Unveiling the CCBE1 role on human iPSC-derived cardiac fibroblasts differentiation and functionality

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Thesis to obtain the Master of Science Degree in

Biomedical Engineering

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Preface

The work presented in this thesis was performed at Animal Cell Technology Unit, on IBET and ITQB-NOVA, during the period February-October 2019, under the supervision of Dr. Maria Margarida de Carvalho Negrão Serra, and within the frame of the NeoCoronary project (Ref. nº 029590) and iNOVA4Health - UID/Multi/04462/2013, financially supported by the Fundação para Ciência e Tecnologia (FCT), Portugal and co-funded by FEDER under the PT2020 Partnership Agreement. The thesis was co-supervised at Instituto Superior Técnico by Prof. Maria Margarida Fonseca Rodrigues Diogo.

Part of the work presented in this thesis has been included in poster communications.

Poster Communications:

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.
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Abstract

Cardiovascular diseases are the leading cause of mortality worldwide. This group of disorders, including myocardial infarction (MI), arises from the widely and irreversible loss of functional cardiac muscle, which is replaced by a fibrotic tissue mainly composed by activated cardiac fibroblasts (CF). Alternative therapies have been focusing on the direct cardiac muscle tissue regeneration through cell-free approaches, thus making crucial to identify key molecules acting on the cardiac microenvironment. In this context, CCBE1 (collagen and calcium-EGF binding domain 1) was identified being expressed in the epicardium during mouse embryonic development. However, its functional role during CF specification and functionality is still unknown. Therefore, the main aim of this thesis was to unveil CCBE1 role in CF specification, by using a modified hiPSC line displaying the CRISPR interference technology (CRISPRi) to selectively knockdown (KD) CCBE1 gene expression. Both wild-type and modified CRISPRi cell lines were differentiated into CF, showing clear morphological and phenotypical changes throughout the differentiation process. Daily doxycycline addition from iPSC to CF was identified to affect the cells’ growth, but not their morphology, viability and phenotype. Using a fibroblast-specific medium combined with a TGF-β inhibitor impacted allowed to generate a higher number of quiescent fibroblasts. The derived CF were able to maintain their phenotype for up to 50 days in culture, to be activated into myofibroblasts and to migrate towards a wound. By unveiling the CCBE1 key role on CF differentiation and activation (mimicking MI) we expect to provide new insights towards the development of CCBE1-targeted regenerative therapies.

Keywords: Cardiovascular Disease; Cardiac Fibrosis; Cardiac Fibroblasts; human induced Pluripotent Stem Cells (hiPSC); CCBE1; CRISPRi
Resumo

As doenças cardiovasculares são a principal causa de morte em todo o mundo. Em particular, no enfarte do miocárdio (EM), o músculo cardíaco funcional lesado é posteriormente substituído por um tecido fibrótico composto por fibroblastos cardíacos (CF) ativados. Diversas terapias alternativas têm sido atualmente desenvolvidas, utilizando o secretoma das células para ajudar a regenerar o miocárdio, tornando, portanto, importante identificar as moléculas-chave que atuam no microambiente cardíaco. Neste contexto, a CCBE1 (collagen and calcium-EGF binding domain 1) foi detetada em percursores cardíacos durante o desenvolvimento embrionário em ratinhos. Assim sendo, o principal objetivo desta tese foi investigar o papel desta proteína ao longo da diferenciação em CF, usando uma linha hiPSC modificada através da tecnologia de interferência CRISPR (CRISPRi) para reduzir a expressão do gene CCBE1. Quer a linha celular hiPSC-WTC como as linhas celulares modificadas com o sistema CRISPRi foram diferenciadas em CF, tendo exibido claras alterações morfológicas e fenotípicas ao longo do processo. A adição diária de doxiciclina durante a diferenciação impactou o crescimento das células, mas não na sua morfologia, viabilidade e fenótipo. Ao utilizar-se um meio específico de fibroblastos suplementado com inibidor de TGF-β gerou-se um maior número de fibroblastos inativos. Os CF diferenciados conseguiram manter o seu fenótipo durante 50 dias em cultura e mostrar características típicas de fibroblastos, como poderem ser ativados e conseguirem migrar. Ao revelar o papel principal do CCBE1 na diferenciação e ativação dos CF (simulando o EM), esperamos conceder novas ideias para o desenvolvimento de terapias regenerativas direcionadas à CCBE1.

Palavras-chave: Doenças Cardiovasculares; Fibrose Cardíaca; Fibroblastos Cardíacos; Células estaminais pluripotentes induzidas humanas (hiPSC); CCBE1; CRISPRi
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<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS3</td>
<td>A Disintegrin and Metalloproteinase with Thrombospondin Motifs-3</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>AT1</td>
<td>Angiotensin II type 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CPC</td>
<td>Cardiac Progenitor Cell</td>
</tr>
<tr>
<td>CM</td>
<td>Cardiomyocytes</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
</tr>
<tr>
<td>CCBE1</td>
<td>Collagen and calcium-binding EGF domain-1</td>
</tr>
<tr>
<td>CRISPRi</td>
<td>CRISPR interference technology</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6'-diamino-2-fenil-indol</td>
</tr>
<tr>
<td>dCas9</td>
<td>Deactivated CRISPR associated protein 9</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGFb</td>
<td>basic Fibroblast growth factor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GATA4</td>
<td>Transcription factor GATA-4</td>
</tr>
<tr>
<td>gRNA</td>
<td>guideRNA</td>
</tr>
<tr>
<td>HF</td>
<td>Heart Failure</td>
</tr>
<tr>
<td>hiPSC</td>
<td>Human induced pluripotent stem cells</td>
</tr>
<tr>
<td>hiPSC-CF</td>
<td>Cardiac Fibroblasts derived from hiPSC</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IWP2</td>
<td>Inhibitor of WNT Production-2</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KRAB</td>
<td>Krüppel-associated box</td>
</tr>
<tr>
<td>LEC</td>
<td>lymphatic endothelial cells</td>
</tr>
</tbody>
</table>
MESP1  Mesoderm Posterior BHLH Transcription Factor 1
MI  Myocardial infarction
MMPs  matrix metalloproteinases
Nanog  Homeobox Protein Nanog
Nkx2.5  NK2 Homeobox 5
Oct4  Transcription factor octamer 4
PFA  Paraformaldehyde
POSTN  Periostin
PBS  Phosphate-buffered saline
RT-qPCR  Reverse transcriptase quantitative polymerase chain reaction
RPLP0  Ribosomal Protein Lateral Stalk Subunit P0
SSEA-1  Stage-specific embryonic antigen-1
SSEA-4  Stage-specific embryonic antigen-4
TGF-β  Transforming Growth Factor beta
Thy-1 (CD90)  Thy-1 Cell Surface Antigen
TNNT2  Cardiac muscle troponin T
TRA-1-60  Human embryonal carcinoma marker antigen 60
VEGF-C  Vascular endothelial growth factor
VEGFR-3  Vascular endothelial growth factor receptor 3
WT1  Wilms Tumor 1
WNT  Wingless-related integration site
1. Introduction

1.1. Cardiac Vascular Diseases

Cardiovascular diseases (CVD) comprise a group of disorders that affect the heart and circulatory system, being accountable for 17.9 million deaths worldwide per year, a number that is expected to rise to more than 23.6 million, by 2030 (Figure 1.1). Within the past decades, the leading cause of mortality has been the Ischaemic Heart Disease, which is commonly manifested through cardiac arrhythmias, stable or unstable angina pectoris and myocardial infarction (MI).

Particularly in MI, also known as heart attack, the prolonged ischemia and lack of nutrients caused by the occlusion of a coronary artery causes the death of more than a billion of cardiomyocytes (CM) on myocardium, either by cell necrosis or apoptosis. This large-scale loss, together with the increased loading conditions, activates a signaling cascade responsible for modulating the heart regeneration after the injury. Since an adult human heart has a reduced regenerative capacity, during this activated healing process the necrotic and damaged myocytes are replaced with fibrotic tissue. Consequently, some pathological changes, which will be further mentioned, can arise from this scar formation and impair the heart's normal function, ultimately leading to heart failure (HF).

Most of MI therapeutic approaches available nowadays are mainly focused on preventing the disease progression and improving patient’s welfare. One example of existing cardioprotective solutions is pharmacotherapy, which uses specific renin-angiotensin inhibitors, β-blockers and mineralocorticoid-
receptor antagonists to slow down or reverse the cardiac remodeling process\textsuperscript{7}. According to different patients’ necessities, some revascularization interventions (e.g., coronary artery bypass) can also be performed to replace the blood supply on the myocardium ischemic area\textsuperscript{8}. In addition, mechanical circulatory support devices (e.g. LVAD) can help to manage HF in extreme cases, while end-stage HF patients wait for heart transplantation, which remains as the best long-term medical treatment capable of completely reestablish the normal heart function\textsuperscript{9}. However, this transplantation solution is associated with high costs, surgical complexity and potential organ rejection, which along with the reduced number of available donors worldwide, hamper the possibility of performing it as a standard therapy\textsuperscript{7}. Moreover, scientists have been focused on alternative therapies; different approaches to promote heart regeneration can be cell-based (e.g. using non-cardiac cells, cardiac derived cells and pluripotent stem cells) or cell-free (e.g. using growth factors, microRNAs, and extracellular vesicles including exosomes)\textsuperscript{7}.

1.2. Cardiac Fibrosis: from MI to scar tissue

In MI, a coronary artery obstruction is responsible for blocking, partially or totally, the nutrients and oxygen supply to the myocardium, which consequently leads to a massive loss of CM. Upon this ischemic injury, a multiphase fibrotic response is triggered, when the necrotic myocytes are removed and replaced with non-contractile collagen-based scar tissue (\textit{Figure 1.2 - Dynamic environment within the infarcted heart, along the overlapping phases of Cardiac Fibrosis (adapted from \textsuperscript{13}). Figure 1.2}). The scar formation process, characterized by large amounts of extracellular matrix (ECM) proteins that are secreted and deposited, is named cardiac fibrosis and can be categorized into two types: replacement fibrosis and interstitial fibrosis\textsuperscript{10–12}. After MI, the heart’s remodeling starts occurring by replacement fibrosis, a process which is accountable for initially support myocardium structural and morphological integrity and prevent any ventricular wall rupture. However, some of the proinflammatory cytokines and profibrotic factors secreted during this fibrosis form are spread to remote zones to the infarction as well, including uninjured myocardium areas. Together with the increased loading conditions post-MI, this leads to the expansion of connective tissue throughout cardiac interstitium remote regions, comprising thus the named interstitial fibrosis\textsuperscript{10–12}. Although the fibrotic scar formation comprises a remodeling process for the heart to repair itself and maintain its compliance, some adverse pathological consequences can arise from it. The abundant secretion of ECM proteins during the scar tissue formation, along with the high number of cardiac fibroblasts (CF) within the matrix responsible for it, can impact CM mechano-electric coupling during the remodeling process\textsuperscript{10}. This results in higher ventricular stiffness and can affect myocardium contractility, thus impairing the normal heart’s function\textsuperscript{10,11}.

The cellular response which mediates the infarcted heart repair can be divided into three different, but overlapping phases: the inflammatory, the proliferative and the maturation phase (\textit{Figure 1.2})\textsuperscript{12–15}. Initially, at the inflammatory phase, the release of a wide range of endogenous molecules by necrotic cells is responsible for stimulating innate immune pathways, such as TLR/IL-1 and RAGE-dependent pathways\textsuperscript{12,13}. Their activation in CF, endothelial cells and non-infarcted CM triggers an
inflammatory reaction in the infarction region, as well as in the recruited leukocytes subpopulation, responsible for cleaning the dead cells and matrix debris originated\textsuperscript{12–15}. Both reactive oxygen species (ROS)\textsuperscript{16}, HMGB1\textsuperscript{17}, S100A8/A9\textsuperscript{18}, interleukin-1α\textsuperscript{19} and heat-shock proteins\textsuperscript{20}, as well as ATP and RNA molecules are included in this group of triggering intracellular contents, named as alarmins or damage associated molecular patterns (DAMPs). Moreover, along this phase, the cardiac ECM is also degraded to open new paths for the injured area repopulation with migrating and proliferating cells, which causes a consequent release of matrix protein fragments\textsuperscript{12}.

While the injured area is being cleaned, during macrophages’ phagocytosis of dead cells, some anti-inflammatory mediators are released in large-scale, such as IL-10 and TGF-β cytokines\textsuperscript{21,22}. When the cleansing of the wound becomes over, pre-programmed neutrophils enter in apoptosis and start releasing inhibition mediators which prevent any further neutrophil recruitment. As a consequence of these events, some anti-inflammatory pathways are activated and the CF are transdifferentiated into myofibroblasts, crucial cellular effectors for scar tissue formation that infiltrate the injured area. These

\textbf{Figure 1.2} - Dynamic environment within the infarcted heart, along the overlapping phases of Cardiac Fibrosis (adapted from \textsuperscript{13}).
activated fibroblasts are responsible for enriching the ECM with matricellular proteins, which bind to growth factors and cell surface receptors and modulate cellular responses\textsuperscript{23}. Simultaneously, during this \textit{proliferative phase}, an abundant microvascular network is also developed so the reparative cells can access to oxygen and nutrients\textsuperscript{12,13,15,24}.

