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Modeling the contribution of cardiac fibroblasts in dilated cardiomyopathy using induced pluripotent stem cells (iPSCs)

Grace R. Mazarura and Terence E. Hébert

Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, H3G 1Y6, Canada

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Running Title: iPSCs to model fibrosis in heart disease.

*To whom correspondence should be addressed.

Dr. Terence E. Hébert, PhD,

Department of Pharmacology and Therapeutics,

McGill University,

3655 Promenade Sir-William-Osler, Room 1325

Montréal, Québec, H3G 1Y6, Canada

Tel: (514) 398-1398

E-mail: terence.hebert@mcgill.ca

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Abbreviations

Abbreviation	Definition
2D	2-dimensional
3D	3-dimensional
α-SMA	α-smooth muscle actin
ACM	Arrhythmogenic cardiomyopathy
Ang II	Angiotensin II
AT1R	Angiotensin II type I receptor
CAR	chimeric antigen receptors
CDM	Cell-derived matrix
CF	Cardiac Fibroblast
CHIR99021	Chemical compound that inhibits GSK3
CM	Cardiomyocyte
c-myc	MYC Proto-Oncogene, BHLH Transcription Factor
COL1A1	Gene encoding alpha-1 type I collagen
CTGF	Connective tissue growth factor
cTNC	cardiac troponin C
DAMP	damage-associated molecular pattern
DCM	Dilated cardiomyopathy
DMD	Duchenne Muscular Dystrophy
ECM	Extracellular matrix
EHT	Engineered heart tissue
EMT	Epithelial-mesenchymal Transition
EndoMT	Endothelial-mesenchymal Transition
Epi-CFs	epicardial-derived cardiac fibroblasts
EC	Epicardial cells
ERK1/2	Extracellular signal regulated kinase 1/2
FAP	gene encoding fibroblast activation protein alpha respectively
FGF	Fibroblast growth factor
GPCR	G protein-coupled receptor
GTP	Guanosine-5’-triphosphate
HCM	hypertrophic cardiomyopathy
HEK 293	Human embryonic kidney 293 cell line
IL-6	interleukin-6
IL-16	interleukin-16
iPSC	Induced pluripotent stem cell
iPSC-CF	Induced pluripotent stem cell-derived cardiac fibroblast
iPSC-CM	Induced pluripotent stem cell-derived cardiomyocyte

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Klf4	Krüppel-like factor 4
LMNA	Lamin A/C
MAPK	Mitogen-activated protein kinase
MF	Myofibroblast
MMPs	Matrix metalloproteinases
MT	microtissues
Oct-04	Octamer-binding transcription factor 4
PBMCs	Peripheral blood mononuclear cell
PDGF	Platelet-derived growth factor
POSTN	Gene encoding periostin
PPDT	PPARα agonist, palmitate, dexamethasone and T3 hormone
p38	p38 mitogen-activated protein kinases
sc-RNA-seq	single-cell RNA-sequencing
SHF-CF	second heart field-derived cardiac fibroblasts
sn-RNA-seq	single-nucleus RNA-sequencing
Sox2	SRY-Box Transcription Factor 2
TIMPs	Tissue inhibitors of metalloproteinases
TGFβ1	Transforming growth factor-beta 1
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
Wnt	Wingless-related integration site

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Abstract

Fibrosis is implicated in nearly all forms of cardiomyopathy and significantly influences disease severity and outcomes. The primary cell responsible for fibrosis is the cardiac fibroblast, which remains understudied relative to cardiomyocytes in the context of cardiomyopathy. The development of induced pluripotent stem cell-derived cardiac fibroblasts (iPSC-CFs) allows for the modeling of patient-specific disease characteristics and provides a scalable source of fibroblasts. iPSC-CFs are invaluable for understanding molecular pathways that affect disease progression and outcomes. This review explores various aspects of cardiomyopathy, with a focus on dilated cardiomyopathy (DCM), that can be modeled using iPSC-CFs and their application in drug discovery, given the current lack of approved therapies for cardiac fibrosis. We examine how iPSC-CFs can be utilized to study heart development, fibroblast heterogeneity, and activation, with the ultimate goal of developing better therapies for patients with cardiomyopathies.

Significance statement: Here, we explore how iPSC-CFs can be used to study the fibrotic component of DCM. Most research has focused on cardiomyocytes, despite the significant contribution of fibroblasts to disease outcomes. iPSC-CFs serve as a valuable tool to elucidate molecular pathways leading to fibrosis, and paracrine interactions with cardiomyocytes, which are not fully understood. Gaining insights into these events could aid in the development of new therapies and enable the use of patient-derived iPSC-CFs for precision medicine, ultimately improving patient outcomes.

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Introduction

Cardiomyopathies constitute a broad spectrum of diseases of the heart muscle which compromise the heart's ability to efficiently pump oxygenated blood; the term encompasses both acquired and congenital/inherited forms of disease. Such diseases often involve maladaptive remodeling of the heart morphology, leading to changes in structure and function of the heart. These changes can include dilation, hypertrophy, and stiffening of the heart muscle and ultimately result in muscle loss and impaired myocardial function. Consequently, cardiomyopathies can be broadly classified into five categories based on morphological and functional criteria; namely dilated, hypertrophic, arrhythmogenic, restrictive, and unclassified cardiomyopathies. Among these, hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are the most prevalent, with DCM alone accounting for 60% of all cases and more than half of all heart transplants (Khayata et al., 2019; Lee et al., 2017; Teshnisi et al., 2020). Distinct types of cardiomyopathies can have different underlying causes including viral infection, genetic mutations, exposure to certain chemotherapeutics, as well as inflammation-mediated events (Maron et al., 2006). Despite this diversity in etiology, fibrosis emerges as a common predictor of disease outcome (Deirdre et al., 2012; Eijgenraam et al., 2020; Looi et al., 2010; Mandawat et al., 2021; Stephanie et al., 2011; Tevfik et al., 2014). Fibrosis, which is an excessive deposition of extracellular matrix (ECM), contributes to contractile dysfunction, electrical abnormalities, exacerbation of cardiac function, and ultimately heart failure (Aimo et al., 2024; de Bakker et al., 1996; Travers et al., 2016b; Vasquez et al., 2010).

