA-seq	single cell RNA sequencing
Snai1	snail family transcriptional repressor 1
TCF21	transcription factor 21
TIMP	tissue inhibitor of metalloproteinase
TGF-β	transforming growth factor-beta
TAK	TGF-beta-activated kinase
TNFα	tumor necrosis factor alpha
UV	ultraviolet
vSMC	vascular smooth muscle cell
WB	Western blot
WT	Wilms' tumor protein
YAP	yes-associated protein
3D	three dimensional

1 INTRODUCTION

1.1 Cardiac fibroblasts and the cardiac extracellular matrix

The mammalian heart is composed of several different cell types, including cardiomyocytes (CMs), endothelial cells (ECs), vascular smooth muscle cells (vSMCs), and cardiac fibroblasts (CFs)[1]. CFs constitute one of the most abundant cell types in the heart, but despite their abundance and their importance in cardiac physiology and pathology, CFs are poorly characterized molecularly. One reason is the relative absence of specific fibroblast markers. Several proteins have been suggested to label CFs, but they either lack in sensitivity (e.g. CD90/Thy-1, DDR2, and FSP1)[2] or specificity (vimentin)[3]. The challenge in the identification of specific markers of CFs is due to the fact that CFs are not a homogeneous cell population, but rather heterogeneous regarding their phenotype, function and cellular origin. In the absence of specific markers, CFs have been often identified based on morphological characteristics and/or proliferative potential. CFs are flat, elongated and spindle-shaped cells with multiple processes protracting from the cell body, and a main characteristic that distinguishes CFs from many other cells is that they lack a basement membrane[4].

CFs are the main source of the cardiac extracellular matrix (ECM) molecules in the heart, a complex molecular network in which cardiac cells are surrounded and interconnected. It provides not only the scaffold for cardiac cells, but also participates in signaling pathways, conveys mechanical signals to cardiomyocytes and facilitates electrical activity[5].

Collagen types I and III are the predominant components of the cardiac ECM, but the ECM also contains other molecules, including collagen types IV, V and VI, fibronectin, laminin, elastin, proteoglycans and glycoproteins[6]. In addition to being the primary source of these ECM molecules, CFs also produce ECM regulatory proteins, such as matrix metalloproteinases (MMPs), which degrade ECM, and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs), contributing to the maintenance of the ECM integrity[7]. Thus, CFs are not only the source of ECM components, but they also are able to modify the ECM by secretion of regulatory proteins controlling degradation and production of different components of the cardiac ECM[8]. ECM, secreted by CFs, provides structural support for the

cells of the heart during cardiac development in the embryo and tissue homeostasis in the adult, but also contributes to fibrosis in response to cardiac injury[9]. In the healthy heart, CFs are not only responsible for maintenance of the connective tissue of the heart, thereby indirectly affecting the biophysical properties of the contracting organ[10]. They also produce cytokines and growth factors[11], paracrine factors activating intracellular signaling pathways in receiving cells[12]. Furthermore, CFs are interconnected with CMs via gap junctions, and therefore they also contribute to the electrical and mechanical functions of the heart[13].

1.2 Structure, synthesis and maturation of collagen

Collagen type I is the most abundant protein in the cardiac ECM, and in response to injury, it represents the main component of fibrotic tissue[14]. It is secreted by CFs and synthesized as a collagen precursor (procollagen) and processed to form mature collagen (tropocollagen) that self-assembled into fibrils. The structure of collagen type I is composed of three polypeptides, two α-1 chains and one α-2 chain, folded in a triple helical domain flanked by globular N- and C-terminal pro-peptides[15] (Figure 1).

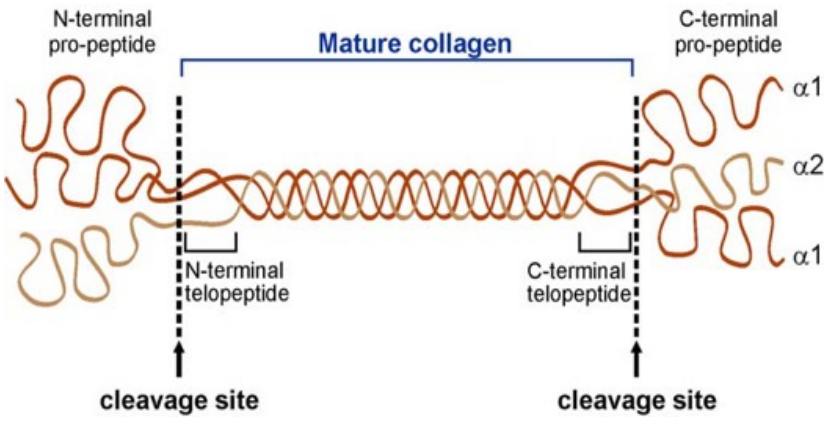

Figure 1: Molecular structure of collagen type I. Taken from Fan et al., *Fibrogenesis & Tissue Repair 2012*. Collagen type I is composed of three polypeptides (two α-1 chains and one α-2 chain) that are arranged in a triple helix flanked by globular pro-peptides at the N- and C-terminals. After cleavage of pro-peptides, mature collagen is formed.

Procollagen is synthesized in the endoplasmic reticulum (ER), and after post-translational modifications, it is transported across the Golgi and secreted into the extracellular space, where it is cleaved by peptidases through proteolytic removal of the N- and C-terminal pro-peptides. Trimming off the pro-peptides facilitates the cross-linking of collagen molecules that are then able to assemble into fibrils by lysyl oxidase (LOX)[16] (Figure 2)[17].

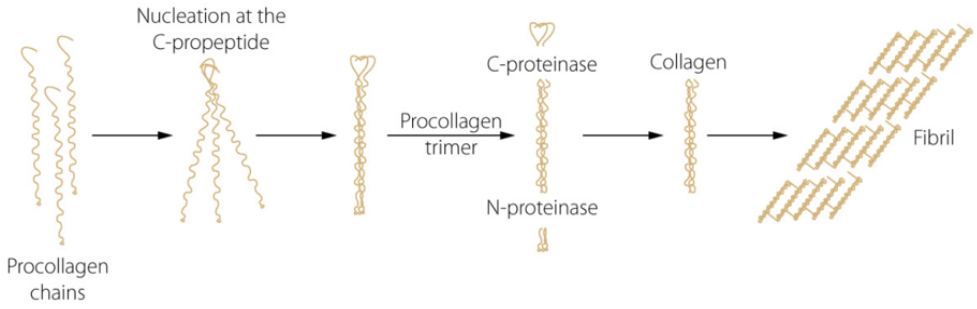

Figure 2: Schematic overview of collagen synthesis by fibroblasts. Modified picture from Canty and Kadler, *J Cell Sci 2005.*

The self-assembly of collagen fibrils is a key event in the formation of fibrotic tissue, since it is required for collagen to exert its structural effect on a tissue. Alternatively, collagen molecules can be degraded by MMPs, which are important for maintenance of ECM degradation[18]. TIMPs are extracellularly secreted proteins that inhibit MMPs, thereby constituting another layer of control over ECM formation, alteration, and remodeling[19].

Thus, collagen synthesis is a complex process that can be divided in intracellular and extracellular pathways, including upstream events that trigger fibrosis, formation of procollagen, trimming of procollagen by proteinases, and collagen fibril formation through cross-linking[20] (Figure 3). All of these key points could be potentially targeted to develop new treatments for cardiac fibrosis, and thus reducing secretion and deposition of collagen in the cardiac ECM that causes tissue stiffness and myocardial dysfunction.

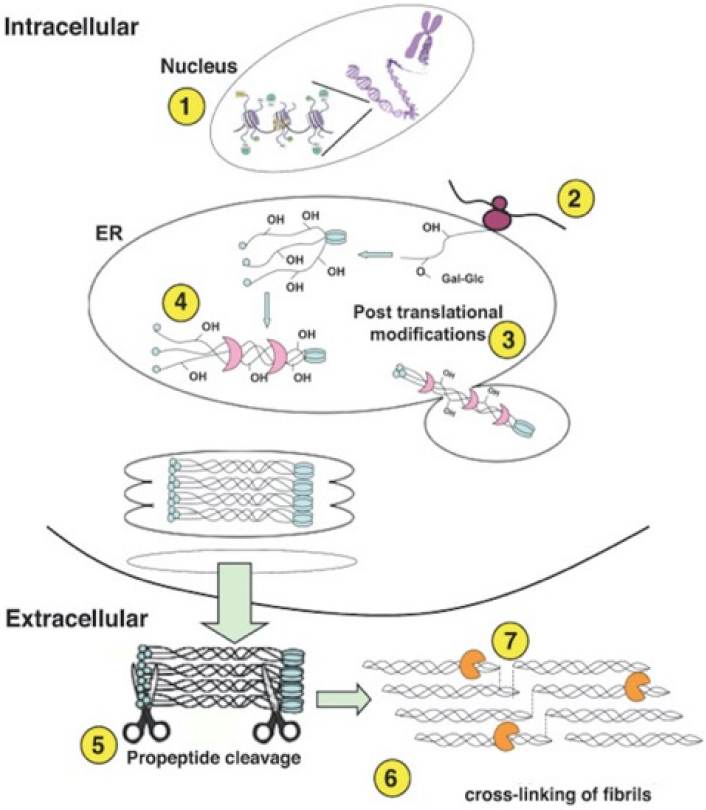

Figure 3: Key points of collagen synthesis. Taken from Chen and Raghunath, *Fibrogenesis & Tissue Repair 2009.* 1) Epigenetic level; 2) Post-transcriptional level; 3) Post-translational modifications; 4) Formation of procollagen triple helix; 5) Post-secretional level: cleavage of pro-peptides; 6) Cross-linking of collagen fibrils; 7) Collagen degradation by matrix metalloproteinases.

1.3 Developmental biology of cardiac fibroblasts

The embryonic origin of CFs has been a matter of debate, but recent lineage tracing studies in genetically modified mice have shed light on this issue[21]. CFs are cells of mesenchymal origin that can originate from different cellular sources. Current opinion is that the majority of CFs are derived from the proepicardial organ (PEO), a transient structure in the embryo that forms the epicardium, the outer layer of the heart, that plays a central role in cardiac development and in response to injury[22]. During embryonic development, mesenchymal cells migrate from the PEO to cover the developing heart, where they undergo epithelial–mesenchymal transition (EMT), allowing them to migrate into the myocardium and differentiate into CFs. A portion of cells derived from the PEO remains on the surface of the heart and form epicardial cells. In addition to CFs, PEO derivatives invading the heart also

differentiate into vSMCs and, possibly, ECs and a minor population of CMs[23]. However, if the PEO contributes to the CMs and ECs of the mammalian heart is still controversial[24].

In addition to the PEO, there are other cell populations that have been suggested to contribute to CFs in the adult heart. It has been shown that a subset of endothelial cells, located in a region dubbed the endocardial cushion, migrates from the endothelial lining of the developing heart into the myocardium through a process known as endothelial-to-mesenchymal transition (EndMT) to constitute a second smaller population of CFs[25].

Taken together, it is now widely accepted that cells of the PEO and of the endocardial cushion delaminated from their respective structures, start expressing a mesenchymal genetic program, and differentiate into CFs essential for the formation of the adult heart[26] (Figure 4).

