Pedro Miguel Gomes Gião Vicente

Degree in Cell and Molecular Biology

Disclosing CCBE1 role in Cardiac Differentiation of Human Pluripotent Stem Cells

Dissertation to obtain Master Degree in Biotechnology

Supervisor: Dr. Margarida Serra, Senior Scientist, IBET/ITQB-NOVA

Jury:

President: Prof. Doctor Susana Filipe Barreiros
Arguer: Doctor José Manuel Café Inácio
Supervisor: Doctor Maria Margarida de Carvalho Negrão Serra

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Preface

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Part of the work presented in this thesis has been included in poster communications and a manuscript is under preparation.

Poster Communications:

- Silva MM**, Vicente P** et al. CCBE1 role along cardiac commitment of human pluripotent stem cells: exploring gene editing tools on loss-of-function studies. Frontiers in Cardiovascular Biology, April 20th – 22nd, 2018, Vienna, Austria.

- Silva MM, **Vicente P** et al. CCBE1-induced cardiac commitment on human pluripotent stem cells: exploring gene editing tools on loss-of-function studies. Workshop on Cardiovascular Research @ Bayer, May 31st – June 3rd, 2018, Cape Cod, USA.

Conference proceeding:

- Silva MM, **Vicente P** et al. (2018) CCBE1 role along cardiac commitment of human pluripotent stem cells: exploring gene editing tools on loss-of-function studies. Cardiovascular Research. 114. S20-S20. 10.1093/cvr/cvy060.038.

Manuscript:

- Silva MM, **Vicente P** et al. (2018) Unveiling CCBE1 role as a modulator of human pluripotent stem cells cardiomyocyte differentiation. *In preparation.*

Abstract

Cardiovascular diseases (CVD) are the leading cause of death worldwide. Within CVDs, myocardial infarction (MI) is associated with a massive and irreversible loss of cardiomyocytes (CM). An in-depth comprehension of key cellular mechanisms and molecules involved in cardiogenesis is fundamental to improve cardiac therapies by exposing novel therapeutic targets. CCBE1, a collagen and calcium-EGF biding domain 1 protein, was identified to be expressed in mouse heart precursors. Mutations in CCBE1 have been associated with Hennekam syndrome, which is characterized by abnormal lymphatic system and congenital heart defects. However, the CCBE1 functional role in cardiac specification is still unknown. Therefore, the main aim of this thesis was to unveil CCBE1 role in CM and Endothelial cells (EC) specification. For this purpose, a modified hiPSC line displaying the CRISPR interference technology (CRISPRi) was used to selectively knockdown (KD) CCBE1 gene expression along CM and EC differentiation.

We showed that CCBE1 downregulation did not affect hiPSCs growth, morphology and stemness. Nonetheless, a significant reduction on gene expression of cardiac troponin T2 gene (*TNNT2*) and lower gene expression ratios of cardiac troponin I isoforms (*TNNI3:TNNI1*) and myosin heavy chains (*MYH7:MYH6*) were detected in CMs derived from CRISPRi-CCBE1 KD cell line at day 15. Ultrastructural changes were also observed in this condition, CMs presented lower sarcomere length and alignment, indicating a more immature state. In contrast, EC differentiation was not affected by CCBE1 KD, with no impact on EC morphology or gene expression levels. Therefore, CCBE1 seems to have a key role on CM specification and maturation. Moreover, we successfully selected hiPSC clonal populations with higher level of CCBE1 KD for future studies. This work may contribute with new insights towards the development of CCBE1-mediated therapeutic strategies for cardiac regenerative medicine.