Once the structural matrix of the scar tissue becomes formed in the infarct region, a transition into the \textit{maturation phase} begins, during which both cellular and ECM elements of the scar start suffering modifications. Along this phase, lysyl oxidase and similar enzymes catalyze collagen and elastin cross-linking reaction, which insolubilizes the ECM proteins and helps its stabilization\textsuperscript{25}. In addition, the number of myofibroblasts within the injured area becomes reduced, as most fibroblasts undergo apoptosis, which also consequently leads to the downregulation of matricellular proteins. However, on the other hand, some remaining myofibroblasts in the infarct zone turn quiescent. During this stage, as angiogenesis becomes inhibited, vascular cells also enter in apoptosis, causing the regression of the created microvessels\textsuperscript{12,13}.

\subsection*{1.2.1. CFs and Myofibroblasts: the cellular impact during Cardiac Fibrosis}

The activation of several mechanisms and pathways involved in the heart remodeling requires the presence of wide-range of cell types, throughout the distinct cardiac fibrosis phases\textsuperscript{15}. Depending on the injury’s underlying cause, diverse cell lineages contribute differently during fibrosis. Some cell types, such as leukocytes, mast cells, dendritic cells, endothelial cells, vascular smooth muscle cells and pericytes are engaged in this process, by secreting fibrosis mediators upon CM death. However, the key cellular effector involved in the fibrotic response are the fibroblasts, which produce the ECM proteins constituting the collagen-based scar tissue\textsuperscript{15}.

In several studies regarding the cardiac cellular composition, where murine and rat models are used, non-myocyte cells have been stated to be the most abundant cell population within the adult mammalian heart, comprising approximately 70-80\% of the cardiac tissue\textsuperscript{26-28}. However, a number or percentage capable of representing CF actual population within the heart’s composition remains uncertain and unknown. To clarify this matter, Pinto \textit{et al.} published a recent study, where a complete and refined analysis of cardiac cellular composition shows that CF comprise less than 20\% of the non-myocyte population, being outnumbered by the endothelial cells occupying more than 60\% of the non-CM group\textsuperscript{29}. All these studies’ conflicting results might be related with the different species evaluated and their distinct genetic backgrounds, age and gender, as well as with the different strategies used for cell isolation and identification\textsuperscript{26-29}. To better define the cardiac cellular composition and identify CF density and phenotype in normal adult human hearts, further studies need to be performed using healthy human cardiac tissue.

Regardless their abundance, most of the existing CF are primarily derived from an epithelial layer named \textit{epicardium}, whose cells differentiate into the mesenchymal fate during cardiac development. Some of these mesenchymal cells develop certain migration features that enable them to
invade the myocardial interstitium, where cells will be later differentiated into the fibroblast lineage and become resident CF which maintain the myocardium structurally compact. In addition, other subsets of resident CF can also be derived and arise from distinct embryonic cardiac layers, such as endocardium or cardiac neural crest. CF multiple origins during the human heart development, together with pieces of evidence stating the existence of various phenotypes and specific functions within different CF subsets, make the fibroblasts an extremely heterogeneous population. Since these cells also change their phenotype after an injury and during cardiac fibrosis as well, identifying all the existing CF subsets by labeling them with specific and reliable markers becomes complicated and challenging. Therefore, in the absence of accurate labeling, fibroblast populations are generally characterized using a combination of fibroblast-related markers, or using different lineage-specific proteins, to further discover the CF population by exclusion criteria. A summary of the current known biomarkers in determining the cardiac fibroblast and myofibroblasts is presented in Table 1.1.

CF present a well-defined morphology, despite their different origins, uncertain abundance and heterogeneous population that limit their study and characterization. In homeostasis or inactive state, these cardiac cells are defined for their spindle-shaped and dendritic-like branched cytoplasm, comprising a speckled nucleus which usually holds 1 or 2 nucleoli. When activated, CF become larger in size, contractile microfilaments and stress fibers arise throughout the cytoplasm and their Golgi apparatus appears more prominent than usual. CF can be also distinguished from other existing cardiac cells by their characteristic lack of basement membrane and, within the heart, are usually located between cardiac muscle fibers, in the form of strands and sheets.

During cardiac homeostasis, quiescent fibroblasts secrete minimal but enough amounts of ECM proteins capable of maintaining the cardiac matrix homeostasis and preserving its structural cohesion. Certain cytokines, growth factors, and Matrix metallopeptidases (MMPs) involved in synthesis and degradation of connective tissue components are also produced by inactive CF, helping to maintain the ECM equilibrium as well. Besides the fibroblasts contribution for stabilizing the ECM, this interstitial network has the reverse responsibility of shielding the resident CF from any injurious mechanical stress and be a scaffold for all the other cardiac cells. Moreover, quiescent CF establish a bidirectional crosstalk with CM through paracrine mediators, direct cell-cell interactions, and indirectly via ECM. This close association between fibroblasts and myocytes, whether in the healthy or remodeled myocardium, is crucial to determine the cardiac muscle structural and electro-mechanical characteristics. Particularly, CF have the capability to couple with neighboring fibroblasts or with CM through gap-junctions, which according to the different possible fibroblast–myocyte interactions, can give rise to distinct functionally-relevant coupling effects on the myocardium electrophysiology.
Table 1.1- Summary of current biomarkers used for identifying CFs and myofibroblast cells (adapted from 35).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Location</th>
<th>Role</th>
<th>Fibroblast</th>
<th>Myofibroblast</th>
<th>Expression in other cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-smooth muscle actin (α-SMA)</td>
<td>Cytoskeletal</td>
<td>Cell contraction</td>
<td>No</td>
<td>Yes</td>
<td>Vascular smooth muscle cells and smooth muscle cells</td>
</tr>
<tr>
<td>Angiotensin 1 (AT1) receptors</td>
<td>Surface</td>
<td>Initiates intracellular signaling pathways involved the renin-angiotensin system</td>
<td>Yes</td>
<td>Yes, increased expression</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>DDR2</td>
<td>Surface</td>
<td>Growth, migration, and differentiation</td>
<td>Yes</td>
<td>Yes</td>
<td>Endothelial and vascular smooth muscle cells</td>
</tr>
<tr>
<td>ED-A fibronectin</td>
<td>Secreted</td>
<td>Adhesive glycoprotein</td>
<td>No</td>
<td>Yes</td>
<td>Smooth muscle cells, endothelial cells, leukocytes, and macrophage</td>
</tr>
<tr>
<td>Fibroblast specific protein 1 (FSP-1/S100A-4)</td>
<td>Cytosolic</td>
<td>May function in motility and tubulin polymerization</td>
<td>Yes</td>
<td>Unknown/not tested</td>
<td>Vascular smooth muscle cells, endothelial cells, leukocytes, and cancer cells</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>ECM</td>
<td>Acidic phosphoprotein adhesion molecule: vascular smooth muscle remodeling and thought to contribute to fibroblast to myofibroblast differentiation</td>
<td>Yes</td>
<td>Yes, increased expression</td>
<td>Bone (osteoblasts and osteocytes), ovarian carcinomas, and dendritic cells</td>
</tr>
<tr>
<td>Periostin</td>
<td>ECM</td>
<td>Cardiac development, remodeling, cell trafficking, and ECM organization</td>
<td>No (not expressed in resting fibroblast cells, but is expressed in development)</td>
<td>Yes</td>
<td>Osteoblasts and cancer cells (glioblastomas)</td>
</tr>
<tr>
<td>Platelet-derived growth factor receptor α (PDGFR α)</td>
<td>Surface</td>
<td>Enhancing migratory and proliferative responses and extracellular matrix (ECM) synthesis</td>
<td>Yes</td>
<td>Unknown/not tested</td>
<td>Platelets</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>ECM</td>
<td>Upregulated in inflammation and tissue remodeling</td>
<td>No (not detectable in normal adult hearts but is expressed during embryonic development)</td>
<td>Yes, increased expression after MI</td>
<td>Smooth muscle cells, neurons, glial cells, and breast carcinoma</td>
</tr>
<tr>
<td>Tensin</td>
<td>Intracellular</td>
<td>Expressed during wound healing</td>
<td>No (not completely confirmed)</td>
<td>Yes, increased expression</td>
<td>Mesangial cells (kidney), myocytes, and most cells in the body</td>
</tr>
<tr>
<td>Thymus antigen 1 (Thy 1/CD 90)</td>
<td>Surface</td>
<td>Cell matrix and cell-to-cell adhesion</td>
<td>Yes</td>
<td>No</td>
<td>Endothelial cells and leukocytes</td>
</tr>
<tr>
<td>Transcription factor (Tcf) 21</td>
<td>Nuclear</td>
<td>Embryonic development</td>
<td>Yes</td>
<td>Yes, Tcf21-positive fibroblasts can convert to myofibroblasts</td>
<td>Smooth muscle cell lineages</td>
</tr>
<tr>
<td>Transforming growth factor-β type II receptor</td>
<td>Surface</td>
<td>Regulates transcription of TGF-β responsive genes</td>
<td>Yes</td>
<td>Yes, increased expression</td>
<td>Myocyte cells and endothelial cells</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Cytoskeletal</td>
<td>Motility and cell shape</td>
<td>Yes</td>
<td>Yes, increased expression</td>
<td>Endothelial and vascular smooth muscle cells</td>
</tr>
<tr>
<td>Wilms tumor (WT) 1</td>
<td>Nuclear</td>
<td>Cardiac development</td>
<td>Yes</td>
<td>Unknown</td>
<td>Endothelial cells and epicardial cells</td>
</tr>
</tbody>
</table>
How do CF react when cardiac homeostasis is threatened, in acute injury circumstances? As before mentioned, immediately after MI, the necrotic myocytes start releasing certain damage-associated molecular patterns that trigger specific innate immune pathways, responsible for initiating the inflammatory phase of the infarct remodeling. Particularly in CF, the activation of these pathways induces a large-scale production of proinflammatory cytokines and chemokines. During this phase, diverse immune and reparative cell types are recruited to help in the healing process and repopulate the infarcted area. For the migration and proliferation of these cells, new paths must be opened throughout the myocardium crosslinked matrix, by ECM degradation and fragmentation. However, this has some side effects, since it consequently affects the matrix structural integrity and exposing the resident CF to injurious mechanical stress.

Following these changes in the myocardium mechanical microenvironment, fibroblasts enter in the proliferative phase of heart remodeling, as their resident population is stimulated and induced to differentiate into proto-myofibroblasts. In addition to these local fibroblasts, other cell populations detaining a similar phenotype are recruited to transdifferentiate into proto-myofibroblasts, where some epithelial and endothelial cells are included, as well as some circulating cells named fibrocytes. However, despite all these existing origins for activated fibroblasts, each population relative contribution to the activated CF cell population remains to be better studied and established. This immature form of myofibroblasts are defined for exhibiting cytoplasmic β-actin and γ-actin stress fibers and focal adhesions, but not expressing α-smooth muscle actin (α-SMA). When exposed to TGF-β1 and ED-A fibronectin, these mediators are responsible for triggering the proto-myofibroblasts differentiation into myofibroblasts. The TGF-β presence within the cardiac interstitium is responsible for activating the Smad3 signaling cascade, which consequently promotes higher α-SMA transcription in fibroblasts. The activated Smad3-dependent pathway in CF induces integrin expression as well, accountable for fibroblast organized arrays present at the infarct border zone. The myofibroblasts also display a crucial role in the formation of scar tissue, by synthesizing and depositing increased proportions of collagen, fibronectin and laminin fibers, as well as non-fibrillar collagens (e.g. collagen VI) in the cardiac ECM. Once the synthesized fibrotic scar is completely formed and starts to mature, myofibroblasts that don’t undergo apoptosis return to their quiescence phenotype.

1.2.2. Cardiac fibrosis disease models

To better understand MI’s pathophysiological scenario and be able to develop efficient anti-fibrotic therapeutic approaches, a deeper understanding regarding the biological mechanisms and pathways involved in the cardiac fibrosis remodeling process is needed. Since these molecular channels are difficult to functionally assess in live human organisms, researchers commonly use disease animal models, which, ideally, closely reflect the complex pathological mechanisms found in humans. Choosing the more adequate animal model for the designed research study specifically is the key action to enable obtaining a more accurate outcome and translational purpose for it. Nevertheless, certain HF and myocardial fibrosis aspects cannot be fully reproduced in animal models. For example, in coronary artery
disease, atherosclerosis gradually causes the arteries’ narrowing, however, in animal models this is impracticable, being the infarction alternatively provoked by sudden artery occlusion\textsuperscript{40}.