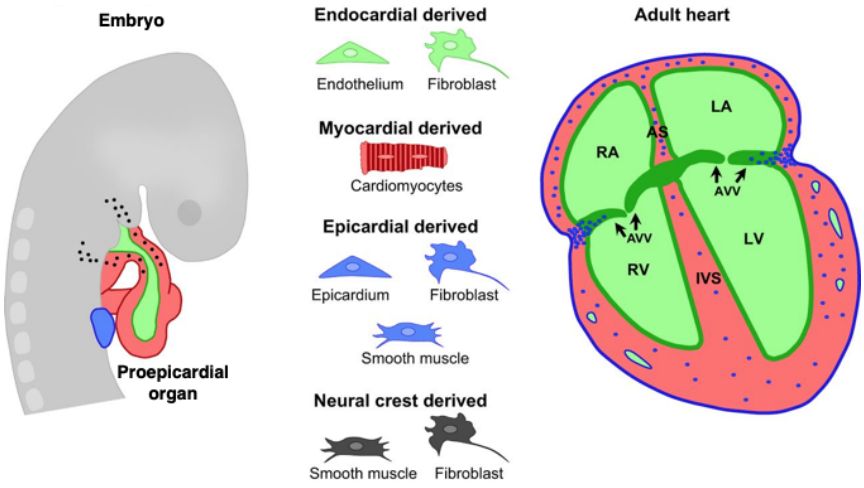

Figure 4: Development origin of cardiac fibroblasts in the embryo. Taken from Furtado et al., *Development 2016.*

Circulating bone marrow-derived cells, monocytes and fibrocytes, have been suggested to constitute additional sources of CFs in response to injury[27], but such claims have been contested[28]. Finally, a small fraction of CFs has been reported to be derived from the neural crest[29].

Taken together, the origin of CFs is still not entirely clear, and further experimental evidence, using stringent genetic models of lineage tracing, will be required to finally settle the ongoing debate on CF ontogeny.

1.4 Heart failure and cardiac fibrosis

Heart failure (HF) is a chronic and progressive condition in which the heart is not able to pump a sufficient amount of blood and oxygen to fulfill the metabolic needs of the rest of the body[30]. It is one of the major leading causes of death worldwide with 38 million affected individuals globally[31]. The prevalence of HF is increasing because of an ageing general population, a more sedentary lifestyle, and due to improved treatment of acute coronary disease in patients, resulting in prolonged survival, but with impaired cardiac function[32]. With the exception of heart transplantation, there are no effective treatments for severe HF, and symptomatic HF is associated with a poor prognosis worse than for many forms of cancer[33].

A key component of the pathogenesis of HF is cardiac fibrosis. Cardiac fibrosis can be viewed as a response to injury or stress, and in contrast to other organs, such as skin, the gastrointestinal system and bone marrow, the adult mammalian heart has a very limited regenerative capacity[34]. Instead, a common response to cardiac injury is fibrosis, and the specific form of cardiac injury molds the fibrotic response into two main different types: replacement fibrosis and reactive interstitial fibrosis[35]. Replacement fibrosis is most commonly caused by a myocardial infarction, in which cardiomyocytes are lost due to hypoxia and are replaced with fibrotic scar tissue[36]. In contrast, interstitial fibrosis is frequently a result of pressure overload and is not associated with a significant loss of cardiomyocytes. In this case, the interstitial space expands because of the deposition of collagen and other ECM molecules between cardiomyocytes[37]. In both cases, normal tissue is converted into a fibrotic tissue that, if it persists, affects the structural and functional properties of the heart by increasing tissue stiffness due to excessive deposition of ECM proteins, and cardiac remodeling that consists of thickening (hypertrophy) of the left ventricular wall[38]. The result is impaired cardiac contraction and relaxation of the heart, thereby affecting cardiac output and organ function, and ultimately leading to HF[39] (Figure 5).

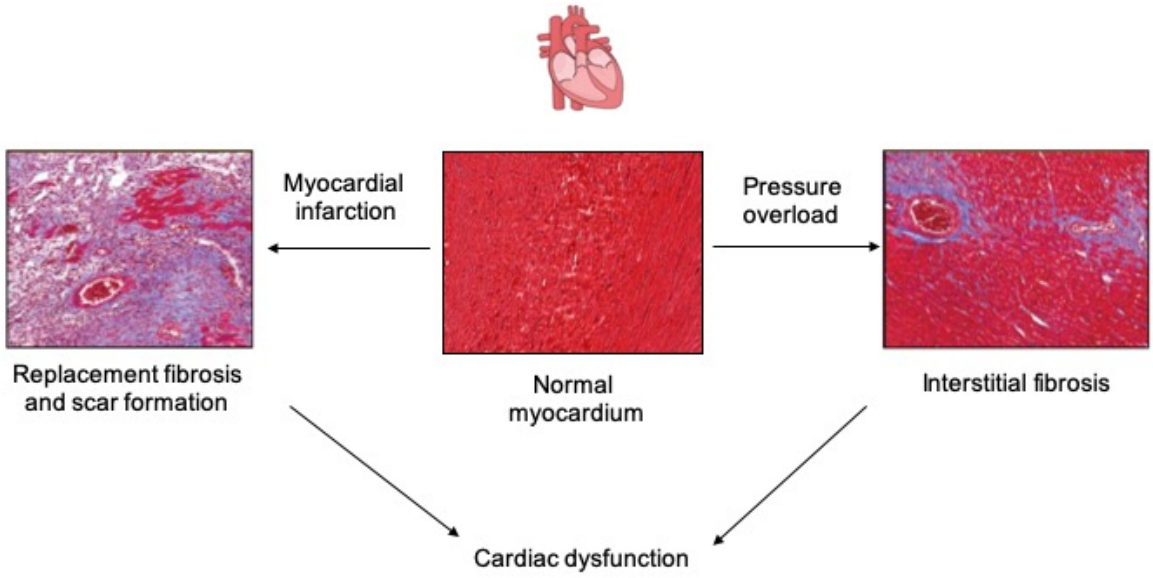

Figure 5: Different forms of cardiac fibrosis. Fibrosis occurs in response to cardiac injury: myocardial infarction and pressure overload. Histopathologic illustrations of normal myocardium (middle), replacement fibrosis (left) with cardiomyocyte loss or death and significant expansion of ECM and collagen deposition, and interstitial fibrosis (right) characterized by increased collagen synthesis with minimal or no loss of cardiomyocytes.

Recently, there has been considerable interest in augmenting cardiomyocyte regeneration as a novel form of therapy in HF. However, conceptually appealing as that idea is, it is clear that cardiac fibrosis would constitute a barrier to regenerative approaches to treat HF.

Effective anti-fibrotic therapies would be of interest in at least some forms of cardiac pathology that eventually lead to HF, but the cellular and molecular mechanisms of cardiac fibrosis have been less investigated[40]. Therefore, a more thorough understanding of the cardiac response to injury and the pathology of HF are necessary to design novel modes of therapy.

1.5 Inflammation and mediators of cardiac fibrosis

Here, I describe the key steps in the cardiac response to injury using the most common disease of the heart, myocardial infarction, as an illustrative example. In a myocardial infarction, cardiac ischemia leads to cardiomyocyte death, which in turn triggers a response to injury. In the heart, the response to injury can be divided into three distinct but overlapping phases: 1) an inflammatory phase, 2) a proliferative phase, and 3) a phase of maturation[41]. During the inflammatory phase, local production of cytokines, chemokines, and growth factors induces infiltration of inflammatory cells into the injured area. Neutrophils and macrophages clear the damaged area from dead cells and matrix debris. In particular, macrophages secrete mediators of inflammation, such as Transforming Growth Factor (TGF)-β, Interleukin (IL)-10 and Tumor Necrosis Factor (TNF)α[42]. During the proliferative phase, as inflammatory cells decrease in number through apoptosis, the expression of inflammatory mediators is suppressed, and instead CFs proliferate and are stimulated to produce ECM in the injured heart. Finally, maturation of the scar follows, where large areas of myocardium are replaced with a fibrotic scar tissue containing cross-linked collagen fibers that are formed during cardiac injury[43]. Thus, cardiac fibrosis is mediated by pro-fibrotic factors, that are released in response to cardiac injury and exert their effect on CF proliferation, differentiation, and ECM deposition. Among these, ligands of the TGF-β family, secreted by macrophages, but also by platelets and activated CFs, are probably the best characterized pro-fibrotic signaling molecules. There are three isoforms of TGF-β (TGF-β1, β2 and β3) in mammals, encoded by three different genes. TGF-β1 is the prevalent isoform and is found in almost all tissues, whereas the other isoforms are expressed in a limited number of cell types[44]. In the heart, TGF-β1 is produced by several cell types including CFs, vSMCs and macrophages[45]. TGF-β is secreted in a latent form, which is activated by binding to integrins and a protease, which cleaves and releases active TGF-β. The active cleaved form of TGF-β binds to a heterodimer of type I and II TGF-β receptors presented on the cell surface of cells expressing these proteins. Binding of ligand to receptor activates intracellular signaling pathways, that fall into two broad classes; the Smad-dependent canonical and the Smad-independent non-canonical pathways[46] (Figure 6).

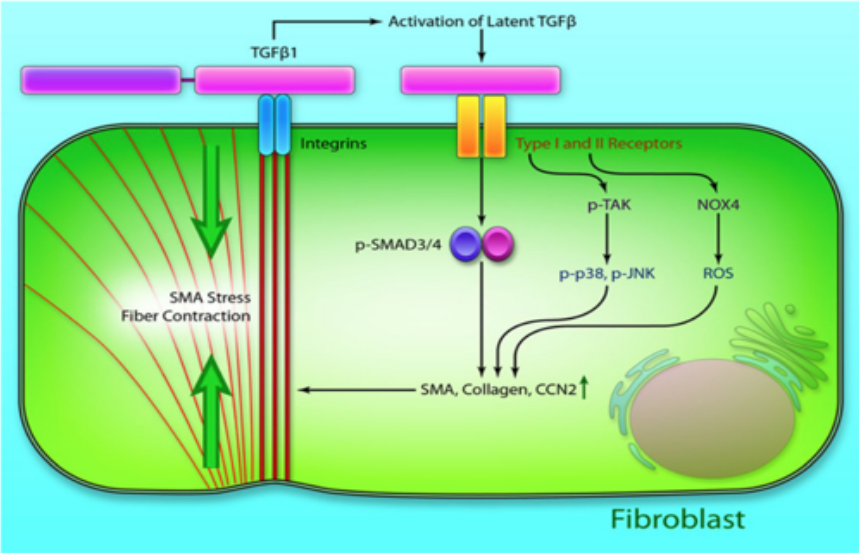

Figure 6: Canonical and non-canonical pathways of TGF-β signaling in fibroblasts. Taken from Leask et al., *Circulation Research 2015.*

In the canonical pathway, TGF-β binds to its receptors, resulting in phosphorylation of the adaptor proteins Smad2/3, that thereafter bind to Smad4, also referred to as co-Smad. The Smad2/3-Smad4 complex thereafter translocates to the nucleus, where it binds DNA and induces transcription of downstream target genes. In CFs, these genes include fibrogenic genes, such as α-smooth muscle actin (α-SMA), collagen, and CCN2 (connective tissue growth factor), which increase deposition of collagen and other ECM proteins[47]. In the non-canonical pathway, TGF-β instead activates TGF-β–activated kinase (TAK) 1 and p38 mitogen-activated protein kinase (MAPK), which have been implicated in phenotype conversion of CFs into myofibroblasts[48]. Thus, TGF-β activates different intracellular signaling pathways that together control multiple important steps in the pathogenesis of cardiac fibrosis; including CF proliferation and phenotype conversion, increased ECM production, decreased ECM degradation, and upregulation of genes promoting ECM maturation. Therefore, inhibition of TGF-β signaling pathways would be a valid therapeutic target for treatment of HF to reduce cardiac fibrosis[49]. However, members of the TGF-β superfamily of ligands, which are expressed throughout the body, are important for the maintenance of homeostasis in many organs[50], and therefore possible side effects limit their use as a therapeutic target for cardiac fibrosis[51].