Keywords: Cardiovascular Disease; human induced Pluripotent Stem Cells (hiPSCs); CCBE1; CRISPRi; Cardiomyocytes; Loss-of-function Studies;

Resumo

As doenças cardiovasculares são das principais causas de morte, sendo o enfarte do miocárdio associado à perda excessiva e permanente de cardiomiócitos (CM). Uma melhor compreensão dos mecanismos celulares que desempenham um papel crucial na cardiogénese é fundamental para identificar novos alvos terapêuticos e melhorar as terapias cardiovasculares. A expressão da proteína CCBE1 (collagen and calcium-EGF binding domain 1) foi detetada nos percursores cardíacos durante o desenvolvimento embrionário em ratinhos. Para além disso, mutações nesta proteína estão associadas ao síndrome de Hennekam, apresentando deficiências no sistema linfático e defeitos congénitos no coração. Dado que, o papel funcional da CCBE1 na diferenciação cardíaca não é conhecido, o principal objetivo desta tese foi investigar a função desta proteína, utilizando células estaminais pluripotentes induzidas (hiPSC) e métodos de modificação génica (tecnologia de interferência CRISPR) para induzir a perda de função do gene em causa durante a diferenciação em CM e células endoteliais (CE).

A Inibição da expressão da CCBE1 não afetou o crescimento, morfologia nem a pluripotência das hiPSC. Por outro lado, durante a diferenciação em CMs observou-se um decréscimo da expressão do marcador cardíaco *TNNT2* e diminuição dos rácios de expressão das isoformas de troponina I (*TNNI3:TNNI1*) e das cadeias pesadas de miosina (*MYH7:MYH6*). Estes CMs também apresentavam alterações ultraestruturais, nomeadamente no tamanho e alinhamento dos sarcómeros, indicadores de um fenótipo mais imaturo comparativamente com a cultura com níveis de CCBE1 normais. No entanto, durante a diferenciação em CE não foi observado qualquer efeito da diminuição da expressão da CCBE1, tanto a nível morfológico como na expressão génica. Estes dados sugerem que esta proteína pode ter uma função importante no fenótipo dos CM. Adicionalmente, foram selecionados clones com maior nível de inibição da expressão da CCBE1 para estudos futuros. Este trabalho poderá contribuir para o desenvolvimento de novas terapias cardíacas baseadas no papel da CCBE1.

Palavras-chave: Doenças Cardiovasculares; Células estaminais pluripotentes induzidas humanas (hiPSCs); CCBE1; CRISPRi; Cardiomiócitos; Estudos de perda de função;

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Abbreviations

ADAMTS3	A Disintegrin and Metalloproteinase with Thrombospondin Motifs-3
BM	Bone marrow
BMP	Bone morphogenic protein
BSA	Bovine Serum Albumin
Cas9	CRISPR associated protein 9
CCBE1	Collagen and calcium-binding EGF domain-1
CMs	Cardiomyocytes
CO_2	Carbon dioxide
CPC	Cardiac Progenitor Cell
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSC	Cardiac Stem Cell
cTnI	Cardiac muscle troponin I
cTnT	Cardiac muscle troponin T
CVD	Cardiovascular diseases
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
DSB	Double strand break
EC	Endothelial Cell
EdU	5-ethynyl-2´-deoxyuridine
Endo	Endocardium
Epi	Epicardium
FBS	Fetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor
FHF	First Heart Field
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA4	Transcription factor GATA-4
gRNA	guideRNA
hESC	Human embryonic stem cells
HF	Heart Failure
hiPSC	Human induced pluripotent stem cells
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IWR	Inhibitors of Wnt response
KD	Knockdown
KDR	Kinase insert domain receptor
KRAB	Krüppel-associated box