There are numerous rodent models available for the modeling chronic HF main causes, such as hypertension, diabetes, metabolic syndrome, or even the combination of these various factors\textsuperscript{40}. The animal models for MI differ between themselves according to their availability and ease of use, but also to cardiac fibrosis mechanism of action, time course, and severity. Usually, to model cardiac fibrosis researchers use rodents subjected to surgical interventions\textsuperscript{41}. Ligating the mice coronary artery to reproduce and modeling MI, followed by reperfusion, leads to scarring, cardiac remodeling, and fibrosis\textsuperscript{42}. However, since the rodent models’ direct translation to clinics is problematic, generally for translational research large animal models are used, as they share a higher genetic homology with humans than rodents do. Indeed, pigs have a very similar cardiac anatomy and circulation physiology to humans and are suitable for translational cardiology\textsuperscript{43,44}. In porcine models, MI is modeled by occluding the coronary arteries using ameroid constrictors placed during open heart surgery\textsuperscript{45,46}.

Alternatively, there have been also advances in modeling in vitro cardiac fibrosis, opening new avenues for drug assessment, since it is associated with low cost, good controllability and availability, as well as the convenience for operation as compared to the animal models\textsuperscript{47,48}. Recent work has been published using hiPSC as a cell source to generate differentiated CF, by modulating Wnt signaling\textsuperscript{48}. hiPSC are a powerful tool in regenerative medicine, drug discovery and cell-replacement therapy since they present self-renewal ability and potential to differentiate into specialized cells, namely CF\textsuperscript{49}. On the protocol recently published by the Joseph Wu laboratory, Zhang and colleagues were able to efficiently generate quiescent CF for in vitro modeling of MI and drug screening.

### 1.2.3. Fibroblasts as therapeutic targets in myocardial infarction

As already mentioned in previous sections, CF are the key cellular effectors involved in heart remodeling. Besides modulating the inflammatory reaction upon the injury, the fibroblasts secrete a large quantity of ECM proteins as well, which constitute the fibrotic scar that replaces the dead myocytes. Since multiple signaling pathways contribute to fibroblasts essential role throughout this fibrotic response post-MI, targeting them and intentionally activate or inhibit some of their crucial stages can help attenuate the myocardium remodeling and, hence, improve the cardiac function. For example, targeting the renin–angiotensin–aldosterone system with angiotensin-converting enzyme inhibitors and angiotensin II type 1 (AT1) receptor blockers can attenuate cardiac fibrosis and, thus, preventing the HF development and reducing the associated mortality\textsuperscript{50}. Additionally, the cardiac fibrosis in patients with acute MI can also be attenuated by decreasing the amount of pro-fibrotic markers circulating in plasma via the administration of aldosterone antagonists\textsuperscript{51}. On the other hand, approaches interfering with fibrogenic growth factor mediated cascades, such as TGF-β1/Smad3 signaling or FGF-2 can be promising therapy for preventing the fibrotic cardiac remodeling\textsuperscript{52,53}. Nonetheless, experimental animal studies suggest carefulness when applying strategies that may impact on matrix metabolism post-MI.
Patients who survive an MI can differ in a pathophysiological manner, depending on their age, gender, genetic susceptibility, possible existing comorbidities and the use of pharmaceuticals which can affect fibrogenic signaling. However, due to heterogeneity between patients (distinct biomarkers), more mechanistic-guided approaches should be developed to treat MI patients\textsuperscript{54,55}. On the other hand, individuals with overactive TGF-β responses, which might benefit from Smad inhibition therapeutic strategies, can be identified through biomarkers reflecting the matrix synthesis. Despite all the scientific advances already made in this field, further investigation is needed to achieve more efficient therapeutic approaches, targeting CF and their role during cardiac fibrosis.

1.3. CCBE1 on lymphangiogenesis and heart development

In MI circumstances, as already mentioned in above sections, myocytes die in a large-scale due to a coronary artery obstruction which blocks, partially or totally, the nutrients and oxygen supply to the myocardium. Simultaneously, excessive fluid accumulation is caused within the cardiac interstitium, as the hyper-permeabilization of the myocardial microvasculature, resultant from MI, turns the capillary fluid filtration faster than the lymphatic fluid removal\textsuperscript{56}. Consequently, this myocardial edema leads to a strong impairment and damaging of the microvascular and cardiac function\textsuperscript{57}. As a matter of fact, some studies state that small changes in the amount of water within the myocardial tissue are responsible for increasing the heart’s total weight and leading to an abrupt decrease in heart’s ability to maintain a normal cardiac output\textsuperscript{56,58}. Additionally, as the organism senses the fluid accumulation, as well as the dead cells and ECM debris, a fibrotic response is triggered to consequently help the heart remodeling\textsuperscript{59}.

Initially, an inflammatory reaction takes place, during which some innate immune pathways are activated, together with the recruitment of several immune cells’ subpopulations, such as neutrophils and macrophages, to clean the infarcted area. However, some proinflammatory cytokines and ROS involved in this cardiac fibrosis phase as inflammation mediators negatively impact the lymphatic system, through the decrease or temporary surcease of the lymphatic pump flow\textsuperscript{60,61}. In addition to this interference on the cardiac lymphatic transport, during the ischemic injury, not only do CM die on a large-scale, but the lymphatic vessels are also greatly affected by the destruction of cardiac tissue\textsuperscript{59}. As result, a robust endogenous lymphangiogenic response is triggered to restore the basal lymphatic vasculature density and its usual interstitial flow. However, the dense lymphatic network resultant from this remodelling process comprises inadequate small vessels, whose lumens are not wide enough to improve the drainage of the excessive proteins and fluid in the cardiac interstitium. Consequently, the myocardial edema persists and starts to become chronical, while the cardiac function also continues being compromised\textsuperscript{59,62-64}. Despite the lymphangiogenesis of the small, blind-ended lymphatic capillaries being the most obvious change and gathering more promptly the attention, a more therapeutically relevant target in this injurious scenario may be maintaining the function of deeper collecting lymphatic vessels\textsuperscript{57,59}.

At the beginning of this century, it has been demonstrated that the administration of exogenous vascular endothelial growth factor C (VEGF-C) has a therapeutic effect in restoring the lymphatic
function in chronic, acquired lymphedema. More recently, some studies have exploited this knowledge to investigate the lymphangiogenesis influence on the heart’s function and microenvironment after MI. Interestingly, these studies in infarction animal models have demonstrated that applying a VEGF-C treatment improves pre-collector and collector lymphatic vessels remodeling and capillary lymphangiogenesis. Consequently, the myocardial edema and cardiac inflammation reduce and become eventually resolved, thus helping to recover a normal cardiac function. All these findings taken together support the therapeutic stimulation of cardiac lymphangiogenesis with VEGF-C, suggesting it as a promising novel approach to prevent adverse cardiac remodeling post-injury. Besides being a key mediator of lymphangiogenesis, VEGF-C was recently stated to also impact on myofibroblasts’ growth and function, by stimulating TGF-β1 production and ERK phosphorylation. Further studies may be performed to deeper understand VEGF-C functional role either in cardiac lymphangiogenesis, as well as in cardiac fibrosis.

Around the same time VEGF-C crucial impact on lymphedema circumstances was discovered, its role as VEGFR-3 ligand also started to gather greater attention. Further studies about this matter revealed that, by binding VEGF-C to this specific receptor tyrosine kinase, VEGFR-3 becomes activated and induces lymphangiogenesis in adult tissues, through the stimulation of lymphatic endothelial cell migration and proliferation. However, to exert its key ligand function, the secreted VEGF-C precursor molecule itself needs to be activated. Collagen and calcium-binding EGF domain-containing protein 1 (CCBE1) exerts its pro-lymphangiogenic effect during the VEGF-C maturation process. CCBE1 has already been demonstrated to enhance A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) enzyme’s protease role, which is responsible for removing the N-terminal pro-peptide from pro-VEGF-C and consequently form a fully active and mature VEGF-C. Indeed, while CCBE1 C-terminal plays a crucial role along the proteolytic processing, its N-terminal domain helps CCBE1 be colocalized, along with ADAMTS3 and pro–VEGF-C, on lymphatic endothelial cells’ surface, which can help turning VEGF-C activation more efficient.

In addition to the research performed using mice and zebrafish models, some studies suggest that mutations in CCBE1 gene are related with a severe form of lymphatic vessels dysplasia, lymphedema-cholestasis syndrome and fetal hydrops, thus supporting CCBE1 important role during embryonic and adult lymphangiogenesis. Nonetheless, some research groups have recently reported that CCBE1 also performs other crucial functions, when present in completely different pathological settings from the abovementioned. Barton et al. have suggested CCBE1 as a promising tumour suppressor in ovarian cancer, since CCBE1’s reduced expression in ovarian cancer cell lines and primary carcinomas can enhance these cells migration and survival, and hence, promote ovarian carcinogenesis. This protein was also considered as a potential GIST’s therapy target due to its capability to promote angiogenesis and mediate drug resistance of gastrointestinal stromal tumour cells to imatinib (a tyrosine kinase inhibitor used in certain cancers treatment). Moreover, CCBE1 appeared downregulated in lung cancer patients with lymph node metastasis and correlated with a poorer clinical outcome, suggesting CCBE1 may be a possible biomarker for lung cancer prognosis.
Figure 1.3 – Lymphangiogenesis: VEGF-C activation by CCBE1 and ADAMTS3 complex. CCBE1 promotes the proteolytic cleavage of pro-VEGF-C form by the disintegrin/metalloprotease ADAMTS3, thus originating mature form of VEGF-C that further activates VEGFR-3 receptor. Most of the VEGF-C cleavage mediated by CCBE1 and ADAMTS3 occurs on the lymphatic endothelial cell (LEC) surface (adapted from 87).

The close relationship between lymphatic and cardiovascular systems, together with the recently unveiled CCBE1 role during lymphangiogenesis, caused researchers to question and investigate any impact this protein could have along the mammalian cardiogenesis. In a first study, Facucho-Oliveira and colleagues identified a CCBE1 higher expression, between day E7.0 to E9.5, on early cardiac precursors from the first and second heart field (FHF and SHF, respectively) and the proepicardium88. Additionally, this protein appeared downregulated as the progenitor cells started differentiating into more specific cardiac lineages88. Further studies using chick embryos allowed to corroborate the previously discovered CCBE1 expression profile in vivo, as well as uncover that CCBE1 protein expression later becomes restricted to the SHF89. Furthermore, CCBE1 was also shown to be involved, in mice, with the development of the sinus venosus–derived subepicardial coronary vessel and arteries, independently of their embryonic origins90. Although these findings support CCBE1 as a strong target as cell-free therapeutic approaches in CVDs patients, little is known about its impact on differentiated cardiac cells, such as CF, or in MI specific circumstances. Indeed, further studies should be performed to assess CCBE1 role within the different cardiac lineages, as well as towards the characteristic conditions of cardiac fibrosis.
2. Aim of the Thesis

CCBE1 has been identified as an essential protein during embryonic and adult lymphangiogenesis, being particularly involved in the maturation process of VEGF-C growth factor. Due to the close relationship between the lymphatic and cardiovascular systems, recent studies have been exploring CCBE1 role along the mammalian cardiogenesis. Despite existing evidence of a potential involvement in cardiac commitment, little is known about CCBE1 function in the cardiac fibroblasts (CF). Therefore, the aim of this thesis was to unveil the role of CCBE1 on CF differentiation and functionality, by using gene editing tools (CRISPR interference) and human induced pluripotent stem cells (hiPSC).

With this purpose, a differentiation protocol for generating CF was first implemented, by using a wild-type hiPSC cell line (hiPSC-WTC) and temporally modulating Wnt/β-catenin signaling pathway via small molecules. Then, to evaluate the role of CCBE1 along the commitment and CF fate, two modified hiPSC-CRISPR interference lines (control and CCBE1 KD) were further differentiated into CF using the previously implemented protocol. The impact of CCBE1 KD, exploring distinct Dox-treatments, on cells’ morphology and phenotype along the different stages of differentiation was assessed. Moreover, to reduce the percentage of activated CF being generated at the end of differentiation for further functionality studies, a distinct fibroblast growth medium combined with a TGF-β inhibitor was used. Finally, the differentiated CF were characterized through several passages in culture, TGF-β activation into myofibroblasts and migration capacity.

Overall, this work aims to provide novel perceptions on CCBE1 role in CF function, which could hopefully potentiate the use of CCBE1 as an alternative therapeutic molecule in cardiovascular regenerative medicine.