1.6 Myofibroblasts

In response to injury, CFs become activated and undergo phenotype conversion to myofibroblasts (myoFBs)[52], a cell type not found in normal myocardium, but present exclusively in injured hearts[53]. MyoFBs constitute, just like CFs, a molecularly rather poorly defined cell type, but are known to be more mobile cells, exhibiting a contractile capacity and a greater ability to synthesize and secrete ECM proteins compared to CFs[54]. MyoFBs exhibit a typical phenotype characterized by stress fibers and also express α-SMA and other contractile proteins[55]. They secrete a variety of cytokines (IL-1α, IL-1β, IL-6, IL-10 and TNFα), which in turn help to maintain the inflammatory response to injury[56]. MyoFBs have been demonstrated to play a critical role in cardiac fibrosis. Because of their ability to produce large amounts of ECM proteins, they are considered to be the main driver of excessive secretion and deposition of ECM, mainly collagen type I, contributing to tissue stiffness and loss of contractility of the heart[57]. Phenotype conversion from CFs to myoFBs can be induced by TGF-β, cytokines, and other growth factors secreted as a response to cardiac injury[58]. Although it is well established that cytokines and growth factors, secreted by inflammatory and immune cells after cardiac injury, can induce some of the phenotypic changes in CFs undergoing phenotype conversion to myoFBs, the molecular mechanisms controlling this transition are not completely understood (Figure 7).

Figure 7: Activation and phenotype conversion of fibroblasts to myofibroblasts after cardiac injury. CFs become activated and undergo phenotype conversion to myoFBs in response to cardiac injury. These cells express α-SMA and secrete high levels of ECM proteins, contributing to cardiac fibrosis.

1.7 Resident cardiac fibroblasts and progenitor cell activation after injury

The classical view of cardiac fibrosis holds that scar-forming myoFBs are generated by proliferation, activation, and phenotype conversion of resident CFs in the injured heart[59]. However, recent data indicate that also other cell types have the ability to generate myoFBs after cardiac injury, complementing the resident CFs formed during embryogenesis and present during homeostasis. Two cellular sources for injury-induced formation of new myoFBs are: 1) epicardial cells surrounding the outside of the heart[60], and 2) a subset of perivascular cells that are located within the myocardium close to the blood vessels[61] (Figure 8).

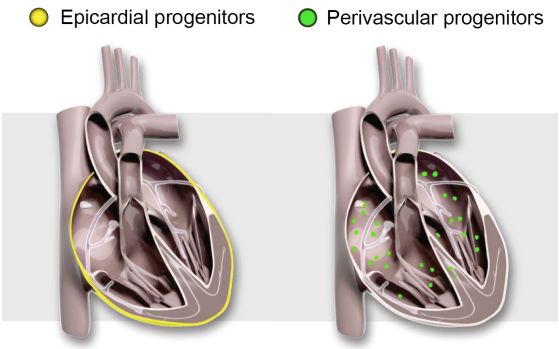

Figure 8: Cell sources of cardiac fibroblasts after injury. Epicardial progenitors (yellow) are surrounding the outer layer of the heart, while perivascular progenitors (green) are located inside the heart close to the vasculature.

Following cardiac injury, such as a myocardial infarction, cells from the epicardium proliferate and form a local thickening of the epicardial layer covering the surface of the heart. Thereafter, they migrate into the myocardium and differentiate mostly into myoFBs, but also into vSMCs[62]. Interestingly, epicardial cells, activated in response to injury, express WT1 (Wilms' Tumor protein 1), a transcription factor important for PEO formation and differentiation during cardiac development in the embryo. Therefore, it appears that epicardial cells after injury in the adult heart revert to a transcriptional program active during embryonic development as a repair mechanism, leading to myoFB formation.

In the mouse heart, it has been shown that perivascular cells expressing Gli1 (Glioma-associated protein 1), a transcription factor regulating the Hedgehog signaling pathway, reside close to blood vessels and co-express some markers of pericytes, including PDGFR-β (platelet-derived growth factor receptor-β), NG2 (neuron-glial antigen 2), 3G5 and nestin.

Following injury, these cells start to proliferate and differentiate into myoFBs, thereby contributing significantly to fibrosis and scar formation[63]. It is not known where these Gli1+ cells come from, when they appear in the mouse heart or whether a similar cell population exists in the human heart (Figure 9), although it is tempting to speculate that they constitute a subpopulation of pericytes derived from PEO and/or endocardial cushion that are present in all mammalian species.

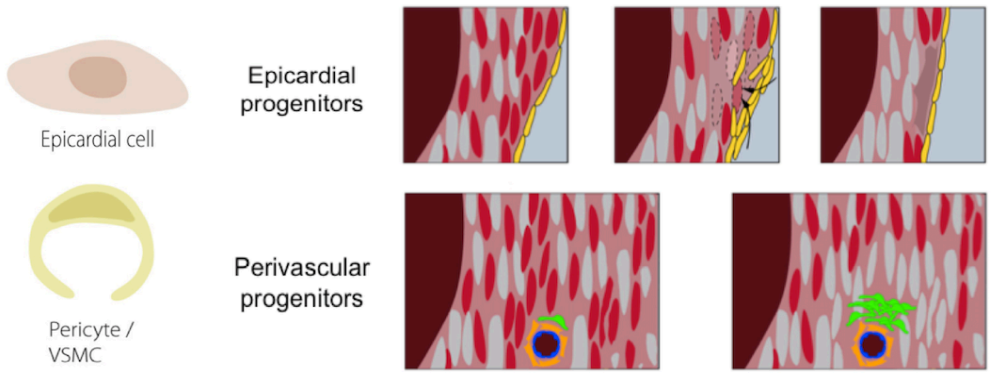

Figure 9: Origin and mechanisms leading to myofibroblast formation. Following injury, epicardial progenitors (yellow) proliferate and migrate within the myocardium, where cardiomyocytes (red) are replaced by scar tissue. Perivascular progenitors (green) proliferate and differentiate by activation of fibroblasts (grey).

The relative contribution of myoFBs generated from resident CFs versus myoFBs generated from other cellular sources to scar formation in different forms of cardiac injury is unknown. Furthermore, the molecular mechanisms controlling epicardial and perivascular cell proliferation and differentiation are poorly understood.

Taken together, the origin of CFs and myoFBs in the injured heart is still controversial and the relative contribution of each of these cell populations to collagen-producing cells after cardiac injury remains unclear.

1.8 Cellular heterogeneity and plasticity of cardiac fibroblasts

Another characteristic of CFs is their plasticity. Following injury, CFs become activated and undergo phenotype conversion to myoFBs, resulting in secretion of high levels of ECM proteins, thereby promoting a fibrotic cardiac remodeling[64]. Would it be possible to exploit the plasticity of CFs and coax them to adopt a cell fate more beneficial for cardiac function? This is a fairly old idea that has garnered more interest the last decade.

A classic study by Davis et al. in 1987 demonstrated that skin fibroblasts could be converted to myoblasts through forced expression of a single transcription factor, MyoD, which activates muscle-specific gene expression leading to a fate switch in transfected cells[65]. At that time, the idea that one single "master" gene could so drastically alter the identity of a cell was completely new, and the initial work on MyoD lead to intensive investigation of cell plasticity. However, the case of MyoD in fibroblasts to some extent proved to be somewhat of an anomaly, and with the exception of a few examples from the hematopoietic system, including work on the role of Pax5 for B cell identity[66] and C/EBP-α and -β as reprogramming factors for B cells to macrophages[67], there were few reported reprogramming studies analogous to MyoD. This changed when Yamanaka and co-workers showed that forced expression of four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc, in fibroblasts resulted in reprogramming to the pluripotent state[68]. The groundbreaking work by Yamanaka and colleagues spurred a huge interest in identifying other combinations of reprogramming factors that could induce conversion to other cell types. Factors promoting the reprogramming to many other cell types, including neurons[69] and endocrine cells of the pancreas[70] followed, and subsequent work in the cardiac field led to the identification of a "transcription factor cocktail", composed of Gata4, Mef2c, and Tbx5, with addition of Hand2 in some studies (referred to as GMT/GMTH), being sufficient to convert mouse fibroblasts into functional cardiomyocyte-like cells both *in vitro*[71,72] and *in vivo*[73]. Work from other laboratories have identified microRNAs, small non-coding RNAs that regulate gene expression in the heart, as putative reprogramming factors. MiR-1, miR-133, miR-208 and miR-499 have been identified as promoting reprogramming of CFs into CMs[74].

2 AIMS

The overall aim of this thesis was to further our understanding of cardiac fibroblasts, with particular emphasis on their embryonic development, their plasticity, and the therapeutic challenges and opportunities presented by cardiac fibroblasts.

The specific aims were:

Study I	To study the cellular heterogeneity and the molecular programs driving cellular diversification in the mesenchymal lineage of the mouse heart.
Study II	To develop a chemically-induced reprogramming protocol to convert mouse CFs into CPCs and differentiate them into other cardiac cell types more beneficial for cardiac function.
Study III	To establish a cell culture protocol that supports extracellular deposition of mature collagen from primary human CFs and to use this protocol to develop an *in vitro* cardiac fibrosis assay to identify compounds with anti-fibrotic activity.
Study IV	To develop a new approach for cell encapsulation in microparticles that can be used therapeutically for example in heart disease.

3 METHODOLOGY

In this section, I briefly describe some principles of the methodology used in the studies of this thesis. For more detailed information, please refer to the Material and Method section of the respective publications.

3.1 Primary cell cultures

Primary HCFs have been cultured routinely for many years, and this technique has provided important insights into their physiology. However, under conventional conditions they do not deposit significant amounts of mature collagen, one of the hallmarks of activated fibroblasts in heart disease. Many previous studies have tried to achieve deposition of mature collagen in culture from cultured fibroblasts[75,76]. We screened the literature and tried many of the published protocols on how to achieve this. It is previously been proposed that the inefficient processing of procollagen to mature collagen in cell culture could be due to the relatively low concentration of procollagen and enzymes in the culture medium. Using cell lines from other organs and forcing them to grow under super-confluent conditions (macromolecular crowding), processing of collagen to mature forms has been reported[77,78]. We hypothesized that a similar effect could be seen in cardiac fibroblasts in culture. Cells were grown under macromolecular crowding conditions by treatment with dextran sulfate (DxS), a negatively charged molecule with a high molecular weight (500 kDa). It is a polysaccharide polymer of glucose that minimizes the distribution volume of secreted molecules into the culture medium, bringing substrates and enzymes closer together, thereby conceptually facilitating enzymatic processes such as the conversion of procollagen to mature collagen[79] (Figure 10).