LVEF	Left ventricular ejection fraction
MESP1	Mesoderm Posterior BHLH Transcription Factor 1
MI	Myocardial infarction
MSC	Mesenchymal stem cells
MYH6	Myosin heavy chain 6, α isoform protein
MYH7	Myosin heavy chain 7, β isoform protein
MYL2	Myosin light chain 2, ventricular/cardiac muscle isoform (MLC2v)
MYL7	Myosin light chain 7, atrial isoform (MLC2a)
Nanog	Homeobox Protein Nanog
Nkx2-5	NK2 Homeobox 5
Oct4	Transcription factor octamer 4
PAM	Protospacer-adjacent motif
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PSC	Pluripotent stem cells
RNAi	RNA-mediated interference
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SHF	Second Heart Field
SIRPα/β	Signal-regulatory protein alpha/betta
SMs	Skeletal myoblasts
SSEA-1	Stage-specific embryonic antigen-1
SSEA-4	Stage-specific embryonic antigen-4
TALENs	Transcription activator-like effector nucleases
TNNI1	Troponin I1, Slow Skeletal Type
TNNI3	Troponin I3, Cardiac Type
TNNT2	Cardiac muscle troponin T
TRA-1-60	Human embryonal carcinoma marker antigen 60
TRA-1-81	Human embryonal carcinoma marker antigen 81
VCAM1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin, CD144
VEGF	Vascular endothelial growth factor
WNT	Wingless-related integration site
ZFs	Zinc Fingers

1.Introduction

1.1.Cardiovascular Diseases: Prevalence & Treatment

Cardiovascular diseases (CVD), a group of disorders of the heart and blood vessels, persist as the leading cause of death worldwide, accounting for 17.7 million deaths per year, a number that is expected to grow even further, to 23.6 million deaths by 2030 [1]. In Europe CVDs were responsible for almost 45% of all deaths in 2016 (**Figure 1.1**). In particular, myocardial infarction (MI) or heart attack results in cardiac muscle loss, due to cardiomyocyte (CM) death either by apoptosis or necrosis. Moreover, the limited capability of the heart tissue to regenerate makes this loss largely irreversible and a scar tissue constituted by fibroblasts is formed, resulting in loss of contractility and decreased heart function. This consequently leads to the development of heart failure (HF) [2]. Although adult CMs do not proliferate, evidences of a resident cardiac progenitors' cells (CPC) population able to differentiate into CMs may provide some endogenous regenerative capacity in the adult heart, however at insufficient rates to compensate for the massive cell loss caused by MI [3].

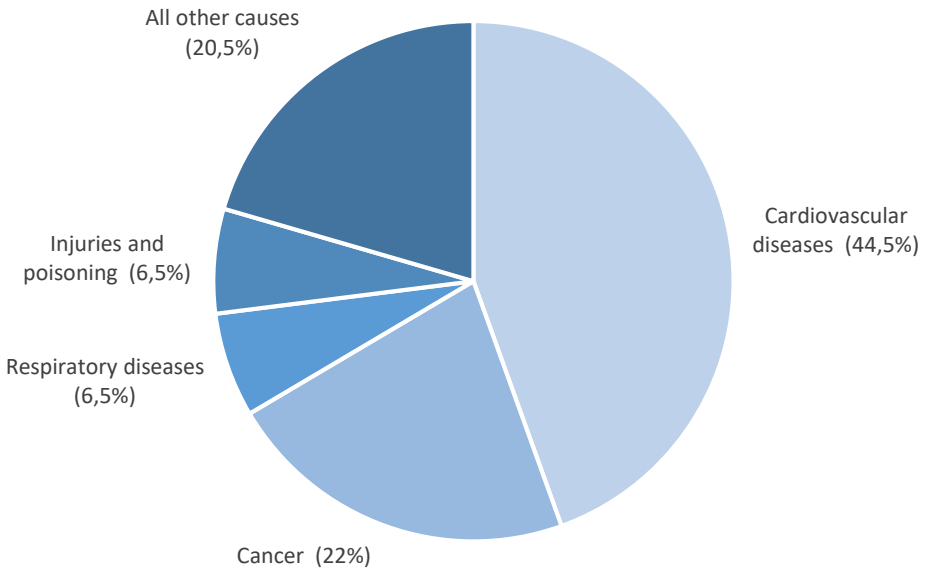

Figure 1.1 – Death causes of European population in 2016. Adapted from [4].