The myocardium is comprised of a heterogeneous population of cells that work in concert to facilitate continuous supply of oxygenated blood to the body. Among these cells are cardiomyocytes, which are the muscle cells that are responsible for the contraction of the heart; non-cardiomyocyte cells including cardiac fibroblasts, which aid in repairing and maintaining the structural integrity of the heart as well as regulating cardiomyocyte function via paracrine

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signaling or direct cell-cell contacts. Additionally, non-cardiomyocytes include pericytes and endothelial cells that are vital for forming blood vessels supplying the heart with blood and maintaining the interior milieu of the myocardium – among other cells (Cui et al., 2019; Tucker et al., 2020). The structural remodeling that takes place in cardiomyopathies typically occurs at the level of the cardiomyocyte (via cardiomyocyte death or altered cardiomyocyte structural/contractile properties) or due to fibrosis (Azevedo et al., 2016). Regarding the latter, fibroblasts are the main contributor to fibrosis (Travers et al., 2016b). Activated cardiac fibroblasts transition into myofibroblasts (MFs) and produce excessive ECM components, contributing to adverse cardiac remodeling in cardiomyopathy (Eijgenraam et al., 2020). However, current therapies mainly focus on cardiomyocytes, with no therapies approved for targeting cardiac fibrosis yet available on the market.

Over the last few years, protocols to faithfully differentiate induced pluripotent stem cells (iPSCs) into different cardiac cell types including atrial and ventricular cardiomyocytes (CMs) (Lian et al., 2013), endothelial cells (Sayed et al., 2020), epicardial cells (ECs) (Bao et al., 2016), smooth muscle cells (Shen et al., 2021), and cardiac fibroblasts (Zhang et al., 2022; Zhang et al., 2019a; Zhang et al., 2019b) have been developed. While strides in mapping the human genome and methods such as whole-genome sequencing and genomic-wide association studies have deepened our understanding of inherited cardiomyopathies, relying on genetic screening alone may not be sufficient to entirely capture disease complexity (Manolio et al., 2009; Walsh et al., 2023). With the advent of iPSCs, we can generate patient-specific cardiac cell-types which can recapitulate disease phenotypes in a dish. iPSC technology has given researchers a platform to distinguish between disease-linked genetic mutations from the “noise” of genetic variation and better understand how genetic variants affect the functions of different cell types in the heart. Moreover, this approach enables us to understand disease

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pathology better and paves the way to patient-specific medicines, personalized drug screening models as well as assessing the effects of different mutations on different cardiac tissues (Caudal et al., 2023). While several reviews have explored the use of iPSC derived cardiomyocytes (iPSC-CMs (Bourque et al., 2022; Brodehl et al., 2019; Karakikes et al., 2015; Li et al., 2022; Paik et al., 2020; Thomas et al., 2022)) in modeling cardiomyopathy, here we describe the particular role of using iPSC-derived cardiac fibroblasts (iPSC-CFs) to better understand fibrosis given its important role in predicting disease outcomes (Looi et al., 2010; Mandawat et al., 2021). The inability to reverse fibrosis remains a major challenge in both standard treatments and novel precision therapies for cardiomyopathy. We will highlight how iPSC-CFs have been harnessed as a tool to understand the role of fibroblasts in cardiomyopathy, and chart future research directions into developing therapies that target fibrosis, as yet no FDA approved drugs exist to treat cardiac fibrosis (see **Figure 1**).

The role of cardiac fibroblasts in health and disease

Cardiac fibroblasts are dynamic cells that can change phenotype in response to environmental cues (Travers et al., 2016b). Cardiomyocytes make up approximately 70-85% of the cardiac volume, as measured using stereological methods, flow cytometry or single cell gene expression (reviewed in (Tang et al., 2009; Trager et al., 2023; Zhou and Pu, 2016)). However, in terms of cell number they are estimated to make up only 25-35% of the cells in the heart. By combining single-cell RNA-seq (sc-RNA-seq) and single-nucleus RNA-seq (sn-RNA-seq) data, ventricular cell composition was shown to be 25.7% cardiomyocytes, 18.3% fibroblasts, 16.6% endothelial cells, 15% mural cells, the remainder consisting of immune cells, endocardial cells, adipocytes and neural cells (Koenig et al., 2022). Cellular composition also varies slightly between atria and ventricles (Litviňuková et al., 2020).

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While cardiomyocytes are responsible for the contraction-relaxation cycle in the heart, fibroblasts are part of the stroma and they provide trophic support to cardiomyocytes and other cell types, as well as maintaining the structural framework of the heart (Furtado et al., 2014; Haniffa et al., 2009). Along with mesenchymal stem cells, fibroblasts and immune cells, the stroma also contains blood vessels and extracellular matrix. The ECM is a complex network which comprises fibrous proteins such as collagen (in its many different forms) and elastin which are necessary for the structure and elasticity of the myocardium; matricellular proteins like periostin and thrombospondins necessary for modulating cellular responses and adhesion (Frangogiannis, 2012). Additionally, it is also comprised of glycoproteins like fibronectin and laminin that mediate anchoring of cells, mechanotransduction in the heart as well as growth factors and metalloproteases (Theocharis et al., 2016). All these serve the common function of maintaining tissue homeostasis. Far from being a passive component in the function of the myocardium, the ECM regulates the abundance of receptors and growth factors available to cells, influences various cell behaviours such as proliferation and cell survival, and plays a critical role in higher-level modalities like tissue plasticity and architecture. For more in-depth review of the ECM the reader is referred to (Frangogiannis, 2012; Yue, 2014).

Apart from structural support and providing nutrients, the stroma can modulate tissue stiffness, provide tensile strength and aid in force transmission in cardiac tissue (Rossi et al., 1998). Signaling cues provided by stroma-parenchymal interactions regulate myocardium function and development. These cues include bidirectional communication via paracrine signaling, for example, growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (Gallini et al., 2016; Tao et al., 2016; Zymek et al., 2006), cytokines (Fix et al., 2011; Palmer et al., 1995), chemokines and micro-RNAs (Bang et al., 2014)). Additionally, biochemical and biophysical signals from the extracellular matrix, along with direct cell-cell interactions between stroma and CMs, play a role in regulating biomechanical

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and electrical signaling. For more comprehensive descriptions of stroma-parenchymal interactions, readers may consult these reviews (Bang et al., 2014; Pellman et al., 2016; Picchio et al., 2022; Walters and Gentleman, 2015).