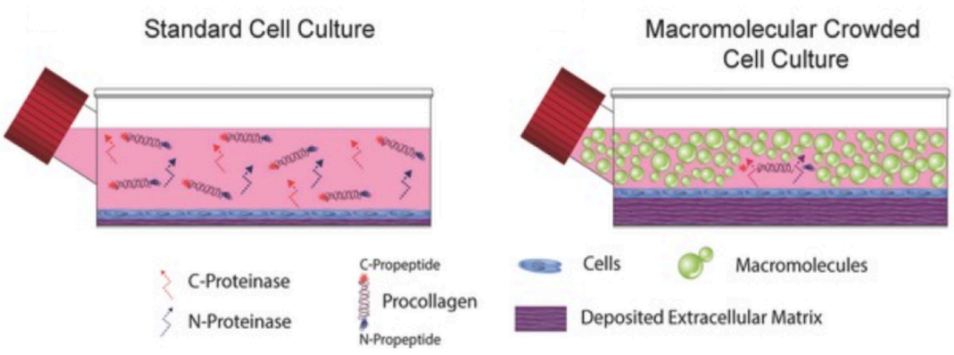

Figure 10: Macromolecular crowding in cell cultures. Taken from Satyam et al. *Advanced Materials 2014.*

3.2 Cardiac fibroblast reprogramming and differentiation

Direct reprogramming is a technique that converts a differentiated cell into another, without passing through the pluripotent state. This process generally relies on forced expression of exogenous genes, most often transcription factors that control cell identity and function. Direct reprogramming of mouse CFs has been showed using different methods: 1) forced expression of cardiac transcription factors (Gata4, Mef2C, and Tbx5)[80]; 2) delivery of miRNAs involved in cardiac development (miR-1, -133, -208, and -499)[81]; and 3) overexpression of reprogramming factors for induced pluripotent stem cells (Oct4, Sox2, Klf4, and c-Myc) followed by exposure to cardiogenic media, thereby escaping reprogramming to pluripotency[82]. However, all these approaches depend on exogenous gene expression, require genetic manipulation and/or viral transduction. Recently, it has been shown that a cocktail of small molecules can be used to convert CFs into other cardiac cell types through direct reprogramming, albeit with low frequency[83].

Our approach relies on the use of a small chemical inhibitor of the TGF-β signaling pathway. Combining this treatment with a defined culture medium in hypoxic conditions, small clones of expandable cells formed, and these cells could thereafter differentiate to cells expressing markers specific for other cardiac cell types. This is an example of reprogramming without forced expression of exogenous genes and does not rely on viruses or modification of the host genome, factors that hamper transfer of other reprogramming methods to therapeutic use.

3.3 Lineage tracing and reporter mouse lines

In developmental biology, lineage tracing experiments and reporter mouse lines are used to label specific cell types to study their progeny. One very powerful technology for lineage tracing studies is the Cre-loxP system. The Cre-loxP system relies on introduction of cDNA encoding the bacterial recombinase Cre and a transgenic cassette including sequences known as loxP sites. Cre recombinase is an enzyme that, when binding two loxP sites in the DNA sequence of the genome, creates an inversion, translocation or deletion of the gene of interest flanked by loxP sites through recombination. Practically, a lineage tracing experiment relies on two mouse lines, one carrying Cre expressed from a cell-specific promoter of choice and another line carrying a Cre-dependent reporter allele, that are crossed to generate mice carrying both transgenic alleles. In these transgenic mice, Cre recombinase will cut out a stop sequence flanked by loxP sites upstream of a cDNA encoding a reporter protein, for example

EGFP. When the stop sequence is removed, the reporter protein will be expressed (Figure 11). Importantly, this is an irreversible event. Thus, as Cre recombinase is only expressed in a cell type of choice, reporter protein expression will be confined to that cell type and its progeny.

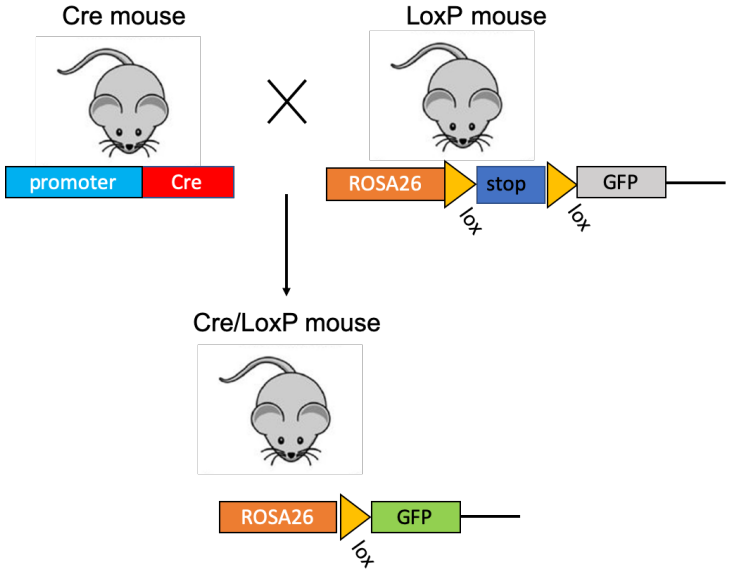

Figure 11: Schematic illustration of the Cre/LoxP system.

A later iteration of the Cre-loxP system permits temporal control of Cre-mediated recombination. Here, Cre is fused with an engineered form of the estrogen receptor (ER), resulting in CreERT. The engineered estrogen receptor localizes to the cytoplasm, but when its ligand tamoxifen is present, it translocates to the nucleus, permitting Cre to bind and recombine DNA at loxP sites. Since tamoxifen is a synthetic compound, recombination can be controlled by administration of tamoxifen to the transgenic mice.

In our study, we performed lineage tracing experiments to irreversibly label CFs expressing PDGFRα in the adult mouse heart. PDGFRα-CreERT mice were crossed with the reporter mouse strain ROSA26R$^{CAG\text{-}LSL\text{-}EGFP}$. After treatment with tamoxifen, recombination was induced. In this way, only cells expressing PDGFRα were labeled by EGFP+. In the heart, the only such cell type is the CF, whereas other cardiac cells do not express PDGFRα and consequently did not express EGFP. Following reprogramming, some PDGFRα-EGFP+ cells started to express SMC markers, showing that these cells were derived from CFs.

3.4 CRISPR-Cas9 screening

CRISPR-Cas9 is a genome editing technology that enables site-specific, irreversible change of DNA sequences. This method allows genetic material to be added, removed, or replaced at particular locations in the genome. It was adapted for use in mammalian cells from bacteria, where CRISPR-Cas9 functions as an immune system against viruses. The CRISPR-Cas9 system consists of a short RNA that binds to a specific target DNA sequence in the genome, and the Cas9 endonuclease cuts the DNA at that specific location. Once the DNA is cut, the DNA break is generally repaired by non-homologous end joining (NHEJ), an endogenous repair mechanism that often results in insertion or deletion of a few base pairs ("indels"). Alternatively, more elaborate changes to the DNA sequence can be achieved through exploiting homology directed repair, but in this study, we used a CRISPR-Cas9 library to inactivate genes through the creation of indels. This allowed screening of genes to identify be controlled by administration of tamoxifen to the transgenic mice.

In our study, Cas9-expressing CFs were transduced with a genome-wide lentiviral guide-RNA (gRNA) library. Following antibiotic selection, a mutant cell population of mouse CFs was generated, and subjected to reprogramming conditions, as described above in section 3.2. Following reprogramming, cells were enriched and stained using Nkx2-5 as a marker to distinguish between positive and negative populations. In this way, cells were screened by sorting of stained cells. Cells were thereafter subjected to genomic DNA extraction, PCR amplification, and DNA sequencing. Enriched gRNA sequences were then analyzed by a computational algorithm that permits the identification of the most significant genes and assigns a score to each gene as enriched or depleted, resulting in a gene ranking. A positive score indicates that depletion of the gene is advantageous for reprogramming, while a negative score means that such genes are important for the reprogramming process. Candidate genes can thereafter be explored mechanistically for effects on reprogramming efficiency[84] (Figure 12).

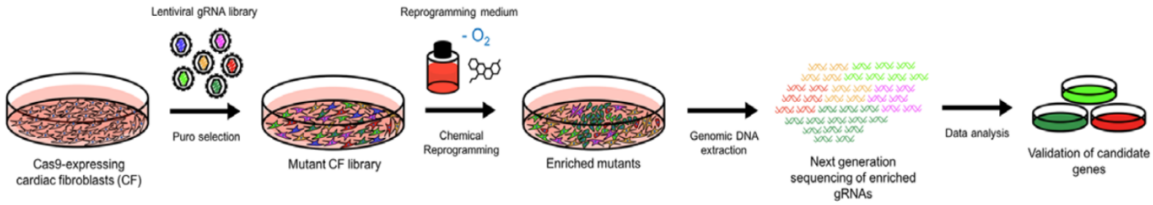

Figure 12: Schematic of CRISPR-Cas9 screening workflow. Taken from Yu and Yuse, *Methods* 2019.

3.5 Bisulfite sequencing and promoter methylation analysis

Bisulfite sequencing is a method used for analyzing the methylation status of DNA. DNA methylation is an epigenetic mechanism characterized by the transfer of a methyl group on cytosine to form 5-methylcytosine. In bisulfite sequencing, genomic DNA is treated with bisulfite, thereby converting all unmethylated cytosines into uracil residues. After PCR amplification, uracil residues become thymine, but 5-methylcytosines remain cytosines, allowing them to be distinguished from unmethylated cytosines. The PCR products can be directly sequenced or sub-cloned and then subjected to sequencing. DNA methylation status can be determined by comparing the results with the original DNA sequence.

In our study, we used bisulfite sequencing to study the methylation status of promoter regions of interest. Genomic DNA was extracted and converted via bisulfite reaction and purified PCR products were cloned into a plasmid and submitted for sequencing.

3.6 Western blot

Western blot (WB) is a common technique used to detect proteins and determine their size. Protein extracts are loaded on a polyacrylamide gel and separated by electrophoresis, thereby separating them by electrophoretic mobility, a function of their molecular weight. The separated proteins are then transferred to a membrane by blotting and detected by antibody staining after blocking to minimize unspecific binding. This method allowed us to detect different forms of collagen using epitope-specific antibodies: 1) anti-collagen I antibody that recognized both unprocessed and mature forms, 2) anti-pro-collagen 1α1 antibody to detect

procollagen, and 3) anti-telo-collagen 1α1 antibody, specific for the C-terminal neoepitope of mature collagen (Figure 13).

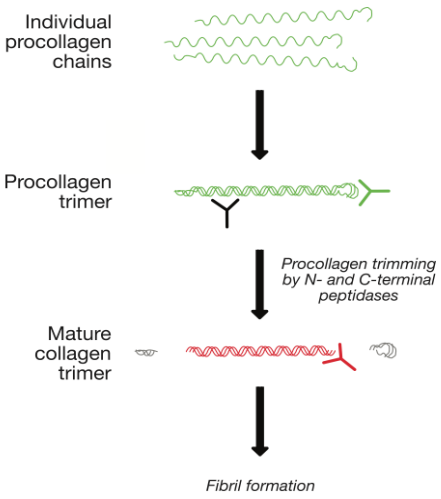

Figure 13: Schematic illustration of collagen maturation. Epitope-specific antibodies recognize different forms of collagen: collagen I antibody detects both forms of collagen (black), pro-collagen 1α1 antibody detects unprocessed collagen (green), and telo-collagen 1α1 detects mature collagen (red).