Figure 2.1 - Schematic representation of the major aims of this thesis. The main aim of this thesis was to unveil the role of CCBE1 on CF differentiation and functionality, hiPSC - human induced pluripotent stem cells; CCBE1 - collagen and calcium-binding EGF domain-containing protein 1; CF – Cardiac Fibroblasts; CRISPRi – CRISPR interference.
3. Materials & Methods

3.1. hiPSC culture & differentiation

3.1.1. hiPSC lines
In this study, two human iPSCs lines with the same genetic background were used, a Wild-type C (hereafter designated as hiPSC-WTC) and a modified cell line CRISPRi Gen1C (hereafter designated as hiPSC-CRISPRi), derived by Mandegar and colleagues\(^91\), which integrates a Tet ON inducible system that modulates the expression of a deactivated Cas9 (dCas9) fused with the repressor KRAB domain. In previous work from our group, the last-mentioned line was transfected, using Neon Transfection System (Thermo Fisher Scientific), with a pgRNA-CKB expression vector (Addgene plasmid #73501), containing mKate2 as a reporter gene and blasticidin as antibiotic selection marker\(^92\). Through this method, two cell lines were generated: CRISPRi-KD cell line, carrying a CCBE1-specific gRNA to target the gene’s transcription start site (TSS) at 37 bp downstream; and CRISPRi-Ctrl cell line, with empty pgRNA-CKB vector. Both cell lines were cultured with blasticidin, to select the nucleofected cells, and its percentage was evaluated by mKate2 expression using an inverted fluorescence microscope (Leica Microsystems GmbH).

3.1.2. hiPSC expansion
hiPSC lines were regularly propagated in static culture systems using T25 and T75 flasks (Falcon™), coated with growth factor reduced (GFR) Matrigel®, Phenol Red Free (BD Biosciences), in mTeSR™1 medium (STEMCELL Technologies), according to the protocol described by Mandegar and colleagues\(^91\). Cells were maintained under humidified atmosphere with 5 % CO2 at 37°C and the culture medium was exchanged daily. At 80-90 % of confluence, cells were passaged using Accutase (STEMCELL Technologies), and seeded at 2-3 × 10^4 cell/cm^2 using mTeSR™1 supplemented with ROCK inhibitor (Y-27632; TOCRIS).

3.1.3. hiPSC cardiac fibroblasts differentiation
hiPSC were differentiated into cardiac fibroblasts (hiPSC-CF) in monolayer culture systems, according to protocol published recently by Bao et al.\(^93\). hiPSC cultures were detached from monolayers by incubation with Accutase for 5 min at 37°C and seeded as single cells (2-3 × 10^5 cell/cm^2) on GFR Matrigel-coated 12-well plates (Falcon™). The protocol is divided into 3 stages as shown in Figure 4.1: cardiac progenitors (hiPSC-CP), pro-epicardium and cardiac fibroblasts. Cell differentiation was initiated three days after cell seeding (day 0). The first stage of differentiation was induced by replacing the expansion culture medium with RPMI 1640 basal medium (Thermo Fisher Scientific) supplemented with 6 µM CHIR99021 (TOCRIS). Twenty-four hours later (day 1), the medium was replaced by RPMI 1640 basal medium. At day 3, cells were incubated with a “combined medium”, containing, per well, 1 mL of the spent medium and 1 mL of freshly prepared RPMI 1640 basal medium supplemented with 2.5 µM
IWP-2 (TOCRIS). At day 5, the medium was replaced with RPMI 1640 medium. At day 6, hiPSC-CPCs were passaged, using Accutase for 5 min at 37°C, and seeded at a density of $8 \times 10^4$ cell/cm² on Synthexam II-SC (Corning®)-coated 12-well plates to induce the second stage of differentiation. The dissociated cells were re-suspended in LaSR medium (Advanced DMEM/F12 medium (Thermo Fisher Scientific), supplemented with GlutaMAX™ (Thermo Fisher Scientific), 100 mg/mL Ascorbic Acid (Sigma-Aldrich)), with 5 µM Y-27632 (TOCRIS) supplemented. From day 7 to day 8, medium was replaced by LaSR medium supplemented with 3 µM CHIR99021 (TOCRIS). From day 9 to day 11, medium was exchanged daily by LaSR medium.

The last stage of differentiation started at day 12. hiPSC-Proepicardial were dissociated into single cell by incubation with Accutase, for 5 min at 37°C, and seeded at a density of $3 \times 10^4$ cell/cm² on 0.1% (w/v) Gelatin (Sigma-Aldrich)-coated 12-well plates with LaSR medium supplemented with 5 µM Y-27632, 2 µM SB431542 (STEMCELL Technologies) and 1% (v/v) FBS (HyClone™). From day 13 until day 18, the medium was replaced daily with LaSR medium supplemented with 10 ng/mL rh-FGFb (R&D Systems). During this period, an alternative fibroblasts culture medium was also tested, FibroGRO™ medium (Millipore Corporation), supplemented with 10 μg/mL rh-FGFb and 2 μM SB431542, according to a recently published protocol.48

From day 18 onwards, for the long maintenance of CF, cells were passaged every 4 to 6 days using 0.05% (v/v) Trypsin-EDTA (Thermo Fisher Scientific), at a density of 5800 cell/cm², on 0.1% (w/v) gelatin-coated 6- or 12-well plates, with LaSR medium supplemented with 10 μg/mL rh-FGFb or in FibroGRO™ medium supplemented with 2 μM SB431542. Cells were maintained under humidified atmosphere with 5% CO₂ at 37°C and medium was replaced every second day.

3.2. Characterization of hiPSC and differentiated cells

3.2.1. Cell concentration and viability

hiPSC and differentiated cells were enzymatically dissociated into single cell suspension, by incubation with Accutase (STEMCELL Technologies) or 0.05% (v/v) Trypsin-EDTA (Thermo Fisher Scientific), respectively, for 5 min at 37°C. All cells were then diluted in 0.1% (v/v) Trypan Blue (Thermo Fisher Scientific) in DPBS (Thermo Fisher Scientific). According to the Trypan Blue exclusion principle, this dye cannot enter the cell through the viable cells’ intact membrane, however, it enters in non-viable cells that possess damaged membranes, allowing the quantitative determination of cells viability and concentration after counting the cells in a hemocytometer counting chamber (Fuchs-Rosenthal).

3.2.2. Cell proliferation capacity

To determine the percentage of proliferating hiPSC-CF, a Click-iT EdU (5-ethynyl-2’-deoxyuridine) Flow Cytometry (FC) Assay Kit (Thermo Fisher Scientific) was used following the manufacturer’s recommended protocol. EdU incorporation into DNA molecules during the cellular replication process enables the detection of cell proliferation, since it corresponds to a thymine analog. Cell cultures were
incubated with 10 μM EdU in culture medium for 24 h at 37°C. For the analysis by Flow Cytometry (FC), cells were washed once with 1 % (v/v) bovine serum albumin (BSA) (Thermo Fisher Scientific) in DPBS (washing buffer) and fixed with the fixative solution (Life Technologies) provided in the Kit for 15 min. Then, cells were permeabilized with 1 % (v/v) saponin in DPBS (saponin-based working reagent) for 15 min. Finally, cells were incubated with Click-iT AlexaFluor® 488 azide, for 30 min, and washed twice before analysis. All incubations periods were performed at room temperature (RT, 20-25°C) and protected from the light. Samples were analyzed in a CyFlow® space instrument (Partec GmbH, Germany) and the quantitative data obtained examined using FlowJo software. At least 10,000 events were registered per sample.

3.2.3. Immunocytochemistry

For the detection of Pro-epicardial and CF markers in differentiated cultures (CRISPRi-Ctrl and CRISPRi-KD), cells were fixed with 4 % (w/v) PFA, for 10-15 min, at RT. After fixation, cells were blocked and permeabilized using 0.1 % (w/v) Tx100 with 0.2 % (w/v) gelatin from cold water fish skin (FSG) in DPBS (+/+) for 30 min at RT, and then, incubated with the primary antibody for 2 hours at RT, protected from the light. Cells were washed three times with DPBS (+/+) and incubated with the secondary antibody, for 1 hr at RT, protected from the light. Finally, cells were washed with DPBS (+/+) and cell nuclei were counterstained with 4',6-Diamidino-2-Phenylindole (DAPI). Preparations were visualized in an inverted fluorescence microscope (Leica DMI6000 B; Leica Microsystems GmbH). Primary and secondary antibodies used are listed in 

Table 3.1.

3.2.4. Flow Cytometry

Undifferentiated and differentiated hiPSC were harvested and dissociated into single cells by incubation with Accutase for 5 min at 37°C. For extracellular markers detection, cells were washed once with 2 % (v/v) FBS (Thermo Fisher Scientific) in DPBS (washing buffer), and then incubated for 1 h at 4°C with the primary antibody. After the incubation period, cells were washed twice with washing buffer. Regarding the non-conjugated primary antibodies, cells were further incubated with the suitable secondary antibody for 30 min at 4°C, followed by two washes with washing buffer. For intracellular markers detection, cells were washed with Intra Buffer (phosphate buffered saline, pH 7.2, with 0.5 % (v/v) BSA and 2 mM EDTA), and fixed using the Inside Fix reagent (Inside Stain Kit; Miltenyi Biotec), mixed 1:1 with Intra Buffer, for 20 min at RT. Cells were incubated with primary antibodies for 30 min in the dark, at RT. Cells were permeabilized with saponin-based working reagent and then incubated with the secondary antibody (30 min in the dark at RT). All samples were analyzed in a CyFlow® space instrument (Partec GmbH, Germany). At least 10,000 events were registered per sample. Quantitative data was analyzed using FlowJo software. All primary and secondary antibodies used are listed in 

Table 3.1.
Table 3.1 - List of all antibodies and dilutions used for immunocytochemistry (IC) and flow cytometry (FC) analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Supplier</th>
<th>Dilution (Application)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRA-1-60</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>1:10 (FC)</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>1:20 (FC)</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Mouse</td>
<td>Dako</td>
<td>1:100 (FC, IC)</td>
</tr>
<tr>
<td>Coll I</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>1:100 (IC)</td>
</tr>
<tr>
<td>CCBE1</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:100 (IC)</td>
</tr>
<tr>
<td>WT1</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:100 (IC)</td>
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<td>Alexa 488, anti-mouse IgG1</td>
<td>Goat</td>
<td>Life Technologies</td>
<td>1:200 (FC)</td>
</tr>
<tr>
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<td>Goat</td>
<td>Life Technologies</td>
<td>1:200 (FC)</td>
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<td>Alexa 488, anti-rabbit IgG1</td>
<td>Goat</td>
<td>Life Technologies</td>
<td>1:200 (FC)</td>
</tr>
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<td>Goat</td>
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<td>BD Biosciences</td>
<td>1:10 (FC)</td>
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<td>BD Biosciences</td>
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<td>BD Biosciences</td>
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<tr>
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<td>2:5 (FC)</td>
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</table>

3.2.5. mRNA Extraction and RT-qPCR

hPSC and differentiated cells were enzymatically dissociated into single cells suspensions, using Accutase and 0.05 % Trypsin-EDTA, for 5 min and 15 min at 37°C, respectively. Cell pellets were washed with DPBS, snap-frozen with liquid nitrogen and stored at -80°C until mRNA extraction. mRNA was extracted using a High Pure RNA isolation Kit (Roche) according to manufacturer’s instructions and quantified in the NanoDrop 2000c (Thermo Scientific). Regarding the cDNA synthesis, it was carried out using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). LightCycler 480 Instrument II 384-well block (Roche) was used for RT-qPCR and the program cycles as follow: pre-incubation for 10 min at 95°C; 45 cycles of amplification with denaturation at 95°C for 15 seconds and annealing at 60°C for 1 min; extension at 72°C for 5 min. The primers and probes used in this work are listed in Table 3.2. The Cycle threshold (Ct) was determined using LightCycler 480 Software version 1.5 (Roche). The results were, then, analyzed as described elsewhere, using the $2^{-\Delta\Delta Ct}$ method for relative gene expression analysis. The gene expression data was normalized using two housekeeping genes,
RPLP0 and GADPH, and represented relative to a control sample (set at 1). The percentage of CCBE1 knockdown (KD) was determined by using the CRISPRi-Ctrl CCBE1 expression as 100% expression, i.e. 0% CCBE1 KD.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Supplier Reference</th>
</tr>
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<tbody>
<tr>
<td>CCBE1</td>
<td>Hs99999905_m1</td>
</tr>
<tr>
<td>Nanog</td>
<td>Hs02387400_g1</td>
</tr>
<tr>
<td>POU5F1</td>
<td>Hs00999632_g1</td>
</tr>
<tr>
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</tr>
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<td>POSTN</td>
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<td>RPLP0</td>
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<td>GAPDH</td>
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</tr>
<tr>
<td>WT1</td>
<td>Hs01103751_m1</td>
</tr>
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Note: Primers and Probe Mix were purchased from Thermo Fisher Scientific.