The cell types responsible for regulating the ECM are the stromal cells (cardiac fibroblasts, mesenchymal stromal cells, endothelial cells, and immune cells). However, the major cell types involved in the synthesis of ECM are fibroblasts (21). One of the strongest determinants of cardiomyopathy severity is the pathological expansion of the extracellular matrix, known as fibrosis, that results in the stiffening of the myocardium (Eijgenraam et al., 2020; Looi et al., 2010). In DCM patients, the ECM has been shown to lose complexity with resulting reduced elasticity in decellularized human ventricular tissue (Perestrelo et al., 2021). As the CF is an important regulator of the ECM, it becomes important to discuss how it modulates ECM in health and disease.

Fibroblast activation in response to injury

In the uninjured heart, CFs mediate homeostatic ECM turnover through secretion of ECM proteins (e.g., collagen, elastin, periostin), growth factors, and critical enzymes such as tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs), responsible for breakdown and buildup of the ECM (Souders et al., 2009). It is important to distinguish between the regular wear and tear in the heart that requires homeostatic ECM turnover and the pathological ECM accumulation that ensues after significant injuries that cause extensive cardiomyocyte death. Following myocardial cell injury, such as that caused by sustained hypertension, myocardial infarction, infection, and toxic insults, there can be a large loss of CMs, leading to the release of damage-associated molecular patterns (DAMPs) by necrotic cardiomyocytes (Zhang et al., 2015). Such DAMPs activate the initial inflammatory

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response whereby resident macrophages and neutrophils release pro-inflammatory chemokines such as interleukin-6 (IL-6), interleukin-16 (IL-16), and tumor necrosis factor α (TNFα) (Daseke et al., 2020; Daseke et al., 2019). Fibroblasts respond to these stimuli by becoming activated and adopting a pro-inflammatory phenotype that contributes to further secretion of inflammatory cytokines that include TNFα (Jacobs et al., 1999) and IL-6 (Wang et al., 2016) at the site of injury. Following this, fibroblasts transition to a proliferative phenotype in the subsequent phase of wound healing. During this phase, they show a decrease in apoptotic genes, such as *CASP3* (the gene encoding caspase 3), along with an increase in angiogenic (e.g., VEGF), proliferative, pro-fibrotic genes, and anti-inflammatory mediators like transforming growth factor β1 (TGFβ1) (Daseke et al., 2020; Mouton et al., 2019; Tille and Pepper, 2002). In the final stage of wound healing, termed maturation, CFs adopt a contractile phenotype which secretes large amounts of ECM proteins to facilitate scar formation (Daseke et al., 2020). Fibroblasts isolated from regions near a myocardial infarction have been shown to exhibit a 2-fold increase in collagen synthesis (Squires et al., 2005). This fibroblast phenotype, characterized by the expression of stress fibers containing α-smooth muscle actin and the secretion of the matricellular protein, periostin, is referred to as the myofibroblast stage, and is associated with pathological fibrosis (Fortier et al., 2021; Frangogiannis, 2021). After this maturation phase, fibroblasts adjust to a new homeostatic state within the myocardium, where the niche of CFs includes a larger number of MFs for the maintenance of scar tissue (Ma et al., 2017).

CMs are terminally differentiated cells that do not normally re-enter the cell cycle, instead, their response to most disease-relevant cues is either to increase rates of contraction, enlarge via hypertrophy or undergo apoptosis (Ahuja et al., 2007). Owing to the lack of regeneration of the myocardium, the heart may compensate for reduced cardiac function through chronic neurohormonal stimulation (Blaxall et al., 2012; Travers et al., 2016a). This increase in neurohormonal stimulation ultimately has deleterious effects on CMs and causes

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fibroblasts to become activated and adopt a MF phenotype thereby leading to pathological expansion of the ECM, termed fibrosis. This dysregulation in ECM increases myocardial stiffness, disrupts electrical coupling between cardiomyocytes in the heart and hampers oxygen and nutrient diffusion into the tissue, leading to cardiomyocyte death (Aimo et al., 2024; Piek et al., 2016; Travers et al., 2016b; Vasquez et al., 2010). These changes lead to remodeling of the heart tissue, which is associated with disease progression and, ultimately, heart failure. Additionally, CFs also respond to mechanical stimuli, and are affected by changes in the stiffness of the myocardium (Braidotti et al., 2024; Stewart and Turner, 2021; Tian and Ren, 2023). For instance, the angiotensin II type-1-receptor (AT1R) is a receptor implicated in cardiac fibroblast activation and its activity is modulated by mechanical strain and substrate stiffness (Niu et al., 2020; Tian and Ren, 2023).

The sources of MFs within the heart remain incompletely understood, and different injuries (e.g., ischemic, hypertensive, diabetic cardiomyopathy, dilated cardiomyopathy) appear to recruit different populations of fibroblasts (33). Sources may include resident cardiac fibroblasts, bone marrow-derived cells (van Amerongen et al., 2008), hematopoietic cells (Bucala et al., 1994), immune cells, pericytes and endothelial cells via endothelial-mesenchymal transition (endoMT). Lineage tracing showed that endocardium-derived fibroblasts are associated with pressure overload-induced heart injury, whereas in ischemic injury, epicardial-derived fibroblasts were a more important driver of fibrosis (Han et al., 2023).

Historically, fibroblasts were primarily seen as reactive modulators or passive responders, reacting to the demands of cardiac muscle which was viewed as the central player in heart failure. Alterations in the extracellular matrix were thought to be secondary to changes in the structure of the heart muscle in evolving disease. However, more recent work has demonstrated that a profibrotic state and stiffening of the myocardium precedes visible indicators of fibrosis or hypertrophic cardiomyopathy as detected by MRI (Ho et al., 2010). This

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was evidenced by an increase in serum C-terminal propeptide of type I procollagen, indicative of elevated myocardial collagen synthesis, in patients with disease-linked genetic variations in the sarcomeric proteins (Ho et al., 2010). Furthermore, elevated mRNA levels of pro-fibrotic proteins such as TGFβ1, fibronectin, and type-1 collagen were observed in mice harboring sarcomeric gene variants linked to HCM, despite the absence of fibrosis or hypertrophy (Kim et al., 2007). Additionally, a recent study showed that myocardial stiffening preceded visible fibrosis in mice with a DCM-linked pathological genetic variation in cardiac troponin gene, where CF number, rather than conversion to myofibroblasts, appeared to be the driver of fibrosis (Bretherton et al., 2023 BioRxiv, https://doi.org/10.1101/2023.01.23.523684). The latter finding goes hand in hand with a study showing that the number of CFs was crucial in modulating the stiffness of engineered heart tissue (Rupert et al., 2020). In this study that used iPSC-CMs and human CFs to construct 3D engineered tissues, it was shown that including 5% of total cell number as CFs was important to improve electrochemical responses and contractile function of engineered cardiac tissues. Conversely, adding 15% CFs (reflecting the concept of the increase in numbers of CFs in a disease context) resulted in tissues with higher spontaneous beating rates (Rupert et al., 2020). Furthermore, in another study, Chaffin et al. used single-cell RNA-seq to show that the number of CFs in the hearts of DCM patients was indeed increased (Chaffin et al., 2022), thus implying that changes in CF number may play a role in disease progression.