3.7 Collagen extraction by pepsin, silver staining, and mass spectrometry

Pepsin is a proteolytic enzyme that cleaves peptide bonds between hydrophobic and aromatic amino acids of proteins, but it cannot digest the triple helix of collagen due to the specific 3D structure of this molecule. Therefore, treating biological samples with pepsin is a convenient way to extract collagen. In our study, we extracted mature collagen through pepsin digestion of samples followed by visualization of precipitated proteins by silver staining.

Silver staining is a colorimetric method based on the binding of silver ions to proteins after electrophoretic separation on a polyacrylamide gel. This technique allowed us to detect bands corresponding to pepsin-resistant proteins and observe that their size matched that of mature collagen using antibody-independent methodology.

Thereafter, protein bands were cut out from silver-stained gels and subjected to in-gel digestion and mass spectrometry. Mass spectrometry (MS) is a sensitive technique used to detect and identify proteins from the mass of their peptide fragments. This method consists of ionizing molecules to generate charged fragments using electrical and/or magnetic fields to

measure their mass-to-charge ratio. A mass spectrum is produced and then analyzed by a computer software using databases for searching the identity of the proteins.

In our study, we used MS to identify pepsin-resistant proteins in silver-stained gels. In this case, we detected collagen α-2(I) chain (CO1A2) in the lower band, and collagen α-1(I) chain (CO1A1) in the upper band. All peptides identified were located in the mature telo-collagen molecule.

3.8 Biotinylation of cell surface proteins

Cell surface biotinylation is a method that enables labeling of cell surface proteins with biotin, a small vitamin that interacts with streptavidin with exceptionally high affinity and specificity. Sulfo-NHS-LC-biotin (sulfosuccinimidyl-6-biotinamido hexanoate) is an ester of biotin that binds to extracellularly primary amines, such as lysine residues, resulting in covalent attachment of biotin to exposed sites. Because Sulfo-NHS-LC-biotin is soluble in water and is charged by the sodium sulfonate group, it cannot permeate the cell membrane. Thus, if the cell remains intact, only primary amines exposed on the surface can be biotinylated. This method allowed us to detect biotinylated proteins, including collagen molecules, that were attached on the cell surface or extracellularly deposited on the cell culture plate before cell lysis (Figure 14).

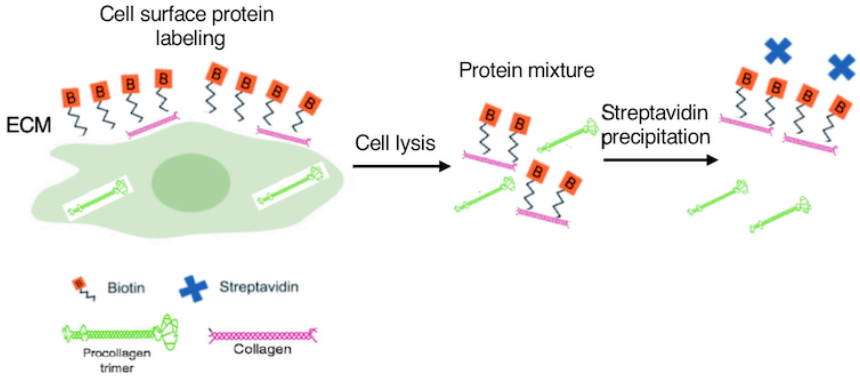

Figure 14: Schematic illustration of biotinylation of cell surface proteins. A protein mixture of unprocessed (green) and mature (pink) collagen molecules on the cell surface can be labeled with biotin and detected by streptavidin after cell lysis.

3.9 ELISA

ELISA (Enzyme-linked immunosorbent assay) is a common method used for protein detection and quantification. This assay is based on the immobilization of an antigen of interest on a solid surface of a multi-well plate, followed by binding to a specific antibody linked to an enzyme. In the final step, a substrate for the relevant enzyme is added, and the subsequent reaction produces a signal that can be detected, often using a calorimetric method.

There are different types of ELISA:

1) Direct ELISA, in which the antigen is immobilized on a plate, and then a conjugated antibody binds to the target protein. Following the incubation, a substrate is added to produce signal that can be quantified.
2) Indirect ELISA, in which the antigen is immobilized on a plate, but an unconjugated antibody is added and binds to the specific antigen. A conjugated secondary antibody that recognized the primary antibody is then added.
3) Sandwich ELISA, in which an antibody, called capture antibody, is coated on the plate and used to immobilize the antigen. Then another antibody, called detection antibody, is added to bind the antigen allowing the signal detection.

There are many modifications of this assay, but, in our study, we used two methods consisting of 1) streptavidin-biotin detection and 2) indirect immobilization of the antigen of interest to the plate (Figure 15).

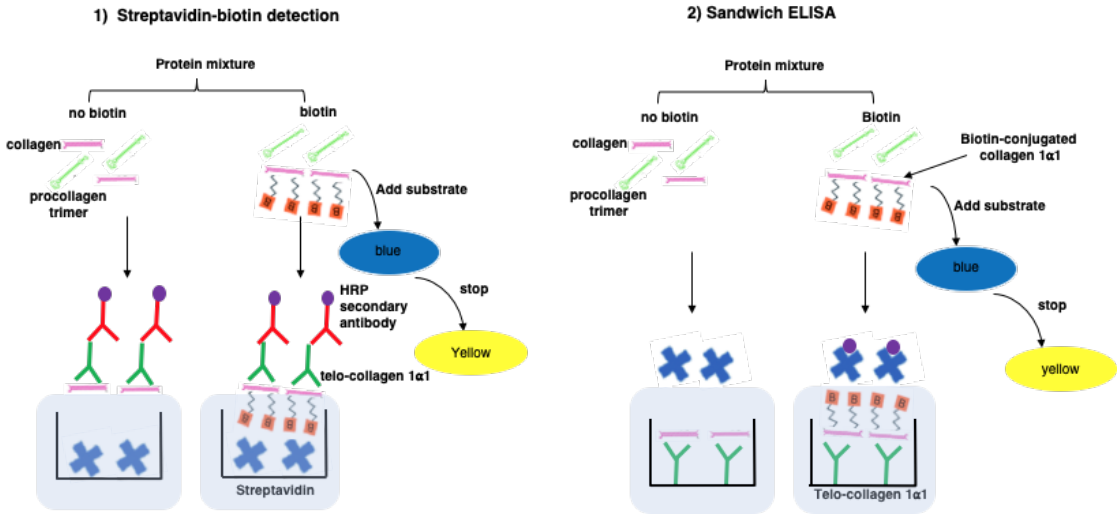

Figure 15: Different ELISA methods. 1) Streptavidin-biotin detection using streptavidin that binds biotinylated proteins, including collagen, and 2) Sandwich ELISA using capture and detection antibodies that both bind collagen.

In the first method, biotinylated proteins, including collagen, labeled as described above in section 3.7, are bound to streptavidin-coated plate. After probing with a primary antibody directed against the epitope of choice, a secondary antibody conjugated to an enzyme (HRP), which is able to catalyze a substrate and change its color, is added to the experiment. The colorimetric reaction can be measured by a spectrophotometer, and the concentration of the protein is determined. In sandwich ELISA, a capture antibody (telo-collagen 1α1), which is bound to the bottom of the plate, and a detection antibody (biotin-conjugated collagen 1α1), both bind collagen forming a sandwich. Sandwich ELISA is a more sensitive method to detect and quantify mature collagen than the WB and also required much less material; however, high sensitivity and specificity depend on the antibodies. Thus, ELISA and WB are both used to complement each other.

3.10 Immunofluorescence

Immunofluorescence is a common technique, based on the use of fluorescent-labeled antibodies and dyes, that allows the detection and visualization of the location and distribution of specific proteins in a sample, most commonly cultured cells or sections of tissues. The fluorescence can be visualized using fluorescence microscopy. Cells were fixed, blocked and permeabilized with an appropriate buffer. Cells were incubated with primary antibodies at 4°C overnight, followed by washes, and then incubated with secondary antibodies conjugated with fluorophores, also followed by washes. Nuclei were counterstained with a dye to stain DNA, and images were acquired by confocal microscopy.

3.11 Confocal microscopy

Confocal microscopy is an optical imaging technique that uses a laser light focused on a single point of the sample and a pinhole in front of the detector to remove out-of-focus noise. Only the light from fluorescence of the focal plane can be detected, and the thickness of the optical sections is determined by the pinhole.

Briefly, a laser is used to provide the excitation light, and using a dichromatic mirror the laser scans across the sample. The dye in the sample fluoresces and emits light. The emitted light passes through the dichromatic mirror and is focused at the pinhole, and is measured by a detector, for example a photomultiplier tube. The detector is connected to a computer that

acquires the image of the sample. The advantages of confocal microscopy over traditional fluorescence microscopy are to reduce the background from the focal plane, acquire serial sections from thick samples, and making high-quality images.

In our study, this technique was used to obtain high-resolution images of cells stained with fluorescent-labeled antibodies and dyes, allowing us to visualize the cells in detail for a better quantification of their phenotypic changes by measuring many cellular parameters, including intensity of the staining, shape, morphology, and texture.

3.12 High-content imaging analysis

High-content imaging analysis is a technology that combines cell-based assays, the use of a confocal microscope, and automated image analysis. It allows automated analysis of multiple images of cells, using fluorescent markers and dyes, to profile similarities and differences among samples based on specific features that can be tracked and analyzed. It is often used for high-throughput drug screening.

In our study, high-content imaging analysis was used to detect and quantify fibrotic phenotypes in an *in vitro* cell culture system. Cells were plated in multi-well plates, treated with the compounds to be tested, stained, fixed, and then imaged by confocal microscopy. Next, an automated image analysis software identifies single cells and measures more than 200 features, such as morphology, size, shape, intensity, and texture. These parameters served as a basis to train a linear classifier that then classified cells into a fibrotic or non-fibrotic phenotype. This methodology represents an attractive complementary analysis to biochemical assays, such as ELISA and WB, and provides more information through multiparametric read-outs at the single cell level.

3.13 Single-cell RNA sequencing

Single-cell RNA sequencing (scRNA-seq) is a technique to study gene expression profiles in individual cells. The basic scRNA-seq protocol consists of dissociation of tissue into a single cell suspension, cell lysis and RNA isolation, reverse transcription to cDNA, PCR amplification, library generation and sequencing (Figure 16).

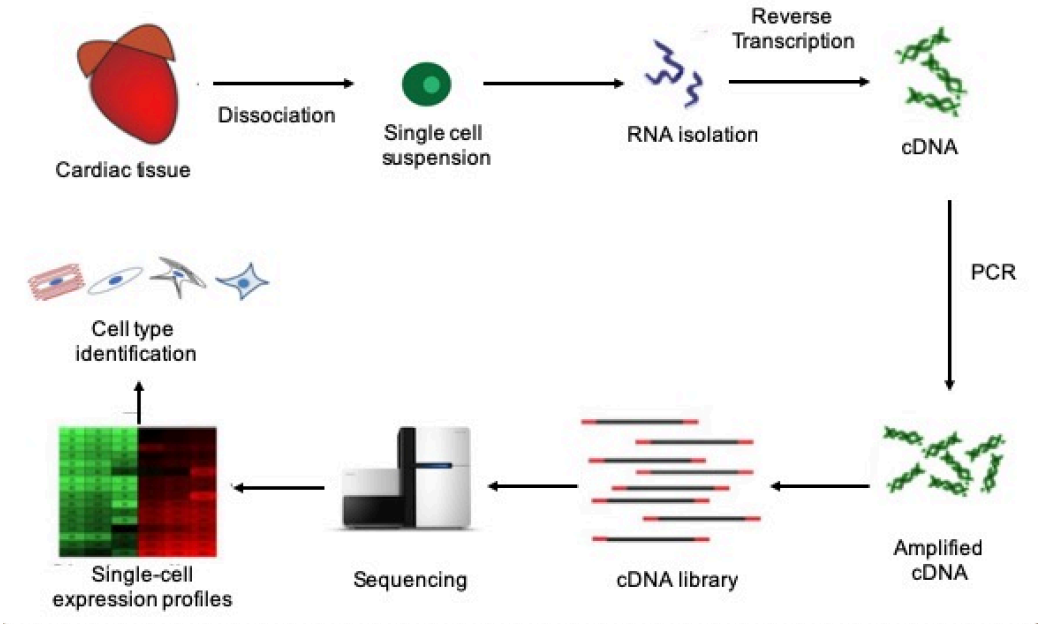

Figure 16: Schematic workflow of the single-cell RNA sequencing on cardiac tissue.