3.2.6. Cardiac Fibroblasts activation

hiPSC-CF were activated into cardiac myofibroblasts in monolayer culture systems, according to the work recently published by Zhang et al.\textsuperscript{96}. Single cell suspension of hiPSC-CF was prepared by incubation with 0.05 % (v/v) Trypsin-EDTA, for 10-15 min at 37°C. Cells were resuspended in FibroGRO\textsuperscript{TM} medium and seeded at a density of 5800 cell/cm\(^2\) in 0.1 % (w/v) gelatin-coated 6-well plates. Culture medium was replaced every second day. When cells reached approx. 80-90 % of confluence, cells were incubated with DMEM medium (Thermo Fisher Scientific), supplemented with 10 % (v/v) FBS and 10 ng/mL rh-TGF\textbeta{}1 (R&D Systems), for 48h at 37°C.

An alternative activation strategy was implemented, where cells were resuspended in FibroGRO\textsuperscript{TM} medium supplemented with 10 \mu{}M SB431542, and seeded at a density of 5800 cell/cm\(^2\) in 0.1 % (w/v) gelatin-coated 6-well plates. SB431542 was daily added to the culture, whereas complete medium exchange was performed every second day. At 80-90 % of confluence, three different conditions were screened: 1) FibroGRO\textsuperscript{TM} medium supplemented with 10 \mu{}M SB431542; 2) FibroGRO\textsuperscript{TM} medium supplemented with 10 ng/mL rh-TGF\textbeta{}1; and 3) FibroGRO\textsuperscript{TM} medium supplemented with 10% FBS. Cells from all three conditions were collected after 6, 24 and 48 h, for further gene and protein analysis.
3.2.7. Scratch Assay

hiPSC-CF migration capability was assessed by a scratch assay, adapted from a protocol published by Liang et al.\textsuperscript{97}. hiPSC-CF single-cell suspension was prepared by incubation of cell monolayers with 0.05 % (v/v) Trypsin-EDTA, for 10-15 min at 37°C. After centrifugation at 200g, cells were seeded at a density of 5800 cell/cm\textsuperscript{2} in 0.1 % (w/v) gelatin-coated 24-well plates in FibroGRO\textsuperscript{TM} basal medium. Culture medium was replaced every second day. At 90-100 % of cell confluence, a straight line was scraped in the cell monolayer with a p200 pipet tip and cells were washed twice in FibroGRO\textsuperscript{TM} medium. All the scratches were visualized and time-analyzed in an inverted fluorescence microscope (Leica DMI6000 B; Leica Microsystems GmbH), at 0, 3, 6, 9, and 24 h after the scratch.
4. Results & Discussion

4.1. hiPSC differentiation into Cardiac Fibroblasts

To assess CCBE1 expression and explore its functional role during cardiac fibroblast (CF) commitment, a differentiation protocol, recently described by Bao et al., was implemented following the temporal modulation of Wnt/β-catenin signaling pathway by small molecules.

Human iPSC-WTC were expanded for three days, and then differentiated into CF through the sequential addition of small molecules and growth factors: firstly, CHIR99021 and IWP2, to direct cells into a cardiac progenitor fate; secondly, CHIR99021, to induce pro-epicardial fate; and finally, bFGF, to guide CF commitment (please see section 3.1.3) (Figure 4.1A). The hiPSC-WTC cells were able to differentiate into all stages of differentiation, showing clear morphological changes throughout, with a fibroid spindle-like morphology on the last day (day 18) (Figure 4.1B). The cell concentration at specific stages of the differentiation was also assessed. The highest fold increase in cell concentration (approximately 8) was observed during CPC commitment into pro-epicardial fate (Figure 4.1C). However, throughout the first and last stage of the differentiation (CPC and CF commitment, respectively), the fold-increase observed in cell concentration was approximately 1 (Figure 4.1C).

Undifferentiated and differentiated cells along CF commitment were also characterized phenotypically, by RT-qPCR and flow cytometry analysis. At day 0 of differentiation, hiPSC-WTC cells’ pluripotency was confirmed by 88.9 % and 98.9 % of cells expressing stemness markers TRA-1-60 and SSEA-4, respectively, along with 0.6 % of cells expressing SSEA-1 (early differentiation marker) (Figure 4.1D). The commitment into CPC was demonstrated by the presence of 35.1 % of Nkx2.5+ cells in culture and the significant gene expression of CPC markers (Nkx2.5 and GATA4) detected on day 6 (Figure 4.1D-E). From day 6 to day 12, an upregulation of GATA4 and WT1 was evidenced by gene expression analysis, showing the differentiation into epicardial lineage rather than cardiomyocyte lineage (similar expression levels of TNNT2 at day 6 and 12) (Figure 4.1E). When comparing to the literature, GATA4 presents a contrasting expression profile, detaining a lower expression at CPC differentiation stage, that might be related to the different genetic backgrounds of the distinct cell lines used. Finally, the differentiation into CF was confirmed by the expression of fibroblast-associated markers, such as GATA4, Periostin (POSTN), CD90 and α-SMA (Figure 4.1D-E). The cardiac transcription factor GATA4 has been reported to be expressed in CPC and CF and we found similar expression levels in CPC and differentiated CF (Figure 4.1E). Periostin is a secreted protein expressed in mouse CF during embryonic development and in CF differentiated from iPSC-epicardial cells. Here, similar levels of POSTN were observed at day 12 and day 18 (Figure 4.1E). Intriguingly, a recent study showed a different expression profile in hiPSC-CF, where CD90 was expressed on only a fraction of hiPSC-CF (30 %) compared to human dermal fibroblasts (90 %). Nevertheless, the use of different fibroblast markers, as we explored herein, allowed us to identify the CF population. In summary, at the end of differentiation, WTC-derived CF presented morphologic and phenotypic properties typical of CF,
as proved by phase-contrast microscopy, flow cytometry, and gene expression analysis (Figure 4.1B-E), similar to what has been reported literature\(^{48,93,96}\).

Figure 4.1 – Differentiation of WTC into cardiac fibroblasts. (A) Schematic differentiation protocol, using small molecules and growth factors for Wnt signaling modulation, in hiPSC lines, as recently published\(^{93}\). (B) Morphology of hiPSC-WTC cell culture, at day 0, 6 (CPC state), 12 (pro-epicardial cell state) and 18 (CF state) of differentiation. Scale bar, 100 µm. (C) hiPSC-WTC fold increase in cell concentration, along the differentiation protocol. (D) Flow cytometry analysis, in hiPSC-WTC, of pluripotency markers, TRA-1-60 and SSEA4, and mesoderm marker, SSEA1 at day 0; of cardiac progenitors’ marker, Nkx2.5, at day 6; of cardiac fibroblasts markers, α-SMA and CD90, at day 18. (E) Relative expression of cardiac progenitor (Nkx2.5 and GATA4), pro-epicardium (WT1), cardiomyocyte (TNNT2), and cardiac fibroblast (POSTN) genes in hiPSC-WTC along the differentiation. Gene expression was quantified using the 2^ΔΔCT method, relatively to day 0, 6 or 12 of differentiation, depending on the gene (housekeeping genes: RPLP0 and GAPDH). (F) Relative expression of CCBE1 gene in hiPSC-WTC line, from day 0 to day 18 of differentiation.
The CCBE1 expression profile was also assessed through RT-qPCR, where the gene showed an upregulation on days 6 and 12 and a downregulation by day 18, when compared to CCBE1 expression level at day 0 of differentiation (Figure 4.1F). This result is consistent with what is described in the literature for mice and chicken embryos. In these studies, CCBE1 is expressed in First Heart Field, Second Heart Field and pro-epicardium (PE), which corresponds to the CPC phase during the heart development68,69.

4.2. Impact of CCBE1 during CF differentiation

4.2.1. CCBE1 knockdown on iPSC-derived CF

To study the impact of CCBE1 on CF, two hiPSC lines (hiPSC-CRISPRi-Ctrl and hiPSC-CRISPRi-KD), harboring an inducible-dCas9 system for CCBE1 knockdown (KD), were used and differentiated into CF, following the protocol implemented with WTC cell line in section 4.1 (Figure 4.2A). This inducible system, in which the deactivated Cas9 (dCas9-KRAB) binds to CCBE1-targeting gRNA to block its expression, as described for other genes elsewhere51, allows us to easily KD the expression of CCBE1 by the simple addition of doxycycline (Dox). Within this context, to understand CCBE1 role on the derived CF, Dox started being added on both CRISPRi cultures only from day 15 of differentiation onwards, throughout 8 days straight (Figure 4.2A). Both of the cell lines used had been previously generated, in our laboratory, to investigate the CCBE1 role on CM differentiation82.

Throughout all stages of the differentiation process, both CRISPRi-Ctrl and CRISPRi-KD cells showed identical morphological changes, presenting a typical fibroid spindle-like morphology on the last day of differentiation (day 18) (Figure 4.2B). Since such observations were similarly detected on hiPSC-WTC in the former experiment, it was possible to conclude that start adding Dox throughout the last stage of differentiation did not impact on hiPSC-CF morphology on day 18. Additionally, both cell lines also displayed very similar growth profiles (Figure 4.2C). Nevertheless, when compared to hiPSC-WTC line, lower fold increase in cell concentration at CF differentiation stage was obtained for CRISPRi-KD culture (Figure 4.2C).

Stage-specific gene and protein expression levels were analyzed as well. At first day of differentiation (day 0), both CRISPRi lines displayed a high percentage (> 80 %) of TRA-1-60+ and SSEA-4+ cells, and a low percentage (< 5 %) of cells expressing SSEA-1 (Figure 4.2D). At day 6, CRISPRi-Ctrl and CRISPRi-KD commitment into CPC was confirmed by the Nkx2.5 expression, together with the decreased percentage (<50%) of TRA-1-60+ cells observed in both cultures (Figure 4.2D, 4.3A). Nevertheless, RT-qPCR results showed very low Nkx2.5 expression level in CRISPRi-KD cells, not correlated with flow cytometry data. Since transcription and translation processes can be differently regulated, mRNAs abundance only partially correlates to the protein abundance (reviewed in 102). A possible interpretation for this difference herein might be related to mRNA low stability. Indeed, Nkx2.5 mRNA molecule was stated to have on its 3’ untranslated region an AU-rich element, a sequence...
motif which has been already related to Nkx2.5 mRNA decay and to the degradation of transiently expressed messengers\textsuperscript{103,104}.

Figure 4.2 – Differentiation of hi-PSC-CRISPRi-Ctrl and -KD cells into cardiac fibroblasts. (A) Schematic representation of doxycycline supplementation mode (2 µM from day 15 onwards) along CF differentiation using both CRISPRi-Ctrl and CRISPRi-KD cell lines. (B) Morphology of hiPSC-CRISPRi-Ctrl and -KD cell culture, at day 0, 6 (CPC state), 12 (pro-epicardial cell state) and 18 (CF state) of differentiation. Scale bar, 100 µm. (C) CRISPRi-Ctrl and CRISPRi-KD hiPSC fold increase in cell concentration, along the differentiation protocol. (D) Flow cytometry analysis, in both hiPSC-CRISPRi line, of pluripotency markers, TRA-1-60 and SSEA4, and early differentiation marker, SSEA1 at day 0; of cardiac progenitors’ marker, Nkx2.5, at day 6; of cardiac fibroblasts markers, α-SMA and CD90, at day 18.

During mouse heart development, WT1 has been detected in PE\textsuperscript{105,106}. Thus, \textit{WT1} expression peak should only appear by day 12 of the differentiation, since it corresponds to the final day of the pro-epicardial commitment stage and it was already seen for the hiPSC-WTC line (Figure 4.1E). However, the \textit{WT1} profile on CRISPRi-KD cells showed a peak of expression at day 6 of differentiation, followed by a downregulation on day 12 (Figure 4.3A). \textit{GATA4} has been identified as an essential factor on PE
formation during the early cardiac development\textsuperscript{107}. In CRISPRi-KD culture, it was also showed a lower expression level on day 12 of differentiation, rather than on day 6 (Figure 4.3A). Therefore, these different profiles of \textit{WT1} and \textit{GATA4} expression in CRISPR-KD culture, together with the lower expression of \textit{NKX2.5} might suggest that the committed CPCs' early differentiated into the pro-epicardial fate. In contrast, the differentiation toward CRISPRi-Ctrl pro-epicardial cells was confirmed by \textit{WT1} and \textit{GATA4} high expression levels on day 12, similarly to what occurred in the hiPSC-WTC culture (Figure 4.3A).