In another study, it was found that pathways including mitogen activated protein kinase (MAPK) and ECM synthesis were upregulated in DCM hearts (Koenig et al., 2022).. The authors identified eight different populations of CFs in the heart and showed that these populations were altered in abundance in diseased hearts. More specifically, they showed that fibroblast populations which expressed *POSTN* (gene encoding periostin) increased in DCM. Moreover, the population of fibroblasts in DCM showed an increase in genes associated with increased activation of fibroblasts (e.g. *COL1A1, CTGF, FAP* – genes encoding alpha-1 type I collagen,

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connective tissue growth factor and fibroblast activation protein alpha respectively) (Koenig et al., 2022). This is in parallel with the study conducted by Chaffin et al. that found that a population of activated cardiac fibroblasts was found in samples for both HCM and DCM hearts but not in non-failing hearts, as revealed by an increase in genes such as *FAP, COL1A1* and *POSTN* in activated CFs (Chaffin et al., 2022),

All these changes in activation state are coordinated by several mediators including neurohormones (angiotensin II, endothelin-1, norepinephrine, and epinephrine), interleukins (e.g. IL-6, IL-10, IL-4 etc.), growth factors such as TGFβ, fibroblast growth factor and platelet-derived growth factor, as well as chemokines of the cysteine-cysteine (CC) or cysteine-any amino acid-cysteine (CXC) families (Frangogiannis, 2019; Frangogiannis, 2021; Mazarura et al., 2022). However, how they are collectively and hierarchically involved remains unresolved, this is further complicated by the mix of different cues e.g., mechano-sensing and the variety of different chemokines that CFs respond to in the myocardium and how all these responses are integrated. One common downstream integrator of these cues is angiotensin II (Ang II) signaling, which has been suggested to be near the top of the hierarchy of events that activate fibroblasts (Mazarura et al., 2022). Gβγ signaling via the angiotensin II type 1 receptor (AT1R) has been proposed to act someway as a “go-or-no-go” braking mechanism for initiation of fibroblast activation (Khan et al., 2023). Through stimulation of AT1R, Ang II mediates secretion of TGF-β (a potent activator of fibroblasts) and collagen, as well as other downstream mediators like endothelin-1 that promote fibrosis (Cheng et al., 2003; Duangrat et al., 2023; Gray et al., 1998). A deeper understanding of the cues that drive disease is crucial for developing therapies to target cardiac fibrosis. With the aid of iPSC-CFs, patient- or disease-specific perturbations can be accurately modeled, providing invaluable insights into disease progression.

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iPSCs as a Model System for Studying Cardiomyopathies

Immortalized cell lines such as Chinese hamster ovary cells and human embryonic kidney 293 cells have been instrumental in our understanding of cellular signaling events. The drawback, however, is that these cells might harbor a wide range of mutations and karyotypic anomalies, and these cell lines can differ significantly between labs at different passages (Stepanenko and Dmitrenko, 2015). Further, receptor signaling is cell-type specific. Taken together, investigating disease progression, therapeutic responses or the relevant signaling events requires the relevant cellular context (Bourque et al., 2023). While animal models are certainly valuable for examining pharmacokinetics, toxicity, and whole organism effects, species-specific differences influence receptor pharmacology, highlighting the need to be cautious about directly applying findings from animal studies to humans (Gao et al., 2022).

Primary animal cardiac cell models have been instrumental in studying cellular mechanisms underlying cardiac disease and cardiac fibrosis. However key differences in receptor expression between rodent and human CFs have been noted. For example, rat cardiac fibroblasts have been shown to express higher levels of the angiotensin II receptor compared to neonatal human cardiac fibroblasts (Gallagher et al., 1998). Additionally, differences in signaling pathways have been observed, such as an increase in intracellular calcium concentrations in rat CFs, a response absent in human CFs. These species-specific variations highlight the importance of considering translatability when interpreting findings from animal models (Gallagher et al., 1998).

iPSC-CMs have also been compared to primary animal cells, with a recent study highlighting differences in gene expression between iPSC-CMs and rat neonatal ventricular cardiomyocytes (RNCMs) (Bourque et al., 2023). The study noted higher levels of endothelin receptor

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expression in iPSC-CMs, while RNCMs showed greater expression of the α1-adrenergic receptor. Furthermore, variations in signaling profiles were observed, with RNCMs showing a stronger nuclear protein kinase A (PKA) response to adrenergic ligands, norepinephrine and epinephrine (Bourque et al., 2023). These differences in expression and signaling between species emphasize the challenges of translating findings across species. Consequently, a recent emphasis has been set on studying receptor signaling using relevant human cell models, with induced pluripotent stem cells (iPSCs) offering a promising avenue for such research.

Given the limited availability of heart tissue from patients, human iPSCs provide a scalable and patient-relevant venue for drug development and studying aspects of disease progression (Funakoshi and Yoshida, 2021; Takahashi and Yamanaka, 2006). This innovation circumvents previous bottlenecks in studying heart disease, which relied on limited *in vitro* cultures of human cardiac cells sourced from biopsies, post-mortem tissue, or non-transplantable hearts. The advantage of harnessing iPSC technology is its capacity to leverage patient-derived samples. By obtaining blood samples or skin biopsies from patients with the disease of interest, then inducing these terminally differentiated cells into pluripotent stem cells, it becomes possible to generate diverse cell types from various tissues. Induced pluripotency can be achieved by inducing the expression of pluripotency genes (e.g., Oct3/4, Sox2, Klf4, c-myc) using episomal or viral delivery methods. iPSCs can be used to obtain different cell types pertinent to the disease of interest (Bao et al., 2016; Lian et al., 2013; Zhang et al., 2022; Zhang et al., 2019a; Zhang et al., 2019b). While the process to generate pluripotency erases many epigenetic marks (Nazor et al., 2012), genomic mutations are preserved and recapitulated in the iPSC-derived cells of interest. Consequently, iPSCs have been employed to model cardiomyopathy associated with specific mutations such as *LMNA* (gene encoding laminin A and C) (Yang et al., 2021), *RBM20* (gene encoding RNA-binding motif protein 20) (Briganti et al., 2020; Wyles et al., 2016), and *TNNT2* (gene encoding cardiac troponin T) (Dai et al., 2020), to

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name a few. For more comprehensive review of studies using iPSCs to study cardiomyocyte function in cardiomyopathy, see (Bourque et al., 2022; Brodehl et al., 2019).