This method is able to quantify the relative abundance of mRNA in a cell, thereby providing a transcriptional profile of individual cells. Computational analysis of the different transcriptional profiles allows the identification of different cell types within a cell population, that subsequently can be further phenotyped using histoanatomical techniques such as immunofluorescence or RNA *in situ* hybridization.

In our study, the scRNA-seq technique allowed us to identify different cell populations in the developing mouse heart to gain a deeper understanding of the mesenchymal cell lineages. We used this method to define the transcriptional profiles for each of the cell types, such as fibroblasts, smooth muscle cells, and pericytes at different developmental stages.

3.14 Cell encapsulation

Cell encapsulation is a technique that consists of the immobilization of cells within a semipermeable polymeric membrane that permits the bidirectional diffusion of molecules, nutrients, oxygen, growth factors and waste products. At the same time, it prevents immune cells and antibodies from passing through the membrane to destroy the encapsulated cells. Cell encapsulation has been used for the delivery of therapeutic molecules in many clinical applications, such as treatment of diabetes[85], cancer[86], and heart disease[87]. There are many

methods to encapsulate cells in microparticles, including the use of polymer membranes made of either a liquid or gel core enveloped by a gel shell structure to form droplets containing live cells[88,89]. However, current methods require step-by-step synthesis, where the core is synthetized in a first polymerization step followed by a second polymerization of the shell[90]. Although all these techniques have been improved, there are many disadvantages related to mechanical instability, lack of permeability, size of particles and cell viability.

In our study, we used a novel approach for cell encapsulation in microparticles made of a gel core and a solid shell structure with a new method of synthesis easy to control. This approach is able to protect the encapsulated cells from the immune reaction, and, at the same time, allows the delivery of secreted therapeutic molecules.

3.15 Ethical considerations

In these studies, primary human and mouse cardiac fibroblasts were used. Human cells were obtained in an anonymized form from commercial providers that have ethical permission from the relevant regulatory board and informed consent from patients. All procedures in animals were performed in accordance with the guidelines and recommendations of animal protection legislation and were approved by the local ethical committee.

4 RESULTS AND DISCUSSION

4.1 Study I

In this study, we defined the transcriptional profiles of cardiac cells of the mesenchymal lineage in the developing mouse heart using scRNA-seq with the goal to better understand the molecular mechanisms controlling their differentiation. We isolated hearts from neonatal mice and sorted individual cells that were analyzed using scRNA-seq. We identified distinct populations of cells in the neonatal heart that clustered together based on differential gene expression, and these putative cell types were further characterized by immunostainings. We observed that mesenchymal cells could be separated into distinct sub-clusters corresponding to vSMCs, pericytes, epicardial cells, and two different groups of CFs. We then performed scRNA-seq to analyze individual cells from mouse embryos of different developmental stages. At embryonic day 9.5 (E9.5), we observed that the PEO and cells undergoing EndMT that will give rise to the mesenchymal cell populations of the heart clustered separately, and cells of the PEO were divided into two classes with one of them corresponding to cells starting to acquire features of epicardium. At E13.5, we found that cells generally clustered close together, indicating a more homogeneous transcriptional profile, and thereafter diverged to cell types easily identifiable in the neonatal mouse, with cells from E15.5 exhibiting an intermediate profile. These results showed that there is a convergence in transcriptional profile from E9.5 to E13.5, followed by divergence to distinct cell types at the neonatal stage. This is in line with a model where progenitor cells that are derived from different cellular sources differentiate into distinct cellular subtypes through a common progenitor cell during cardiac development.

To gain further insights the mechanisms driving cellular diversification in the mesenchymal lineage, we performed clustering analyses on cells of each developmental stage using only the differential expression of genes encoding transcription factors. The rationale for this was that transcription factors directly control gene expression and have been shown to be crucial for cell fate acquisition in many different organs and, in some notable cases of cellular reprogramming, expression of a small set of transcription factors even can convert one cell type to another. This re-clustering resulted in clear separation of cells at E9.5, with two clusters corresponding to PEO and EndMT cells. At E13.5, we observed a more limited

separation of clusters. Surprisingly, as mesenchymal progenitors underwent differentiation to CFs, vSMCs, and pericytes, clustering after differential transcription factor expression did not result in distinct clusters of cells. In contrast, clustering based on differential gene expression of transcripts encoding extracellular proteins and their receptors resulted in poor clustering at E9.5, but clustering into distinct subpopulations from E13.5 onwards. These data indicated that differential transcription factor expression is not the main driver of cellular heterogeneity in the mesenchymal lineage, and no distinct "transcription factor code" described in many other cell types could be identified. Rather, cellular diversification is at least in part under the control of external factors, such as secreted paracrine factors and ECM molecules.

In conclusion, we identified different subsets of CFs in the heart and we also defined molecular markers for fibroblasts, vSMCs, and pericytes. These results supported a model where different subpopulations of precursor cells are patterned by transcription factor expression that converge to form mesenchymal progenitor cells. However, as cells differentiated to distinct cell types, this patterning gradually disappeared and only relatively subtle differences in transcription factor expression was seen in cells of the mesenchymal lineage at the neonatal stage. Extracellularly secreted paracrine factors and ECM molecules seemed to play a key role in the differentiation of mesenchymal progenitor cells and maturation to fibroblasts, vSMCs, and pericytes.

4.2 Study II

Direct reprogramming of CFs can be achieved by forced expression of specific cardiac transcription factors or by miRNAs regulating gene expression in the heart. However, current methods require the use of viral transduction and integration into the genome, which compromise their use in a clinical setting. Other strategies include the use of small molecules that are able to induce chemical reprogramming by modifying gene expression in absence of transgenes.

We presented a novel approach to reprogram CFs into CPCs using a single chemical compound inhibiting the TGF-β signaling pathway under hypoxia, avoiding genetic manipulation of the cells and risk of tumorigenesis. This chemically-induced protocol generated two progenitor populations with different potency: an initial population that was able to differentiate spontaneously into CMs and ECs, and a second population that was more lineage restricted and gave rise to myoFBs and SMCs.

Primary mouse CFs were isolated from hearts of transgenic mice and cells were cultured in reprogramming conditions under hypoxia and using a chemical compound to induce *in vitro* reprogramming. They formed colonies of CPCs that spontaneously differentiated into CM and EC lineages with low frequency. CPCs were then expanded and could thereafter be differentiated towards the SMC lineage, either partially to myoFBs or directly to SMCs.

We next investigated the mechanisms involved in cardiac reprogramming using CRISPR-Cas9 technology to identify key factors that regulate the conversion of CFs into CPCs. Using a genome-wide CRISPR-KO screen, we generated a mutant CFs that was then subjected to reprogramming. Given that Nkx2.5 is upregulated during reprogramming, we used Nkx2.5 as a marker to distinguish between reprogrammed and non-reprogrammed cells. We sorted the cells and extracted genomic DNA and then obtained gRNAs. To evaluate whether individual gRNAs were enriched or depleted, we used a computational tool that assigns a score for each gene. A positive score indicates that the loss of a particular gene facilitates reprogramming, while a negative identifies genes that are positive regulators of the reprogramming process. In this way, we identified candidate genes, such as Dmap1, Ryok2, and Ebi3, that did not encode for structural proteins. Among these, the gene encoding Dmap1, a component of the DNA demethylation machinery, was found to be a key factor regulating reprogramming. In fact, loss of Dmap1 enhanced Nkx2.5 expression in CFs, thereby increasing reprogramming to progenitor cells.

To further explore the mechanism of reprogramming, we analyzed the methylation status of the Nkx2.5 promoter and observed that loss of Dmap1 decreased Nkx2.5 promoter methylation. These data suggested that Nkx2.5 promoter methylation is a key mechanism by which CF properties are controlled. We next demonstrated that loss of Dmap1 also influences smooth muscle (SM) differentiation. We found that expression of SMC markers was decreased in absence of Dmap1. In fact, when we introduced a cDNA encoding Cas9-resistant Dmap1 into the cells, we found a suppression of CPC markers, such as Nkx2.5, while differentiated cells showed a partial expression of SMC markers, such as α-SMA and Cnn1. We observed differences in cell morphology with Dmap1-deficient cells acquiring a more epithelial phenotype. Therefore, we analyzed the expression of epithelial markers, such as E-cadherin and Snai1. We found that E-cadherin expression was increased in absence of Dmap1, suggesting that Dmap1 blocked E-cadherin expression, which was necessary for full differentiation potential of reprogrammed cells.

In conclusion, we showed that mouse CFs can be reprogrammed into CPCs under hypoxia using a single chemical compound that inhibited the TGF-β pathway. These reprogrammed cells spontaneously differentiated into an initial progenitor population that gave rise to CM and EC lineages, but also to another progenitor population that could be differentiated into myoFBs and SMCs[91] (Figure 17).

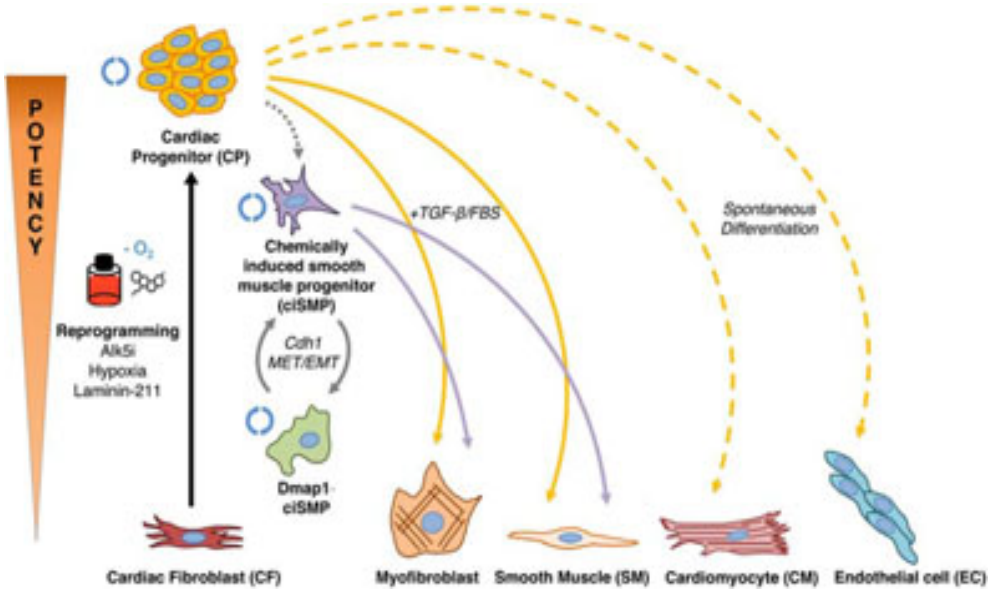

Figure 17: Chemically-induced reprogramming using TGF-β inhibition and hypoxia. Taken from Yu et al. *Stem Cell* 2019.