Besides all the possible and existing differences, both lines have shown identical levels of \textit{TNNT2} expression on days 6 and 12, demonstrating their tendency for differentiating into epicardial lineage rather than CM (Figure 4.3A). In addition, by day 18, the CRISPRi lines finally presented a CF typical phenotype, with both populations displaying high percentages of CD90\textsuperscript{+} and \textalpha{-SMA}\textsuperscript{+} cells, as well as high expression levels of \textit{POSTN} (Figure 4.2D, 4.3A). Concluding, despite some inconsistent results along hiPSC-CRISPRi-KD differentiation, in contrast to hiPSC-CRISPRi-Ctrl identical behavior to hiPSC-WTC line, at the end of differentiation both CRISPRi-derived CF presented morphologic and phenotypic CF typical characteristics (Figure 4.2B-D, 4.3A).

To induce dCas9-KRAB expression and thus enable the KD of \textit{CCBE1} gene expression, Dox must be added for 7 days minimal, as described by in the literature\textsuperscript{91} and confirmed in our laboratory previous work\textsuperscript{92}. Therefore, an 8-day Dox-treatment was initiated for both CRISPRi lines at day 15 of differentiation (Figure 4.2A). As mCherry reporter gene is under the control of the same promoter as dCas9-KRAB, fluorescence microscopy images were taken to evaluate these cells' responsiveness to Dox. For both CRISPRi lines, all images captured on day 18 and 22 showed low mCherry intensity signal (Figure 4.3B). However, Mandegar and colleagues already showed that KD can occur even if cells do not display detectable mCherry expression, since just a small quantity of dCas9-KRAB protein is needed to induce the KD\textsuperscript{91}. Indeed, through RT-qPCR analysis for both CRISPRi lines it was possible to observe a 58\% knockdown of \textit{CCBE1} gene expression in CRISPRi-KD line (Figure 4.3C). Additionally, CRISPRi-KD cells had an identical \textit{CCBE1} expression profile along CF differentiation when compared to hiPSC-WTC culture, while CRISPRi-Ctrl culture presented a continuous downregulation from day 6 to 18.

Despite the moderate KD obtained here, previous experiments from our group and others have demonstrated higher KD percentages (80 \% - 90 \%) in differentiated cells, either for \textit{CCBE1} gene\textsuperscript{92} or others specific-lineage markers\textsuperscript{91}. Such observations raise the question if Dox should be added earlier in the differentiation process, more specifically during its iPSC expansion stage, in order to obtain a higher KD of \textit{CCBE1} expression on CF.
Figure 4.3 – Phenotypical characterization of hi-PSC-CRISPRi-Ctrl and -KD cells along the differentiation. (A) Relative expression of cardiac progenitor (NKX2.5 and GATA4), pro-epicardium (WT1), cardiomyocyte (TNNT2), and cardiac fibroblast (POSTN) genes in hiPSC-CRISPRi lines along the differentiation. Gene expression was quantified using the 2^{-ΔΔCT} method, relatively to day 6 or 12 of differentiation, depending on the gene (housekeeping genes: RPLP0 and GAPDH). (B) Detection of mCherry reporter in CRISPRi-Ctrl and CRISPRi-KD -derived CF, at day 22, to assess dCas9 induction with Dox efficiency. Scale bar represent 100 μm. (C) Relative expression of CCBE1 gene in hiPSC-CRISPRi lines, from day 6 to 18 of differentiation, along with the percentage of knockdown observed in CCBE1 expression.

4.2.1. CCBE1 knockdown on hiPSC and during CF differentiation

To understand if the addition of Dox from the hiPSC stage to derived CF impacts on the CCBE1 KD, as well as on hiPSC commitment into CF itself, a second differentiation was performed. Both cell lines were differentiated in the presence of Dox since day -2 of expansion, however, at day 2 and 4 of differentiation, there was no Dox addition, since no medium replacement was performed (Figure 4.4A).

Within this context, the CCBE1 expression profile was analyzed throughout all the differentiation stages. From day 0 to day 3, there were observed poor expression levels of CCBE1 gene in both hiPSC-CRISPRi-Ctrl and CRISPRi-KD line. However, an upregulation arises on day 6 of differentiation, which is then followed by a continuous decrease of CCBE1 expression until day 18 (Figure 4.4B). Although CRISPRi-KD presented a lower expression between day 0 to day 3 than CRISPRi-Ctrl did, both cultures showed a similar gene expression profile throughout, concordant with what Facucho-Oliveira and
colleagues had already described. The minor expression levels displayed by CRISPRi-KD along the first days of the differentiation enabled to conclude its CCBE1 gene expression was being knocked down. Indeed, by day 0 and 3, the CRISPRi-KD cell line presented 56% and 77% of KD, respectively. Nevertheless, by day 6, cells only displayed 7% of CCBE1 expression knock downed, and from this day onwards, none KD was furtherly detected (Figure 4.4B).

Such observations on the gene expression analysis are confirmed by the fluorescence microscopy images captured. Although KD can occur even still cells do not display detectable mCherry expression on day 0 of differentiation, the two CRISPRi lines displayed indeed a high mCherry intensity signal, which then abruptly faded from day 7 onwards (Figure 4.4C). When comparing the results with the former experiment, similar fluorescence microscopy images were obtained, however, at day 18, CRISPRi-KD cell line displayed a significant KD of CCBE1 gene expression not observed herein (Figure 4.3B, 4.4C). Considering that Mandegar and his colleagues have not tested inducing the CRISPRi system with repeated doses of Dox over time, it is not known whether the system can fail to be repeatedly induced and therefore affect its efficiency to KD the expression of a given gene. Since in the present experiment we induce the system continuously twice (Dox was added from day -2 to 2 and from day 4 onwards), if the system actually fails to be repeatedly induced, this may help explain the results herein observed for the CCBE1 KD and the loss of Dox responsiveness.

Moreover, to assess how the addition of Dox and the resultant KD of CCBE1 expression could affect hiPSC commitment into CF, both CRISPRi cultures were morphologically and phenotypically analyzed throughout the distinct differentiation stages. CRISPRi-Ctrl and CRISPRi-KD cells were able to differentiate into CF, displaying similar morphological changes as the observed in former experiments. In addition, on the last day of differentiation, both cultures presented cells with a fibroid spindle-like morphology, thus demonstrating that neither the earlier addition of Dox nor the CCBE1 KD had any morphological impact on hiPSC commitment into CF (Figure 4.4C). Similar cell growth profiles were also observed for the two lines, however, in contrast to previous experiments, CRISPRi-Ctrl and -KD cells growth during hiPSC expansion was greater than at the pro-epicardial differentiation stage. (Figure 4.4D).

Furthermore, both cultures’ phenotypic changes throughout hiPSC differentiation into CF were also assessed, using gene and protein expression analyses. On day 0, CRISPRi-Ctrl and CRISPRi-KD cells’ pluripotency was confirmed by the Oct-4 (POU5F1) and NANOG gene expression levels, as well as by the presence of more than 80% of TRA-1-60+ and SSEA4+ cells within the CRISPRi populations. Despite CRISPRi-KD displaying a low expression level of the NANOG gene, the insignificant percentages of SSEA1+ cells helped support their pluripotent phenotype (Figure 4.4E, 4.5). By day 3, hiPSC-CRISPRi-Ctrl and -CRISPRi-KD differentiated into the cardiac mesodermal lineage, with cells displaying high expression levels of the MESP1 gene, a crucial regulator role on cardiovascular development (Figure 4.5).
Figure 4.4 - Differentiation of hi-PSC-CRISPRi-Ctrl and -KD cells into cardiac fibroblasts, with the Dox-treatment starting on hiPSC expansion. (A) Schematic representation of doxycycline supplementation mode (2 µM from day -2 onwards) along CF differentiation using both CRISPRi-Ctrl and CRISPRi-KD cell lines. (B) Relative expression of CCBE1 gene in hiPSC-CRISPRi lines, from day 0 to 18 of differentiation, along with the percentage of knockdown observed in CCBE1 expression. (C) Morphology of hiPSC-CRISPRi-Ctrl and -KD cell culture, at day 0, 6 (CPC state), 12 (pro-epicardial cell state) and 18 (CF state) of differentiation. Scale bar, 100 µm. (D) CRISPRi-Ctrl and CRISPRi-KD hiPSC fold increase in cell concentration, along the differentiation protocol. (E) Flow cytometry analysis, in both hiPSC-CRISPRi line, of pluripotency markers, TRA-1-60 and SSEA4, and early differentiation marker, Nkx2.5, at day 6; of cardiac progenitors’ marker, Nkx2.5, at day 6; of cardiac fibroblasts marker, CD90, at day 18.

The commitment into the cardiac progenitor stage was then demonstrated by high expression levels of Nkx2.5 gene and protein, and lower number of cells expressing TRA-1-60 marker (Figure 4.4E, 4.5). Both GATA4 and WT1 expression profiles, assessed by RT-qPCR analysis, showed an
upregulation on day 12 indicating that both lines’ CPCs have differentiated into the epicardial lineage (Figure 4.5). In addition, also similarly to former experiments, none differentiation into CM has been observed (insignificant expression levels of TNNT2 gene from day 6 to day 12) (Figure 4.5). The GATA4 and CD90 expression levels on day 18, taken together, suggested that the pro-epicardial CRISPRi-Ctrl and CRISPRi-KD cells were able to differentiate in CF afterwards (Figure 4.4E, 4.5). Therefore, despite the low POSTN expression observed at the end of the differentiation, both cultures presented a typical morphological and phenotypical profile of CF, consistent with the reported literature48,93,96. These results suggest that adding Dox from iPSC stage to CF and CCBE1 KD did not affect the differentiation, neither the cells’ phenotype. It is also worth noting that, when considering all the results so far obtained, more phenotypical similarities between the two CRISPRi cultures were found in the present experiment than in the former one.

Figure 4.5 – Gene expression analysis of hiPSC-CRISPRi-Ctrl and -KD cells along the differentiation. Relative expression of pluripotency (POUS5F1 and NANO), cardiac mesoderm (MESP1), cardiac progenitor (Nkx2.5 and GATA4), pro-epicardium (WT1), cardiomyocyte (TNNT2), and cardiac fibroblast (POSTN) genes in hiPSC-CRISPRi lines along the differentiation. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method, relatively to day 6 or 12 of differentiation, depending on the gene (housekeeping genes: RPLP0 and GAPDH).

Although the addition of Dox was shown to not affect cells’ morphology or phenotype throughout differentiation, no clear conclusions were obtained regarding its effect on CCBE1 KD efficiency. Therefore, to unveil any underlying cause to the CCBE1 expression intriguing results, a third CF differentiation was performed, using the same hiPSC-CRISPRi cell lines abovementioned, and the same differentiation protocol as well (Figure 4.6A - Strategy (1)). In this experiment, both cell lines were differentiated on the daily presence of DOX, since day -8 of expansion (Figure 4.6A). Here, the two CRISPRi lines displayed again a mCherry signal of significant intensity on day 0 that continuously fades from day 6 until the end of the differentiation (Figure 4.6B). Despite just a little amount of dCas9-KRAB protein being able to induce KD, as it has been confirmed before and it will be mentioned hereafter, this fading has been detected in every differentiation carried out. Therefore, further studies should be
performed, to better understand what is causing the particular decrease in the reporter mCherry expression from day 6 onwards.

Through phase-contrast microscopy, no impact of the daily Dox addition on cell morphology was observed, with both CRISPRi cultures presenting the typical morphological changes throughout the commitment and a characteristic CF morphology on day 18 (Figure 4.6B). However, on the other hand, daily adding Dox without any interruptions has seemed to impact hiPSC-CRISPRi-Ctrl cells’ growth during CF commitment. When evaluating both cultures growth profile, CRISPRi-Ctrl displayed a higher fold increase in cell concentration along the pro-epicardial differentiation stage (day 6 to 12) than as detected in former experiments, either for the control line as well for hiPSC-WTC (Figure 4.6C). In the case of CRISPRi-KD culture, it was observed a fold increase in cell concentration between days 6 and 12, identical to the wild-type line (Figure 4.6C). Since CRISPRi-Ctrl cells presented twice the growth of CRISPRi-KD culture in this experiment alone, further studies need to be performed to disclose the effect of CCBE1 KD and daily Dox-treatment on both CRISPRi populations’ proliferation.