Fibroblasts derived from iPSCs serve as an evolving approach to study physiological and pathophysiological aspects of fibrosis in human heart disease. The developmental sources of cardiac fibroblasts are diverse, with approximately 80% of CFs originating from the epicardial lineage via epicardial epithelial-mesenchymal transition, while a smaller proportion come from second heart progenitor cells, the endocardium (via endothelial to mesenchymal transition) and the neural crest (Ali et al., 2014; Cai et al., 2008; Gittenberger-de Groot et al., 1998). Protocols have been developed for differentiating iPSCs into both epicardial-derived CFs (Epi-CFs) and second heart field-derived CFs (SHF-CFs) (Zhang et al., 2022; Zhang et al., 2019a; Zhang et al., 2019b).

To generate epicardial-derived CFs, small molecules can be used to modulate Wnt signaling temporally to differentiate iPSCs into cardiac progenitor cells, followed by treatment with retinoic acid to drive cells toward an epicardial lineage, and finally fibroblast growth factor (FGF) to induce differentiation into the fibroblast lineage (Zhang et al., 2019a). This protocol yielded iPSC -CFs that closely resembled primary cardiac fibroblasts functionally - in their response to treatment with pro-fibrotic factors and their ability to become activated into myofibroblasts - as well as in their transcriptional profiles (Bekedam et al., 2024; Zhang et al., 2019a). Additionally, a protocol to differentiate iPSCs into fibroblasts derived from second heart field progenitors involves using small molecules to temporally regulate Wnt and FGF signaling, as described here (Zhang et al., 2019b).

Although iPSC-derived cells typically exhibit a fetal phenotype, several studies have demonstrated their utility modeling disease *in vitro* with ongoing efforts to generate more mature iPSC-CFs to better represent adult phenotypes (Briganti et al., 2020; Kitani et al., 2019; Sayed et al., 2020; Sun et al., 2012; Tang et al., 2020; Wu et al., 2019). Given the relatively simple and

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non-invasive method for sample collection, iPSCs offer us the opportunity of building a large bank of donors to study disease in a larger population. iPSC-derived cell models have uses ranging from investigating disease mechanisms involved in cardiomyopathies, modeling different aspects of fibrosis, and studying how cellular lineage plays a role in cardiomyopathy. Using co-culture systems and three-dimensional (3D) cell culture, it is possible for researchers to probe the interactions between different cells in the heart, and how these interactions evolve during development as well as how they are altered with disease progression.

Understanding the contribution of cardiac fibroblasts in cardiomyopathies and fibrosis using iPSCs

Heart development: iPSC-CFs to study lineage of CFs

The heart is the first functional organ to form *in utero*, originating from the cardiogenic region of the mesoderm layer (Eisenberg and Eisenberg, 2002). Both the lineage and the microenvironmental niches that fibroblasts support may confer distinct functional properties to cardiac fibroblasts. Studies using sc-RNA-seq and sn-RNA-seq have revealed that fibroblasts are comprised of a heterogenous population which can be clustered into different groups based on spatial location within the heart (Cui et al., 2019; Koenig et al., 2022; Tucker et al., 2020). Differences between fibroblasts have also been noted in different chambers of the heart, with some studies identifying between five to nine separate and distinct clusters of CFs within the heart. These studies also revealed distinct markers for fibroblasts derived from epicardium,

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endocardium, SHF or neural crest sources (Asp et al., 2019; Cui et al., 2019; Floy et al., 2021; Koenig et al., 2022; Litviňuková et al., 2020; Tucker et al., 2020).

Studying the implication of fibroblast lineage could help develop better targeted therapies for fibrosis in addition to improving our current understanding of specific functions of different fibroblast populations. In a study by Floy and colleagues, the authors characterized iPSC-derived Epi-CFs and SHF-CFs and found that populations derived from either lineage expressed key markers of fibroblast identity such as VIM, TE7, and CD90 at similar levels as revealed by flow cytometry (Floy et al., 2021). However, some genes were differentially expressed, with Epi-CFs showing higher expression of genes implicated in gap junctions, while cell cycle genes were upregulated in SHF-CFs. At a functional level, Epi-CFs showed a greater propensity to activate when treated with TGFβ1, serum or Ang II compared to SHF-CFs. Consequently, the authors concluded that epicardial-derived cardiac fibroblasts are better suited to the study of cardiac fibrosis, and a tool in anti-fibrotic therapy drug testing, while SHF-CFs are more amenable to investigating the study of cardiac mineralization (pathological calcification of the myocardium, heart valves and extracellular matrix that commonly occurs as a result of injury or inflammation (Pillai et al., 2017; Shackley et al., 2011)), outflow tract development and better suited for cell therapy purposes due to their diminished propensity to activate compared to Epi-CFs (Floy et al., 2021).

When compared to adult and fetal CFs, the authors found that iPSC-derived CFs show a fetal transcription profile. Fetal CFs tend to express more fibronectin, in line with facilitating a suitable environment for cell proliferation, while adult CFs tend to produce more collagen with a higher abundance of matricellular proteins (Floy et al., 2021; Williams et al., 2014). This finding was also confirmed in another study by Fernandes et al who were also able to drive iPSC-CFs to a more adult phenotype using 3D organoids composed of both iPSC-derived cardiomyocytes and iPSC-derived epicardial cells (Fernandes et al., 2023). To enhance the maturation of

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cardiomyocytes, these organoids were co-treated with a combination of the PPARα agonist, GW7475; palmitate; dexamethasone and T3 hormone (referred to as PPDT) under low-glucose cell culture conditions. This treatment facilitated the differentiation of cardiac fibroblasts from epicardial derived cells via epithelial-mesenchymal transition (EMT). Cells treated with PPDT showed greater expression of genes such as *ELN* (encoding elastin) and *FBLN1* (encoding fibulin-1) which are associated with a more mature fibroblast-phenotype. Notably, these results were specific to multicellular organoids, as 2D cultures of epicardial cells did not exhibit the same maturation. This indicates that cardiac fibroblasts mature simultaneously with cardiomyocytes within the organoid structure and respond to the same signals that drive cardiomyocyte maturation (Fernandes et al., 2023). This underscores the significance of cell-to-cell interactions in development.