4.3 Study III

Currently, there are no therapeutic approaches available that specifically target cardiac fibrosis. This is likely due to a limited understanding of the pathological mechanisms of cardiac fibrosis and the lack of predictive *in vitro* models for high-throughput screening for identification of compounds with anti-fibrotic activity. Therefore, new therapeutic strategies need to be developed for treatment and prevention of cardiac fibrosis.

In this study**,** we sought to establish an *in vitro* cell culture system that supported extracellular deposition of mature collagen from primary human CFs. We therefore examined ways to induce processing and maturation of procollagen *in vitro.* Western blotting revealed that macromolecular crowding through treatment with DxS resulted in processing of procollagen. Using a multifaceted approach, employing several independent methods, including western blotting with an antibody specific to telo-collagen 1α1, a pepsin digestion assay coupled with silver staining and mass spectrometry, and scanning electron microscopy (SEM), we provided conclusive evidence for deposition of significantly increased amounts of mature collagen in cultures treated with TGF-β and DxS compared to unstimulated controls.

The *in vitro* cardiac fibrosis assay was successfully adapted to a microwell format and transferred to a robotic cell culture system suitable for high-throughput screening (HTS) with automated cell seeding, medium change and cell lysis. Guided by the comprehensive biochemical characterization of the cultures described above, we developed a high-content imaging platform for detection and quantification of fibroblast activation and collagen deposition, i.e. " *in vitro* cardiac fibrosis". High-content imaging analysis revealed phenotypic changes based on more than 200 cellular features, such as morphology, intensity, and texture of telo-collagen 1α1 and α-SMA signals. These parameters served as a basis to train a linear classifier that classifies cells into a fibrotic or non-fibrotic phenotype. We used unstimulated cells and cells inhibited by SB431542, a TGF-β inhibitor, to define a non-fibrotic phenotype, and stimulated cells for a fibrotic phenotype.

In a proof-of-principle experiment to verify the usefulness of this experimental platform to identify chemical compounds with anti-fibrotic activity, we screened 23 compounds that were previously reported to have anti-fibrotic effects *in vivo* for their effect in our assay system. We selected compounds targeting a diverse set of molecules, both proteins related to the TGF-β signaling pathway as well as proteins not known to be directly associated with this

pathway, and compounds with a mechanistically unclear anti-fibrotic activity. All tested compounds are reported in Table 1.

Matrix remodelling

	Compound	Mode of Action	Activity
1	MG101	CAPNS1/Calpain inhibition	N
2	Pirfenidone	Inhibition of Collagen expression	N
3	UA60	FAP-α inhibition	N
4	RS130830	MMP inhibition	N

TGF-β pathway

	Compound	Mode of Action	Activity
1	SB505124	TGF-β antagonist	Y
2	RepSox	ALK5 inhibition	Y
3	SB431542	ALK5, ALK4 and ALK7 inhibition	Y
4	(5Z)-7-Oxozeanol	TAK-1 inhibition	Y
5	Y27632	ROCK1 and ROCK2 inhibition	Y/N
6	LY294002	PI3K inhibition	Y/N
7	Losmapimod	p38 MAPK inhibition	N

Other

	Compound	Mode of Action	Activity
1	ESI09	EPAC1 and EPAC2 inhibitor	Y
2	Baricitinib	JAK1 and JAK2 inhibition	Y
3	PD156707	Endothelin-1 antagonist	Y
4	AZ3312	HDAC1 inhibition and AR antagonist	Y
5	Takeda139d	GRK2 inhibitor	Y
6	JQ1	BRD1–4 inhibition	Y
7	Gallein	Gβγ–GRK inhibition	Y/N
8	ML290	RXFP1 allosteric partial agonist	Y/N
9	8-pCPT	EPAC1 activator	N
10	hFGF21	FGF21 receptor activation	N
11	RLN2	RXFP1 agonist	N
12	Irbesartan	Angiotensin II receptorblocker	N

Table 1: Cardiac fibrosis compounds.

By high-content imaging analysis, we found that 14 compounds were effective (green) or weakly effective (yellow) in reducing "*in vitro* cardiac fibrosis", whereas the other compounds did not exhibit any effect above threshold levels (red) (Figure 18).

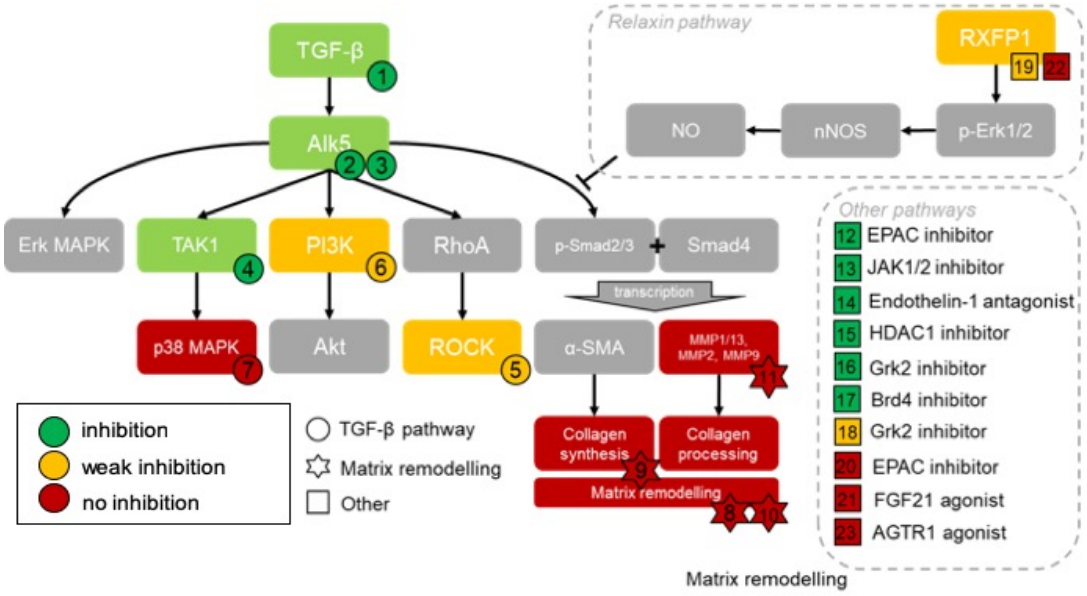

Figure 18: Cardiac fibrosis pathway map.

Both compounds targeting the TGF-β signaling pathway as well as compounds targeting other proteins were reported as conferring anti-fibrotic effects. To verify the results of the high-content imaging assay, sandwich ELISA and WB were performed to assay levels of telo-collagen 1α1 after chemical compound treatment. We found a reduction of telo-collagen 1α1 expression in cells treated with several of the 14 compounds identified as hits in the high-content imaging analysis. However, not all compounds showed a strong reduction in the biochemical analyses. There could be several explanations for this. High-content imaging gives a multi-parametric read-out, providing information on parameters, such as protein localization and distribution, texture, and cell morphology. In contrast, ELISA and WB both are focusing on one specific molecular read-out, in this case telo-collagen 1α1 levels. Thus, it is conceivable that a compound could inhibit one aspect of the fibrotic phenotype, but it has a comparably smaller effect on collagen deposition. Another potential explanation could be due to the lower throughput of biochemical assays. ELISA and WB were run at a single concentration for each compound, while the high-content imaging assay allowed us to run compounds in full dose-response at different concentrations, making it easier to detect anti-fibrotic effects. The high-content imaging assay has several advantages, *i.e.* 1) screening in high-throughput; 2) analyses in microwell format requiring fewer cells and minimizing the amounts of reagents; and 3) quantification of multi-parametric read-outs on single cell basis.

Thus, the *in vitro* cardiac fibrosis assay coupled with high-content imaging analysis is ideal for large-scale screening and machine learning-based drug discovery.

In conclusion, we established an *in vitro* cardiac fibrosis assay that resulted in the extracellular deposition of mature collagen. In a proof-of-principle experiment, the *in vitro* cardiac fibrosis assay identified compounds with anti-fibrotic effects from different cellular pathways. Finally, the *in vitro* cardiac fibrosis assay represents a sufficiently sensitive method for high-throughput screening of anti-fibrotic compounds with the goal of developing new therapeutic strategies for HF.

4.4 Study IV

This work is focused on cell encapsulation for therapeutic use. Here, the concept is that cells secreting specific molecules, encapsulated in microparticles, are introduced in the human body for therapeutic effects. Previous methods for cell encapsulation revealed unresolved challenges related to control of permeability and morphology of the particles, mechanical stability, and immunogenicity. The novelty of our approach is that we used new materials with stable and tunable properties and a new synthesis method consisting of a single UV polymerization step in a microfluid system.

Human cells were encapsulated in microparticles made of a synthetic polyethylene glycol diacrylate (PEGDA) hydrogel core and a solid, but porous, shell of off-stoichiometric thiol-ene polymer (OSTE). PEGDA is a polymer of ethylene oxide and water that polymerizes by exposure to UV light. It has been widely used and characterized for cell encapsulation due to its properties: excellent biocompatibility, stability and permeability, low immunogenicity, easy surface modification, and photopolymerization. OSTE is a polymer composed of a mixture of thiol and ene monomers. OSTE has many good mechanical properties, including high biocompatibility, easy surface modification, and easy polymerization by UV light. These characteristics make these materials suitable for drug delivery system and other clinical applications.

We chose PEGDA hydrogel for the core and a solid OSTE for the shell to produce microparticles with new polymer materials that allowed more permeability and less biodegradation, minimizing the host immune response. We generated droplets containing cells, PEGDA monomers, and UV initiator in a microfluidic system with a continuous flow

of OSTE monomers. After a rapid UV exposure, PEGDA and OSTE monomers were polymerized simultaneously creating particles made of a gel core and solid shell structure in which cells were encapsulated. This resulted in core-shell particles containing live cells. We comprehensively characterized the particles' morphology, providing information on parameters including size, shell thickness and permeability, and showed that they can be easily controlled by varying the UV exposure and flow rate.

Shell permeability was assayed using molecules of different molecular weight. We showed that particles were permeable to low molecular weight molecules (<180 Da), such as water and glucose, but impermeable to higher molecular weight molecules (>420 Da) such as rhodamine B. These results demonstrated that small molecules can diffuse through the shell, whereas particles were impermeable to larger molecules, such as secreted proteins of the immune system, a potentially important aspect for therapeutic purposes.

We next measured cell viability in these particles at different time intervals using cell viability dyes, such as Calcein AM for live cells and ethidium homodimer-1 (EthD-1) for dead cells. We found that the encapsulation process did not induce cell death and cells had >90% viability during 3 h. Although cell viability of encapsulated cells was high, this result required more optimization for longer time to be clinically relevant.

In summary, we demonstrated that our novel approach for cell encapsulation is a promising method for delivery of cells secreting therapeutically relevant molecules, and it could potentially be used as a drug delivery system for treatment of many different forms of pathology including heart disease.

5 CONCLUSION AND FUTURE PERSPECTIVES

The studies presented in this thesis provide some new insights into CF biology. However, there are many unanswered questions, and in this section, I integrate the findings described here with current knowledge and point to future challenges in the field.