As seen in the previous differentiation, from day 0 to day 6, CRISPRi-Ctrl and CRISPRi-KD cultures presented an identical phenotypic shift between the iPSCs pluripotency and CPC typical features. On day 0, the significant expression of pluripotency markers, such as TRA-1-60, SSEA4, POU5F1, and NANOG confirmed both cultures were pluripotent (Figure 4.7A). Afterwards, similar high MESP1 gene expression levels at day 3 of differentiation indicated the cell commitment into the cardiac mesoderm, which entered on the CPC stage by day 6, as demonstrated by Nkx2.5 strong gene and protein expression, once again (Figure 4.6D, 4.7A). Regarding the WT1 and GATA4 expression, similar profiles were detected for both CRISPRi lines between the present and the former experiment. Indeed, taken together with the insignificant expression of TNNT2, their peak of expression on day 12 similarly indicated CRISPRi CPCs have efficiently differentiated into the PE fate (Figure 4.7A). Moreover, by day 18 of differentiation, several fibroblast-associated markers were used to confirm that pro-epicardial cells had committed into fibroblasts. One of the biomarkers used, CD90, marked more than 90% of CRISPRi populations, an observation which is transcendent to all the experiments performed (Figure 4.6D). In addition, both CRISPRi cultures displayed also high percentages of α-SMA+ cells, identical to the ones detected previously (Figure 4.6D). Taken together with POSTN gene expression and morphological analysis, these results confirmed, indeed, the presence of CRISPRi-derived CF in both cultures at the end of the differentiation (Figure 4.6D, 4.7A). Since herein both CRISPRi cultures presented similar morphological and phenotypic changes to the observed in past experiments, it was possible to conclude that the daily addition of Dox from day -8 onwards also did not impact CF differentiation.
Figure 4.6 – Differentiation of hi-PSC-CRISPRi-Ctrl and -KD cells into cardiac fibroblasts, with the daily addition of Dox from hiPSC to CF. (A) Schematic representation of the differentiation protocols carried out, using small molecules and growth factors for Wnt signaling modulation, in hiPSC-CRISPRi lines. The strategy (1) follows a protocol previously published by Bao et al.,93 while the strategy (2) follows a different protocol throughout pro-epicardial cells differentiation into CF, as described by Zhang et al.48. Doxycycline (2 µM) was supplemented supplementation mode from day -8 onwards, every day along CF differentiation. (B) Morphology of hiPSC-CRISPRi-Ctrl and -KD cell culture, at day 0, 6 (CPC state), 12 (pro-epicardial cell state) and 18 (CF state) of differentiation. Scale bar, 100 µm. (C) CRISPRi-Ctrl and CRISPRi-KD hiPSC fold increase in cell concentration, along the differentiation protocol. (D) Flow cytometry analysis, in both hiPSC-CRISPRi line, of pluripotency markers, TRA-1-60 and SSEA4, and early differentiation marker, SSEA1 at day 0; of cardiac progenitors’ marker, Nkx2.5, at day 6; of cardiac fibroblasts markers, α-SMA and CD90, at day 18.
Figure 4.7 – Gene and Protein expression analysis of hi-PSC-CRISPRi-Ctrl and -KD cells along the differentiation. (A) Relative expression of pluripotency (POUSF1 and NANOQ), cardiac mesoderm (MESP1), cardiac progenitor (Nkx2.5 and GATA4), pro-epicardium (WT1), cardiomyocyte (TNNT2), and cardiac fibroblast (POSTN) genes in hiPSC-CRISPRi lines along the differentiation. Gene expression was quantified using the 2-ΔΔCT method, relatively to day 6 or 12 of differentiation, depending on the gene (housekeeping genes: RPLP0 and GAPDH). (B) Relative expression of CCBE1 in hiPSC-CRISPRi lines, from day 0 to 18 of differentiation, along with the percentage of knockdown observed in CCBE1 expression. (C) Detection of CCBE1 protein expression (green), at day 18. Nuclei were counterstained with DAPI (blue). Scale bar represent 100 μm.

To understand if this continuous Dox-treatment would trigger a more efficient KD of CCBE1 expression, a gene analysis was performed for both CRISPRi cultures. When comparing to the results obtained in the last experiment, a similar KD profile was detected during the earlier differentiation stages (Figure 4.4B, 4.7B). However, some differences were observed as CRISPRi-KD cells started displaying, higher percentages of KD from day 6 onwards, presenting 38% of CCBE1 KD by the end of differentiation (Figure 4.7B). CCBE1 KD at day 12 was also observed at protein level, with the immunofluorescent images showing clear differences between CRISPRi-Ctrl and CRISPRi-KD CCBE1 expression (CRISPRi-KD cells appear less stained) (Figure 4.7C). These observations suggest that daily addition of Dox had a crucial impact on increasing the CCBE1 KD efficiency throughout the differentiation. Nevertheless, it is important to notice that on the first experiment performed with CRISPRi lines a higher % of KD on day 18 was observed, although Dox just started being added there on day 15. Further studies need to be carried out to disclose the reproducibility of the present experiment results and assess if a higher KD of CCBE1 expression could be obtained at day 18. In addition to the previous
observations, the only experiment where cells presented an identical level of POSTN expression to the detected herein, was at the first commitment carried out using hiPSC-CRISPRi lines (Figure 4.3A). Since this higher expression of POSTN on day 18 is accompanied, in both these differentiations, by a greater % of CCBE1 KD, such observations suggest that lowering CCBE1 expression can possibly impact directly on POSTN expression, by increasing it. Nevertheless, additional studies must be performed to uncover and better understand this possible connection.

4.3. Impact of differentiation medium on PE commitment into CF

Within the healthy myocardium, fibroblasts usually tend to remain quiescent interstitial cells, however, during the remodelling process upon an injury, some of the released cytokines trigger the CF transdifferentiation into myofibroblasts. Particularly, one of those cytokines, TGF-β, activates the Smad3 signaling cascade accountable for promoting the higher α-SMA expression in fibroblasts110. Indeed, there have been identified a high number of myofibroblasts markedly expressing α-SMA within human and animal myocardial scars formed during MI healing111,112. As an attempt to generate quiescent CF at the end of the differentiation (this is, low percentage of α-SMA+ cells), a distinct fibroblast growth medium combined with a TGF-β inhibitor was used along the last commitment stage. Similar rationale as described by Zhang and colleagues was explored herein.48 Thus, CRISPRi-Ctrl and CRISPRi-KD pro-epicardial cells were differentiated in the presence of FibroGro™ culture medium, daily supplemented with bFGF, SB431542 (TGF-β inhibitor) and Dox (Figure 4.7A – Strategy (2)). In parallel, as a control experiment, the epicardial commitment into CF was also performed following the previously implemented protocol (Figure 4.7A – Strategy (1)).

Using phase-contrast microscopy, it was possible to observe that the use of a fibroblast-specific medium combined with the TGF-β inhibitor did not affect the morphological changes which usually occur during the PE differentiation into CF. Indeed, CRISPRi-Ctrl and -KD cells presented a fibroid spindle-like morphology on day 18. (Figure 4.8A). However, on the other hand, by following this different protocol above described, both cultures' cell growth were affected during the commitment. When comparing to the previous experiments performed using LaSR medium, herein both CRISPRi cultures displayed a greater growth between the days 12 to 18 of differentiation, which can be visually confirmed by their higher cell confluence at day 18 (Figure 4.8A-B). Such observations taken together suggest that using a fibroblast-specific medium supplemented with a TGF-β inhibitor may impact on the final cell concentration, without affecting CF morphology.
Figure 4.8 - hiPSC-CRISPRi-Ctrl and -KD pro-epicardial cells differentiated into cardiac fibroblasts in two distinct fibroblasts differentiation medium. (A) Morphology of hiPSC-CRISPRi-Ctrl and -KD cell culture, at day 12 (pro-epicardial cell state) and 18 (CF state) of differentiation, in both medium conditions. Scale bar, 100 µm. (B) Comparison between the fold increases in cell concentration at the CF commitment stage when using LaSR medium and the specific fibroblast medium, FibroGro™. hiPSC-CF I, II, III and IV correspond to the CF generated on the first, second, third and fourth differentiation performed with hiPSC-CRISPRi lines, respectively. (C) Flow cytometry analysis, in both hiPSC-CRISPRi lines, of cardiac fibroblasts markers, Vim, α-SMA, CD90, and PDGFRα, at day 18. (D) Relative expression of CCBE1, cardiac progenitor (GATA4), pro-epicardium (WT1), and cardiac fibroblast (POSTN) genes in hiPSC-CRISPRi lines on day 18. Gene expression was quantified using the 2^(-ΔΔCT) method, relatively to day 6 or 12 of differentiation, depending on the gene (housekeeping genes: RPLP0 and GAPDH). (E) Comparison between α-SMA expression in both the different medium conditions. (F) Detection of α-SMA marker (green), in both the different medium conditions, at day 18. Nuclei were counterstained with DAPI (blue). Scale bar represent 100 µm. (G) Detection of Collagen I expression (green), in both different medium conditions, at day 18. Nuclei were counterstained with DAPI (blue). Scale bar represent 100 µm.
On day 18, CRISPRi-Ctrl and CRISPRi-KD cultures were phenotypically characterized to confirm the presence of derived CF within both. Similarly to the previous differentiations using LaSR medium, both CRISPRi populations showed more than 95% of their cells expressing CD90 (Figure 4.8C). In addition, VIM (fibroblast-associated marker) and POSTN were highly expressed (Figure 4.8C). Taken together with cells’ low expression of GATA4 and WT1 genes on day 18, these results suggest that both cultures had differentiated into CF (Figure 4.8C-D).

Having in consideration the rationale underlying this present experiment, PDGFRα (CD140) and α-SMA were also analyzed on the protein level. PDGFRα (Platelet-Derived Growth Factor Receptor α) is an essential protein in CF formation during the heart development, which has been also detected iPSC-CF. However, a recent study identified that inhibiting PDGFRα signaling could induce quiescence and reduced self-renewal to fibroblasts. Given the low expression of CD140 marker observed herein for both CRISPRi cultures, this might suggest that our adapted protocol originated a great number of quiescent CF rather than activated ones (Figure 4.8C). Moreover, less than 45% of CRISPRi-Ctrl and CRISPRi-KD cells were expressing α-SMA, approximately 30% less than the observed in previous differentiations using LASR medium (Figure 4.8C, E). Such differences were also detectable in the immunofluorescence images captured. While CF derived within LaSR culture displayed highly organized stress-fibers of α-SMA (characteristic of myofibroblasts), the fibroblasts generated in the present experiment showed some α-SMA expression but no defined filaments of this protein (Figure 4.8F). These observations help support the supposition that using fibroblast-specific medium supplemented with a TGF-β inhibitor can help generate a greater number of quiescent CF.

On day 18 of differentiation, CCBE1 gene transcription was also analyzed for both cultures, with CRISPRi-KD cells displaying CCBE1 expression knock downed in 72% (Figure 4.8D). Although this observation might suggest that using a distinct differentiation medium can affect the CRISPRi system’s KD efficiency, further experiments must be carried out to confirm and validate such a hypothesis. Moreover, as already mentioned before, the fibroblasts are a key element during post-MI heart remodeling due to their ability to secrete the ECM proteins constituting the scar tissue. Therefore, it was important to analyze this ability in the fibroblasts derived under both conditions (LaSR medium and fibroblast-specific medium combined with TGF-β inhibitor), in order to understand how a distinct differentiation medium could affect this CF functionality. For this purpose, CRISPRi-derived CF’s ability to secrete Collagen I (Col I, a structural ECM protein) was analyzed by immunocytochemistry. On day 18, both CRISPRi cultures differentiated in the presence of LaSR medium presented high quantities of Col I being secreted, however, the derived CF were not capable of forming an organized structural network, characteristic from the fibrotic scar tissue (Figure 4.8G). On the other hand, when differentiated in fibroblast-specific medium, both CRISPRi cultures displayed a more organized and homogeneous collagen network. However, between both, some small discrepancies were observed, with CRISPRi-KD collagen fibers appearing less arranged (structurally) than the observed in CRISPRi-Ctrl culture (Figure 4.8G). Taking into account that these CRISPRi-KD cells were also displaying the CCBE1 expression highly knock downed on day 18, such observations might suggest CCBE1 could have a role on regulating fibroblasts’ ability to secrete ECM proteins.
Finally, to assess if the CF generated from the four differentiations carried out would present a typical fibroblast phenotype, the derived fibroblasts were compared to isolated human primary CF (hCF) through gene analysis. For this purpose, the cell cultures were analyzed based on a specific group of fibroblast-associated markers (GATA4, WT1 and POSTN) and CCBE1. From this comparison, it was possible to observe that CCBE1 and POSTN genes were being less expressed in the differentiated fibroblast than in adult CF (Figure 4.9). However, in contrast, both CRISPRi cultures presented a higher expression of GATA4 and WT1 than in primary adult fibroblasts. This may indicate that the generated CF presented a less mature phenotype compared to adult CF.

Figure 4.9 - Comparison between the gene expression of fibroblast-associated markers on hiPSC-CRISPRi-Ctrl and hiPSC-CRISPRi-KD cardiac fibroblasts derived in all the differentiation experiments. hiPSC-CF I, II, III and IV correspond to the CF generated on the first, second, third and fourth differentiation performed with hiPSC-CRISPRi lines, respectively.