In the same study, Fernandes and colleagues observed that they were able to subcluster fibroblasts into 12 transcriptional states (Fernandes et al., 2023). Moreover, the abundance of fibroblasts in the respective clusters changed after inducing injury to the organoids using a combination of isoproterenol/hypoxia and TGFβ1, which is upregulated in heart failure. Studies such as these could help distinguish fibroblasts that promote disease progression, and potentially allowing for targeting treatment with CAR-T cell therapy (Rurik et al., 2022), and those that are beneficial for heart function and potentially useful in cell therapy. Furthermore, developmental studies can help underpin molecular differences between healthy hearts and those from patients with genetic cardiomyopathies to better understand the impact of disease-causing mutations of fibroblast behaviors.

The effect of mechanical strain on CFs

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CFs are also mechano-sensitive cells that respond to mechanical stimuli and are likely affected by changes in the stiffness of the myocardium ranging from 10 pN to 350 nN (Braidotti et al., 2024; Rogers et al., 2021). For instance, the angiotensin II type-1-receptor, implicated in cardiac fibroblast activation, is triggered by mechanical strain and increased substrate stiffness with substantial evidence suggesting that more rigid substrates can induce the conversion of cardiac fibroblasts to myofibroblasts, partly through AT1R activation (Niu et al., 2020; Yong et al., 2016). Moreover, cyclic stretch has been demonstrated to alter the effects of agonists such as TGFβ1, IL-1β, TNFα, and Ang II in rat cardiac fibroblasts (Rogers et al., 2021). For example, cyclic stretch reduced the effect of agonists that upregulated proteases like MMP9, while enhancing the effect of agonists that suppressed several matrix metalloproteinases such as MMP1and MMP8 in CFs (Rogers et al., 2021).

iPSC-CFs have been used to investigate mechanical stimulation, whereby iPSC-CFs were exposed to cyclic stretch at 1 Hz for 72h, mimicking the cardiac strain experienced during rest under physiological conditions (Bekedam et al., 2024). iPSC-CFs were co-treated with TGFβ1 in the presence or absence of cyclic stretch. Cyclic stretch led to a diminished effect of TGFβ1 on increasing expression of ECM-associated genes such as *COL1A1, LOX* (encodes lysyl oxidase), *LOXL2* (encodes lysyl oxidase like 2) – this was also shown to hold for collagen I protein expression. Furthermore, cyclic stretch inhibited TGFβ-induced activation of iPSC-CFs as shown by its ability to reduce α-SMA protein expression and the expression of α-SMA stress fibres that are typical of myofibroblasts, as revealed by western blot and immunofluorescent staining (Bekedam et al., 2024). Taken together, this study underscores the utility of iPSC-CFs in modeling the effect of mechanical stress on CF response to cytokines to enhance our understanding of the mechanisms that influence fibroblast activation.

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The impact of ECM on disease

Fibrosis has conventionally been thought of as a reaction to injury, however, recent studies has brought to light that changes in the myocardium ECM that are mediated by CFs occur even before the onset of fibrosis (Ho et al., 2010; Kim et al., 2007). Thus, suggesting that cardiac fibroblasts work in tandem with cardiomyocytes to drive disease pathology. Mutations that are specific to genes expressed by cardiomyocytes can impact fibroblasts, where fibroblasts perhaps “sense” the consequences of these mutations (e.g., reduced contractility) and then attempt to compensate or adapt accordingly (Ho et al., 2010; Zou et al., 2022) (Bretherton et al., 2023 bioRxiv, https://doi.org/10.1101/2023.01.23.523684).

Given the key role that fibroblasts play in the pathology of cardiomyopathy, it is essential to also study ECM contributions to disease pathology. ECM plays a crucial role in cellular signaling, regulating abundance of growth factors and activating signaling pathways essential for myogenesis and supporting the developing heart through ECM receptors (Bradshaw, 2014; Calabro et al., 2014; Hinz, 2015; Piñeiro-Llanes et al., 2022; Song and Zhang, 2020). Cell-derived matrices (CDMs) have been shown to recapitulate the composition of diseased ECM (Rubí-Sans et al., 2021) and consequently, they have been used in to study the effect of ECM produced by iPSC-CFs from patients with Barth syndrome. This syndrome is a genetic disorder, that affects metabolism and is linked to cardiomyopathy. Barth syndrome is caused by a mutation in the TAZ gene which encodes an acetyltransferase (Piñeiro-Llanes et al., 2022; Spencer et al., 2006; Steward et al., 2010). In a study that compared CDMs derived from ECM produced by iPSC-CFs from individuals with Barth syndrome and healthy donors, mass spectrometry was used to reveal 15 ECM-related proteins with altered expression levels in disease-CDMs compared to healthy CDMs (Piñeiro-Llanes et al., 2022). These proteins included ECM proteins such as thrombospondin 2 (THBS2), implicated in extracellular signal-regulated kinase 1/2 (ERK1/2) signaling and angiogenesis; structural proteins such as collagen

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type XII alpha 1 (COL12A1) and laminin subunit alpha 5 (LAMA5); and metalloprotease inhibitors such as metalloproteinase inhibitor 3 (TIMP3) (Piñeiro-Llanes et al., 2022). The dysregulation of these ECM proteins impacted mitochondrial function, resulting in lower ATP production and higher ROS generation in cells grown on Barth-CDMs compared to healthy CDMs. This study highlights the potential influence of ECM composition on mitochondrial function and cellular processes related to cardioskeletal development (Piñeiro-Llanes et al., 2022).

Studying cellular proteins linked to ECM signaling can elucidate the impact of mutations on CF function, such as dystrophin which plays a role in linking the cytoskeleton to the ECM. For instance, research into iPSC-derived cardiac fibroblasts from patients with Duchenne Muscular Dystrophy (DMD) revealed that loss of dystrophin expression heightened the propensity of CFs to activate into their myofibroblast phenotype when stimulated with TGF-β or Ang II, and impaired mitochondrial bioenergetics compared to control iPSC-CFs (Soussi et al., 2023). This study underscores how proteins linked to ECM-cell signaling can affect cell function, including metabolism and cytoskeletal organization, and amplify the fibrotic response in CFs from DMD patients.