In **study I**, we defined the transcriptional profiles of differentiating cells of the mesenchymal lineage in the developing mouse heart using scRNA-seq. We found that there are limited differences in transcription factor expression between cell types within this family and no distinct "transcription factor code" that can be used to tell them apart. This implies that extracellular factors are important for cellular diversification and fate acquisition of mature mesenchymal cells from a common progenitor cell, and may explain the plasticity of fibroblasts. It will be interesting to further examine mediators of intercellular communication within the heart and how they control cellular differentiation and response to injury within the mesenchymal lineage. It will also be of interest to further characterize the different subtypes of CFs identified in the mouse heart. Does the heterogenicity of these subsets of CFs reflect the functional diversity of CFs in the heart? What are the phenotypic characteristics, fate and identity of these CF subpopulations? Answering these questions is important in order to provide a better understanding of CF biology.

In **study II**, we developed a reprogramming protocol using a single chemical compound to generate progenitor populations capable of differentiation towards other cardiac cells without the use of transgene delivery. However, further investigations will be required to improve this approach, for example, to derive cardiac cells that are fully functional to make them more suitable for therapeutic use. It will also be important to determine whether these induced progenitor cells preserve their differentiation potency while they still retain self-renewal capacity. The fact that they appear to lose their capacity to differentiate into CMs during expansion suggest that potency is diminished as the cellular phenotype stabilizes.

Direct reprogramming of CFs into CMs represents a novel approach to replace dead cells after a myocardial infarction. However, this new therapeutic approach is currently hampered by several factors. Firstly, the efficiency of reprogramming is very low, with only a small proportion of the reprogrammed cells exhibiting features of CMs, and it will need to be improved to have any chance of clinically meaningful results. Secondly, current reprogramming strategies rely on the transduction of CFs with viruses, resulting in integration of their genetic material into the genome of the transduced cells, which is problematic to transfer to the clinical setting. Thirdly, the reprogramming process is not well understood, particularly with regards to the receiving fibroblast population. Are all CFs possible to reprogram, or are only specific subsets prone to respond to the reprogramming cocktail? Is it even possible to reprogram myoFBs, which is probably the most relevant cell type after injury? These are important issues that need to be clarified before reprogramming of CFs into CMs can be a viable option for treating patients with heart disease.

In **study III**, we presented an experimental platform for *in vitro* modeling of cardiac fibrosis to identify anti-fibrotic compounds. This novel assay system is useful to screen a large amount of compounds using high-content imaging analysis. Compared to other traditional approaches, high-content imaging is a multiparametric method, thereby providing a more sensitive assay to identify compounds with anti-fibrotic activity. However, when testing 23 chemical compounds with known anti-fibrotic effects, the platform did not score all these compounds as inhibitory with regards to fibrosis. One possible reason could be that the *in vitro* phenotypic assay might not be able to fully reflect *in vivo* conditions of cardiac fibrosis. For this purpose, future development of a 3D model, such as 3D cardiac organoids, could better mimic anti-fibrotic effects *in vivo*. Finally, from a drug discovery perspective, our novel experimental platform would be part of a multi-assay pipeline to find novel anti-fibrotic drugs. Interesting hits from the *in vitro* fibrosis assay would need to be validated in more complex systems, and here it would also be interesting to use 3D organoids for screening of compounds identified in our 2D system. In this way, the most promising candidates can be identified and selected for further characterization in animal models of cardiac fibrosis, which is appealing from both ethical and economical perspectives.

In **study IV**, the cell encapsulation method we developed offers a promising approach to encapsulate cells in terms of stability, viability, and immunogenicity. Further studies will be required to translate this method into clinical therapies, where a large number of cells and long-term viability are clinically relevant. On the one hand, a possible solution will be to use genetically engineered cells that would allow long-term viability to test pharmaceutical activity of the particles, and on the other, increase the particle synthesis rate or the number of encapsulated cells. For this purpose, a sorting system could help to sort out particles with no or low number of cells to ensure a more efficient method for cell encapsulation.

The epidemiology of cardiac disease has changed significantly during the last decades. Development of effective therapeutic drugs targeting major risk factors for cardiovascular disorders, such as hyperlipidemia and hypertension, have significantly lowered the burden of cardiovascular disease in society. However, cardiovascular disease is still one of the leading causes of death globally, and even though improved treatments of the acute phase of myocardial infarction have reduced the mortality of this condition, it has resulted in more patients surviving cardiac events with a permanently impaired cardiac function. Thus, despite these impressive achievements for modern medicine, the incidence of HF is increasing drastically, and there are currently no treatments available that promote formation of new CMs or reduce cardiac fibrosis. It is clear that new therapeutic strategies will be needed to specifically achieve these objectives.

Regarding cardiac fibrosis, further investigation into the pathogenesis of this condition will be required to identify novel therapeutic approaches. Although some molecules mediating the response to cardiac injury, including the TGF-β signaling pathway and inflammatory cytokines, have been identified, they are rather poor targets for pharmaceutical intervention due to their widespread expression throughout the entire organism[92]. Thus, by delineating molecules and pathways functioning as sensors of cardiac injury and effectors of the response to injury, novel putative targets with a higher degree of cardiac-specific expression may be identified. In parallel to this line of research, unbiased strategies to find novel anti-fibrotic compounds need to be used. This line of work, largely relying on large-scale screening of chemical compounds and cDNA/miRNA molecules, has been hampered by a lack of reliable *in vitro* models for cardiac fibrosis. Therefore, the development of *in vitro* models that better

mimic the key aspects of cardiac fibrosis will be important for identification of anti-fibrotic compounds for therapeutic purposes.

Work in both mice and humans have shown that there is a low but measurable and statistically significant turnover of cardiomyocytes in the heart[93,94]. Formation of new CMs after cardiac injury could conceptually occur through three different mechanisms: 1) cell division, where existing CMs could re-enter the cell cycle and divide, 2) progenitor differentiation, where endogenous CPCs could differentiate into new CMs, and 3) trans-differentiation, where a non-cardiomyocyte, non-cardiac progenitor cell could change its fate and adopt features of CMs. Considerable work has been focused on each of these three points. It has recently been shown that hypoxia can promote a subgroup of CMs in rodents to dedifferentiate and re-enter the cell cycle[95]. Many laboratories have tried to promote cardiomyocyte cell division through forced expression of specific gene products, including components of the cell cycle[96] and the YAP pathway[97]. Although some experiments have provided insights into basic biology of CMs, it is difficult to envision that any of these techniques could be translated into clinical treatments[98].

Even more work has been focused on isolating endogenous cardiac progenitor cells in the adult mammalian heart. Several different cell surface markers have been used for isolation of cells with these properties[99], but there is no consensus in the field as to which cells actually harbor any regenerative potential. Rather, recent studies in mouse using genetic lineage tracing techniques have refuted earlier studies claiming the isolation of endogenous CPCs in the adult mammalian heart[100,101]. So far, there is no agreement regarding which cell types fulfill the criteria of endogenous adult CPCs, the molecular mechanisms controlling their differentiation, or even if they exist at all[102]. Finally, trans-differentiation is a more recent concept, where non-cardiomyocytes in the heart are converted into CMs. After the initial demonstration of fibroblast reprogramming through forced expression of exogenously delivered MyoD[103], the reprogramming to CMs was long considered impossible to achieve, but, after Yamanaka and co-workers demonstration of reprogramming to pluripotency, the floodgates for trans-differentiation through reprogramming opened. After successful adoption for many types of cells, including pancreatic β-cells and neurons, the Srivastava and Olson groups were able to convert fibroblasts into functional cardiomyocyte-like cells both *in vitro* and *in vivo*.

Work in this area is fascinating because cells exhibit a higher degree of plasticity than traditionally believed. However, there are several limitations that preclude this concept from being close to clinical use. For example, the obtained cells, so called "induced cardiomyocyte-like cells", are heterogeneous based on gene expression and contractile properties[104]. Furthermore, the methods currently used for inducing reprogramming are not efficient *in vivo* and not safe for clinical practice because of their potential complications, such as tumor formation[105]. In summary, considerable work is required before any method for generating new CMs *in vivo* can be translated into clinical applications. One interesting twist on cellular reprogramming was reported from the Chien group. Here, a local application of modified mRNA encoding VEGF (vascular endothelial growth factor) in the heart, following a myocardial infarction, resulted in an increase in epicardial-derived endothelial cells at the expense of myoFBs, and it was also shown that this was beneficial for the animal. Such an approach would nullify the many arguments against therapeutic cellular reprogramming since it does not rely on viral transduction or genetic modification of the host cells[106].

In conclusion, the work presented in this thesis provides some new insights into CFs and how they potentially can be targeted or used for therapeutic purposes. I believe this is a promising field of research, but it is clear that a deeper understanding of the basic biology of CFs as well as a detailed comprehension of the molecular mechanisms of cardiac diseases are necessary before therapeutic strategies targeting CFs could be a clinical reality.

6 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people who have helped and motivated me during my PhD studies and make possible to achieve this important goal in my life.

I would like to thank my supervisor **Emil Hansson**, who believed in me, for giving me the opportunity to be your first PhD student at Karolinska Institutet and sharing with me your scientific knowledge and practical guidance, and also teaching me the way of thinking independently in science.

I would thank my co-supervisors: **Andras Simon** for introducing to his group and sharing his knowledge about salamanders at the early period of my PhD studies, and **Kenneth Chien** for introducing me to Emil and I started to work in his lab.

I would thank my mentor **Rainer Heuchel**, for giving me support and your helpful advice, and for always being a caring person, who listened to my complaints and encouraged me to not give up during all these years of my PhD studies.

Thanks to all the members of the group: **Stefanos Leptidis, Alessandra Rigamonti, Nelly Rahkonen, Aldo Moggio**, for sharing scientific knowledge at the meetings and practical skills in lab techniques.

Thanks to my collaborators at AstraZeneca in Gothenburg, especially **Erik Müllers** and **Märta Jansson**, for sharing of reagents and protocols, and having interesting conversations about research projects in a constructive way.

Thanks to the director of the Integrated Cardio Metabolic Centre (ICMC) **Christer Betsholtz**, and all the people at the department for sharing lab space and equipment, providing me a good working environment.

Thanks to my friends at KI, **Parisa** and **Ani**, who encouraged and advised me, sharing with me good and bad times through my PhD life.

Thanks to all my family and friends who are spread all over the world in Sweden, Italy and the USA, for their love and encouragement, especially my dearest friends **Gaetano** and **Rozeta**, who helped me to overcome the difficult moments in my life.

Special thanks to **Francesco**, who taught me to be patient and persistent, guiding me in my personal, professional and spiritual growth.

As last but not least, big thanks to the most important people in my life, my parents **Saro** and **Anna** for always being there and for their unconditional love and support, my sister **Gilda**, who taught me to be strong and wise, and my beloved boyfriend **Jean-Francois** for his unwavering love, understanding, and patience.

I always thought that my PhD was the end of a long journey started many years ago, but only now I have realized that it is just the beginning of my future career in research.
Pursuing a PhD was a very challenging experience, but also very hard and full of obstacles during the path. I have seen my PhD studies as a personal and professional growth for me, however, this would never have been possible without all of you!

I am very grateful to be surrounded by people who loved and supported me, and I want to share with them my happiness in this special moment. I feel accomplished to conclude this chapter of my life and joyful not to be alone but in two!!!

Thanks to everybody!
Grazie a tutti!

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