4.4. CF long-maintenance: LaSR vs. FibroGro™

To further investigate the CF maintenance without losing their typical fibroblast phenotype, both differentiated cultures using the protocol as described in sections 4.2 and 4.3 were propagated for several passages. For this, both CRISPRi lines were propagated in different culture media: LaSR medium supplemented with bFGF or FibroGro™ supplemented with bFGF and SB431542 (Figure 4.10A). Cell cultured in LaSR medium were able to maintain their growth profile and fibroid spindle-like morphology for 4 passages, while in FibroGro™ both cell lines were propagated for 8 passages maintaining the fibroblasts phenotype (cell alignment and morphology) (Figure 4.10B).
Figure 4.10 – Long-maintenance of hiPSC-CRISPRi-Ctrl and -KD cardiac fibroblasts in the presence of two different commitment medium conditions. (A) Schematic representation of the long-maintenance, throughout the time and passages. (B) Morphology of hiPSC-CRISPRi-Ctrl and -KD cell culture, at passage 1 (P1), 2 (P2) and 3 (P3), for the both medium conditions, and additionally at passage 8 (P8) for the FibroGro™ medium condition. Scale bar, 100 µm. (C) Percentage of proliferative cells, for both hiPSC-CRISPRi cell lines, when cultured in different (LaSR and FibroGro™) medium. These results were obtained by performing a Click-iT EdU Flow Cytometry Assay. (D) Cumulative Population Duplication Level (PDL) for hiPSC-CRISPRi-Ctrl and -KD cells cultured in FibroGro™ medium.
The percentage of proliferative cells was also assessed, in both media conditions at passage 2 (Figure 4.10C). Both CRISPRi-Ctrl and CRISPRi-KD lines, when cultured in FibroGro™ medium, displayed a higher percentage of proliferative cells, than in LaSR medium condition (Figure 4.10C). These results demonstrated how a different fibroblast culture medium can strongly influence CF growth and morphology.

Additionally, the total number of times the cells within the population have doubled since their initial inoculum was also assessed for the CRISPRi-derived CFs cultured in FibroGro™ medium. From P1 to P3, both cultures presented a similar growth profile; however, from P4 onwards, discrepancies began to arise, with CRISPRi-Ctrl cells starting to display a higher proliferative capacity than CRISPRi-KD-derived CFs. Indeed, by the ending of the 8 passages, the hiPSC-CRISPRi-Ctrl line presented approximately twice the total number of times the CRISPRi-KD cells have duplicated (Figure 4.10D). These evidences might indicate a potential role of CCBE1 on CF proliferative capacity, which should be further studied in future experiments.

### 4.5. Functional characterization of hiPSC-CF

During the inflammatory and proliferative stage of the fibrotic response upon MI, fibroblasts migrate towards the injured area, where they transdifferentiate into myofibroblasts and form the scar tissue replacing the dying myocytes. TGF-β signaling pathway stimulation and scratch assay may be performed to assess respectively these CF activation and migration capacities in vitro. Therefore, to evaluate if the CF herein generated possess such functional potential and if it would be affected by CCBE1 KD, both these assays were carried out, using the hiPSC-CRISPRi-derived.

In a first activation, three conditions were screened for 48 h on cells differentiated as described in Figure 4.6A – Strategy (1): 1) incubation with FibroGro™ medium (negative control); 2) incubation with DMEM medium only supplemented with 10% Fetal Bovine Serum (FBS); and 3) culturing in DMEM medium supplemented with 10% FBS and 10 ng/mL of TGF-β. Since CRISPRi-Ctrl cells were already highly confluent when the activation assay started, no specific differences in cells’ shape and morphology were possible to observe between all the conditions tested. However, some morphological discrepancies were possible to detect between the three conditions screened for CRISPRi-KD-derived CF. While CRISPRi-KD cells incubated in FibroGro™ medium maintained a typical spindle-like morphology, the fibroblasts cultured in DMEM medium + 10% FBS, with or without 10 ng/mL of TGF-β combined, presented an identical larger shape than at day 0 of the activation (Figure 4.11A). Such observation suggested that CRISPRi-KD fibroblasts were able to be activated even without TGF-β in their presence (in DMEM medium + 10% FBS condition). Nevertheless, despite of this detectable morphological change, the CRISPRi-derived CF did not appear so elongated and aligned as they are observed in the literature. CRISPRi-Ctrl cells presented a higher proliferative capacity than CRISPRi-KD line, however, either in the presence or absence of TGF-β (+ or – TGF-β conditions, respectively), both lines presented similar percentages of proliferative cells, suggesting TGF-β did not have any impact on CRISPRi-derived CF proliferation (Figure 4.11B).
Figure 4.11 – Activation of hPSC-CRISPRi cardiac fibroblasts by the modulation of the TGF-β signaling pathway. (A) Morphology of hPSC-CRISPRi-Ctrl and -KD cells cultured in FibroGro™ medium, DMEM medium + 10% FBS and DMEM medium + 10% FBS + 10 ng/mL of TGF-β, by day 0 and 2 of the activation experiment. (B) Percentage of proliferative cells, for both hPSC-CRISPRi cell lines cultured in the presence of 0 and 10 ng/mL TGF-β, at day 2 of the activation experiment. (C) Flow cytometry analysis, on hPSC-CRISPRi cell lines cultured in the presence of 0 and 10 ng/mL TGF-β, of α-SMA protein expression at day 2 of the activation experiment. (D) Flow cytometry analysis, on hPSC-CRISPRi cell lines cultured in the presence of 0 and 10 ng/mL TGF-β, of CD90 protein expression at day 2 of the activation experiment.

To evaluate the CF activation state, the percentage of α-SMA+ cells in non-treated and TGF-β-treated conditions was assessed. In CRISPRi-Ctrl culture, higher percentage of α-SMA in treated
condition was detected when compared to non-treated, being this small difference coherent to the demonstrated by Zhang and colleagues\textsuperscript{46}. Nevertheless, in CRISPRi-KD no identical differences were observed in both conditions after 48 h (Figure 4.11C). CD90 protein expression was also assessed on day 2 of the activation assay, with both CRISPRi lines displaying identical expression either in the presence or absence of TGF-β. In the case of CRISPRi-Ctrl culture, it was detected high expression of CD90, similar to the observed at the end of the CF differentiation. On the other hand, CRISPRi-KD line presented a significantly lower percentage of CD90+ cells within its CF population (Figure 4.11D). Despite only approximately 30% of CRISPRi-KD cells have been detected expressing CD90, as already mentioned before, on a recent study published by Zhang et al. a similar expression was observed for hiPSC-derived CF\textsuperscript{46}. In further experiments, additional fibroblast markers should be used to confirm cells maintain the fibroblast phenotype, after the activation has been carried out.

Since the results above referred did not provide any clear conclusions on CF activation, as an attempt to implement a robust CF activation protocol, a second activation experiment was performed, using CRISPRi-Ctrl cells differentiated as described in Figure 4.6A – Strategy (2). In this assay, the derived CF were also exposed, for 48 h, to three different conditions: 1) FibroGro\textsuperscript{TM} medium combined with SB (SB condition); 2) FibroGro\textsuperscript{TM} medium supplemented with TGF-β small molecules (TGF-β condition); and 3) FibroGro\textsuperscript{TM} medium combined with 10% FBS (FBS condition). Since in the last experiment the fibroblasts incubated in DMEM medium + 10% FBS appeared activated, the 3\textsuperscript{rd} condition herein used was designed to be a positive control for CF activation.

Along this experiment, CRISPRi-Ctrl culture was analyzed phenotypically by RT-qPCR and immunocytochemistry, for CF activation markers (Figure 4.12A-B). The CF marker POSTN presented high expression levels in SB and FBS condition, while in TGF-β condition, CRISPRi-Ctrl cells displayed similar levels of expression at 6 to 48h. Since this later result is not coherent with the observed in the literature\textsuperscript{46}, further studies on this gene expression need to be performed. The expression of Col1A1 and ACTA2, two of the most studied genes in CF activation, was higher in TGFβ-treated cells at 24 and 48 h post-treatment than in SB and FBS conditions (Figure 4.12A). These observations are consistent with the reported literature\textsuperscript{46} and with fluorescence microscopy images captured for both markers (Figure 4.12B).

Besides expressing α-SMA, myofibroblasts have the ability to incorporate it in stress fibers, which helps increasing fibroblasts’ adhesion strength\textsuperscript{116}. For that reason, the cytoskeleton organization (actin staining) was also assessed in the three conditions, with stress fibers being detected only on TGF-β- and FBS-treated cells (Figure 4.12B). In addition, the expression of tissue inhibitors of metalloproteinases (TIMPs) was also assessed. The expression profile of this factor in SB displayed an intriguing upregulation at 24 h, which should be further investigated. Nevertheless, a higher expression of TIMP1 in TGF-β and FBS conditions was detected at 48 h post-treatment in comparison to control (SB) (Figure 4.12A). Such observations suggest that, since they inhibit MMPs from degrading the ECM proteins (e.g., collagen and fibronectin), less matrix degradation has occurred upon TGF-β and FBS conditions. Indeed, a dense and organized collagen-stained network was observed in the same conditions, which helps corroborating this assumption (Figure 4.12B). Finally, we also assessed the
expression of another fibrotic marker, Osteopontin (SPP1), where no clear differences were observed between TGF-β and SB conditions (Figure 4.12A). These results should be further confirmed, since there are recent evidences on its impact as a TGF-β signaling enhancer\textsuperscript{117}.

Finally, as above mentioned, the migration capacity of CF was also analyzed by a scratch assay, with both CRISPRi lines being monitored along 24 h. After the scratch, it was possible to observe the created wound was occupied by cells, continuously across the time, with both CRISPRi cultures showing the wound fully populated, at 24 h. However, CRISPRi-KD-derived CF seemed to start migrating towards the wound earlier than CRISPRi-Ctrl cells did, as CRISPRi-KD culture presents the wound already greatly occupied at the 6 h after the scratch (Figure 4.13). Nevertheless, further studies need to be carried out, in order to understand if there are proliferative cells fulfilling the wound, instead of migration CF, and how CCBE1 can affect the migration capacity of fibroblasts.
Figure 4.12 – Gene and Protein expression analysis of hiPSC-CRISPRi-Ctrl-derived CF in the second activation experiment. (A) Relative expression of specific CF activation markers (POSTN, COL1A1, ACTA2, SPP1, and TIMP1) in hiPSC-CRISPRi-Ctrl fibroblasts at 6, 24 and 48 h after initiating the activation. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, relatively to day 6 or 12 of differentiation, depending on the gene (housekeeping genes: RPLP0 and GAPDH). (B) Detection of α-SMA (red), Phalloidin (green) and Collagen I (green) markers in the three different conditions tested (SB, TGF-β, and FBS conditions), at 48 h after starting activation. Nuclei were counterstained with DAPI (blue). Scale bar represent 100 μm.
Figure 4.13 – Monitorization of the wound closing at 0, 3, 6, 9, and 24h after the scratch being scraped, in hiPSC-CRISPRi-Ctrl and -KD differentiated CF cultures.
5. Conclusion

In this thesis, a protocol for differentiation of hiPSC into cardiac fibroblasts (CF) was successfully implemented and validated with 3 hiPSC lines. hiPSC showed morphological and phenotypical features similar to CF at the end of the differentiation. The modified hiPSC lines harboring an inducible-dCas9 system for CCBE1 knockdown (KD) were used to evaluate the impact of CCBE1 knockdown throughout the differentiation process and on CF phenotype. Applying the Doxycycline (Dox)-treatment (used to induce the CRISPRi system) at different stages of the differentiation process allowed to observe that Dox did not affect hiPSC expression of stemness, cardiac progenitor and pro-epicardium markers as well as CF properties. Daily addition of Dox from iPSC to CF demonstrated to play a crucial impact on increasing the CCBE1 knockdown efficiency (up to 82%) throughout the differentiation, however further studies would be needed to validate these observations.

A different protocol to differentiate pro-epicardial cells into CF was explored as well, to assess the impact of the medium formulation on CF commitment. Lower percentages of α-smooth muscle actin and PDGFRα positive cells suggested that, by using FibroGro™ medium combined with a TGF-β inhibitor throughout CF differentiation stage, a higher number of quiescent fibroblasts were generated. Additionally, a higher CCBE1 knockdown efficiency was also obtained (up to 72%, on day 18). The functionality of hiPSC-CF generated in FibroGro™ medium was also evaluated, with cells showing the ability to transdifferentiate into myofibroblasts and to migrate toward an injured area (wound).

This work provides new insights regarding the role of CCBE1 on CF commitment and functionality. Additional gain-of-function experiments should be carried out to assess the possible impact a recombinant CCBE1 protein\textsuperscript{118} could have on cells’ phenotype and functionality, whether when provided throughout the commitment or to the derived CF alone. Future work must also include the establishment and characterization of 3D heterotypic cultures of hiPSC-EC and hiPSC-CF, for both CCBE1 KD and control (expressing CCBE1) conditions. Underlying such experiment is the hypothesis that the CCBE1 secreted by CF could possibly promote the development of new vascular networks from endocardial progenitors.
6. References


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