A study of DCM in a dish: modeling molecular pathway perturbations using iPSC-CFs

LMNA is a gene encoding laminin A and C. These are intermediate filament proteins that form part of the nuclear envelope and are essential for nuclear integrity and processes such as regulation of gene expression and signal transduction. Although genetic variants in *LMNA* have been mostly studied in cardiomyocytes, linking these variants to disease phenotypes has proven difficult. To understand molecular mechanisms underlying DCM in families with *LMNA*-related cardiomyopathy, Widyastuti et al. used RNA-seq on primary skin fibroblasts from a

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family bearing a mutation in *LMNA* to evaluate the gene profile that caused DCM (Widyastuti et al., 2020). They were able to show that genes involved in ERK1/2 signaling were dysregulated in DCM patients, as opposed to cells from the control group, thus making ERK1/2 a candidate pathway that may be perturbed and lead to cardiomyopathy (Widyastuti et al., 2020). Another study that complemented this finding showed ERK hyperactivation in induced-pluripotent stem cell-derived cardiac fibroblasts of DCM patients harboring a mutation in the *LMNA* gene. Out of the 7 samples examined, 6 of the iPSC-CF populations from DCM-patients harboring specific *LMNA* variants (M1I, R216C/R399H, R541C, R377H, R399H, R216C) showed an increase in ERK1/2 phosphorylation, while one variant (*LMNA*- R335Q) did not show a significant difference relative to controls (Yang et al., 2021). Thus, perturbations in ERK1/2 signaling may be involved in the molecular mechanisms underlying disease progression, and these perturbations may be distinct depending on the specific mutation in *LMNA*.

Moving into an *in vivo* model, Rouhi and colleagues used a Cre-knock out mouse model to selectively knock out *LMNA* in cardiac fibroblasts (Rouhi et al., 2022). They were able to show that *LMNA*-associated DCM could be recapitulated by ablation of *LMNA* in cardiac fibroblasts, implying that both CMs and non-CMs synergistically cause the DCM phenotype (Rouhi et al., 2022). Knocking out *LMNA* in CFs of these mice led to arrhythmias, myocardial fibrosis, conduction defects and cardiac hypertrophy (Rouhi et al., 2022). These studies all underscore the significance of both cardiomyocytes and other non-cardiomyocyte cells within the heart as crucial contributors to the development of DCM.

One of the reasons mutations in CFs may affect CM function is illustrated by several studies that have highlighted that crosstalk between CMs and cardiac fibroblasts evolves with disease progression. In terms of paracrine crosstalk, one study showed that media conditioned by iPSC-CMs of DCM patients contained exosomes that induced a fibrotic response both *in vivo* and *in vitro*. These exosomes contained the microRNA, miR-218-5p, which activated TGFβ

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signaling by suppressing TNFAIP3, (gene encoding TNFα-induced protein 3, an inhibitor/negative regulator of inflammation and immune response). In contrast, media conditioned by control iPSC-CMs did not trigger this fibrotic response (Fu et al., 2023).

Previous work by Verdonschot and colleagues involved performing RNA-seq analysis on biopsies taken from a cohort of 795 patients with DCM unveiled distinct phenotypic clusters, each characterized by a unique cardiac transcriptomic signature. These differences in molecular pathology delineate variations among phenogroups, while still sharing a common pathophysiology when compared to patients in other phenogroups (Verdonschot et al., 2020). DCM is a heterogenous disease, and treatments have shown varying degrees of efficacy in clinical trials and overall treatment outcomes (Verdonschot et al., 2019; Verdonschot et al., 2020). The use of iPSCs holds promise in establishing a bed-to-bench-to-bed pipeline to improve therapy for cardiomyopathy patients while overcoming the limitation of cardiac tissue availability. For example, efforts have been made to model molecular perturbations of DCM using iPSC-derived cardiac cells and FRET biosensors to probe into signaling pathways disturbed in disease such as ERK1/2 signaling (Bourque et al., 2022) (Bourque et al., 2022a bioRxiv, https://doi.org/10.1101/2022.09.06.506800). This approach seeks to categorize patients for personalized treatment strategies and expedite drug development by prioritizing drug classes tailored to specific phenogroups. Moreover, this methodology can extend to fibrosis drug discovery by creating phenogroups, which can be used to target therapies more effectively.

Using 3D models to model influence of cross cellular talk and to study Arrhythmogenic cardiomyopathy

Understanding the crosstalk between cardiac cell types during development and isolating the contribution of diseased cells to healthy cardiac tissue provides valuable insights into cardiomyopathies. Both 2D and 3D culture techniques serve as useful tools for probing cell-

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to-cell interactions between cardiomyocytes and non-myocyte cell types. Co-culturing iPSC-CMs with iPSC-CFs, for instance, has been shown to enhance iPSC-CM contractility compared to cultures of iPSC-CMs alone (Stempien et al., 2024).

It is well documented that 3D cultures better replicate *in vivo* conditions, including cell morphology, cytoskeletal organization, and cellular processes like proliferation (Pontes Soares et al., 2012). Additionally, 3D culture has been instrumental in promoting maturation of iPSC-derived cells, which typically exhibit a fetal phenotype. Notably, 3D culture has been utilized to study cell interactions between cardiomyocytes, epicardial cells, and cardiac fibroblasts in the context of arrhythmogenic cardiomyopathy (ACM) (Giacomelli et al., 2020). Giacomelli and colleagues demonstrated the effectiveness of 3D culture in investigating such interactions. By coculturing iPSC-CMs, -ECs, and -CFs in self-aggregating microtissues (MTs), they demonstrated that tri-lineage 3-dimensional MTs enhanced iPSC-CM maturation, as evidenced by improved mechanical contraction, sarcomeric structure, and metabolic maturation. This was partially attributed to proposed cross talk between iPSC-CMs, and iPSC-CFs mediated via the gap junction protein, connexin-43 (Cx43) (Giacomelli et al., 2020).

The desmosomal protein, plakophilin (encoded by *PKP2* gene) plays a role in Cx43 trafficking and is associated with Arrhythmogenic Right Ventricular Cardiomyopathy (ACVM) (Agullo-Pascual et al., 2013; Dries et al., 2021; James et al., 2021). Giacomeli and colleagues investigated the effect of diseased iPSC-CFs obtained from a patient harboring the heterozygous c.2013delC *PKP2* genetic variant (referred to as ACM iPSC-CFs) (Giacomelli et al., 2020). When compared to iPSC-CFs derived from a healthy subject, diseased iPSC-CFs showed no notable difference in PKP2 protein expression level. However, in microtissues (MTs), only healthy control iPSC-CFs exhibited sustained Cx43 expression. Additionally, ACM iPSC-CFs adopted a more myofibroblast-like phenotype, as assessed by α-SMA expression. Furthermore, they impaired the ability of microtissues to respond to pacing at frequencies above

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2Hz and induced arrhythmic phenotypes in healthy CMs (Giacomelli et al., 2020). Taken together, iPSC-derived cardiac cells enable us to study the impact of each cardiac cell type on disease by modeling their effect on healthy cells to better understand disease mechanisms. Furthermore, they highlight the importance of investigating how cell-cell contacts evolve during disease progression, and how these changes contribute to the development of cardiomyopathy.

Drug development: From target finding to cardiotoxicity testing using iPSC-CFs

Profiling the cell surface proteins of cardiac cells offers insight into druggable targets that can be useful for drug development as well as key molecular markers in disease progression. The development of CellSurfer, a platform integrating mass spectrometry to generate “surfacesome” maps of various cardiac cells, including iPSC-derived CFs, yielded 436 cell surface markers that were common to both human primary CFs and iPSC-CFs (Berg Luecke et al., 2023). Notably, primary fibroblasts expressed 85 druggable targets that were not found on iPSC-CFs such as AOC3 (as targeted by hydralazine) and NPR2 (as targeted by nesiritide). This discrepancy may stem from the fetal phenotype displayed by iPSC-CFs. However, it is important to note that the protocol used by the authors generated iPSC-CFs from a second heart field progenitor cell lineage, while epicardial derived cells form the majority of CFs found in the heart, which could account for these differences. Nonetheless, this tool offers an avenue to map how the surfacesome of iPSC-CFs evolves along the activation pathway of CFs, and to discover targets specific to fibrosis-linked fibroblasts. The study revealed surfacesome disparities between cardiomyocytes from failing and non-failing hearts, shedding light on altered genes related to cell-to-cell and cell-ECM adhesion in disease states, potentially elucidating key molecular patterns associated with disease progression (Berg Luecke et al., 2023).

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Another study used RNA-seq and mass spectrometry to profile lysed iPSC-CFs of epicardial lineage alongside adult and fetal human CFs (Moita et al., 2022). Upon activation using TGFβ1, all CF sources showed a convergent gene response pattern, with an upregulation in genes related to cell adhesion and migration, as well as ECM protein production and angiogenesis (Moita et al., 2022). Moreover iPSC-CFs showed a lower basal activation level than primary CFs and a larger difference in response between control and treated cells, thus providing a wider assay window. Overall this highlighted the advantage of iPSC-CFs in studying CFs along their activation pathway, and the usefulness of the model in overcoming the difficulties of keeping primary cardiac fibroblasts quiescent *in vitro* (Landry et al., 2019). Primary cardiac fibroblasts are challenging to maintain in culture in a quiescent state, methods such as culturing them on softer substrates (e.g. silicone or collagen) or limiting nutrient or serum concentrate have been used to extend culture times from three days to three passages (Landry et al., 2019; Landry et al., 2021; Sahadevan and Allen, 2022). In another study, a co-culture system that incorporated both iPSC-CMs and -CFs, was used to screen potential anti-fibrotic drugs and model their efficacy *in vitro* (Iseoka et al., 2021). In the study, pirfinedone, ONO-1301, and comostat mesylate reduced ECM expression *in vitro*, and were efficacious in an *in vivo* model, demonstrating the utility of this tool for anti-fibrotic therapeutic screening.

Finally, iPSC models have been useful as models for toxicity screening studies. For instance, iPSC-derived CFs and CMs in monoculture were utilized to screen the cardiotoxicity of 21 FDA-approved tyrosine kinase inhibitors (Sharma et al., 2017). This screen revealed that both CMs and CFs had a similar cytotoxic profile, with sorafenib, regorafenib and ponatinib being the most cardiotoxic drugs and enabled the authors to establish a safety index for their toxicity for these compounds (Sharma et al., 2017). Doxorubicin, a chemotherapeutic known to promote fibrosis and induce cardiac cell death (Tanaka et al., 2020), has been a subject of study for drug-induced cardiotoxicity using iPSC-derived cardiac microtissues (Ergir et al., 2022).

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Exposure to doxorubicin resulted in alterations in the beating profile and shapes of these microtissues, along with reduced viability (Ergir et al., 2022).

Conclusion

Here, we have highlighted how iPSC-CFs serve as valuable tools for modeling genetic perturbations and their effects *in vitro*, while offering a scalable source of cardiac cells which are often difficult to obtain. They recapitulate different disease aspects *in vitro*, including cell-cell interactions, ECM-cell interactions, heart development, perturbations in molecular pathways, and mapping changes during disease progression, facilitating the discovery of drug targets for fibrosis. However, challenges persist, such as the maturity of iPSC-derived cells and the potential loss of disease-specific epigenetic markers during reprogramming. The maturity issues can to some extent be mitigated by using co-culture or organoid models. The loss of epigenetic markers can be prevented by direct conversion of somatic cells into the relevant cell types, but this would have to be done prior to each experiment rather than having iPSC lines that provide a more robust source of cells. Although generation of iPSC-CFs represents a path toward enhancing drug development and translational research, further refinement is necessary to fully realize their potential.

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Figure Legend

Figure 1. *iPSC-CFs as a useful tool for modeling cardiomyopathy.* Cardiac fibroblasts serve as valuable tools for modeling various aspects of fibrosis. Induced pluripotent stem cells (iPSCs) offer the advantage of deriving samples from diverse populations of control subjects and patients and differentiating them into cardiac cell types, including iPSC-derived cardiac fibroblasts. Utilizing 2-dimensional monoculture, co-culture, or 3-dimensional culture techniques enables the modeling of various aspects of cardiac physiology and pathophysiology (A-E), facilitating drug development, testing, and personalized therapy recommendations for patients (F-G). Created with BioRender.com.

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Declaration of Competing Interests

No author has an actual or perceived conflict of interest with the contents of this article

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Data Sharing

This article contains no datasets generated or analyzed during the current study.

Author contributions

Wrote or contributed to the writing of the manuscript: Mazarura G., Hébert T.E.

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Figure 1