Heart transplantation remains the best long-term solution for end-stage HF, however the limited number of donors available, the high costs and possible tissue rejection, makes it unrealistic to be considered a standard therapy. Current treatments can improve patient's survival and well-being, yet they fail to regenerate or repair the damaged heart. For example, pharmacological approaches, such as inhibitors of renin-angiotensin system, aim to reduce myocardial fibrosis, hypertrophy and incidence of heart failure [5]. In addition, revascularization

approaches have also been widely used to improve blood flow after MI [6]. Even though these methods have successfully reduced the mortality rates, they are intrinsically non-curative and hence novel approaches capable to promote heart regeneration and repair are still required.

1.2.Novel Therapies for heart regeneration and repair

1.2.1.Stem cell-based therapy for Myocardial Regeneration

Regeneration of the injured heart by replacing the lost CM population, is an attractive approach to repair the heart and avoid future HF. To date, clinical efforts towards cardiac repair and regeneration have largely focus on stem cell-based therapies. The first generation of cell-based therapies used for this purpose included the transplantation of noncardiac stem cells, such as, mesenchymal stem cells (MSCs), bone marrow (BM)-derived cells and myoblast cells, as they were more easily obtained [7]. One of the first cell types to be tested, with the goal of remuscularization in mind, were the skeletal myoblasts (SMs), although they did not yield any improvements in the randomized phase-II MAGIC trial [8]. In addition, concerns about their arrhythmogenic potential led to the end of further development of cell therapies based on this cell type [9].

Other cell types used in cardiac regeneration trials were BM-derived cells, due to their safety, easy isolation and encouraging initial results [10]. Early clinical trials, namely the BOOST [11] and REPAIR-AMI [12], have shown some beneficial effects in patients with MI through improvement of the ejection fraction in cell-treated groups compared to placebo. Nevertheless, other clinical trials with a wider number of patients, didn't display any beneficial outcomes [13, 14]. The ongoing investigations with these cells aim to end these controversies and to draw a conclusion about their beneficial effects for patients.

The last major noncardiac cell source studied in regenerative therapies are the multipotent adult MSCs, which demonstrated a great potential for cardiac regeneration in preclinical studies, displaying an inherent capacity for self-renew and differentiation into adipocytes, chondrocytes, hepatocytes, osteoblasts, neurons and skeletal muscle cells [15]. When co-cultured with primary CMs or in the presence of the DNA methyltransferase inhibitor 5-azacytidine these cells are also able to differentiate into CMs *in vitro* [16, 17]. Human MSCs can be isolated primarily from bone marrow, but also from other adult and fetal tissues, including adipose tissue, cord and peripheral blood, placental and umbilical tissues [18]. Their availability and high expansion rate, combined with successful cryopreservation and strong paracrine effects, namely their angiogenic, anti-inflammatory and immunomodulatory properties, makes them a very attractive source in autologous or allogenic therapies for heart regeneration [18]. However, in clinical trials such as POSEIDON [19] and MSC-HF [20], transplantation of MSCs resulted only in modest improvements for patients with ischemic heart failure.

In conclusion, the transplantation of noncardiac stem cells has not shown consistent positive results in the treatment of heart diseases yet, and the few favorable effects were likely

due to paracrine mechanisms such as neovascularization and remodeling of the scar, rather than the formation of new CMs and the direct regeneration of the heart [21]. These heterogeneous outcomes could be due to low cell engraftment and limited differentiation potential, therefore, the research and clinical focus shifted to the second-generation of stem cells, comprising cardiac progenitors/stem cells (CPC/CSC) and pluripotent stem cells (PSC).

CPCs/CSCs are a resident population of the heart with multipotent, self-renewal and clonogenic capacity, possessing the ability to differentiate into multiple lineages of the heart: CMs, smooth muscle cells and endothelial cells, without the aptitude for teratoma formation, as observed in PSC [22]. Thus, they offer an appealing alternative for cell transplantation therapies since they can be widely propagated *in vitro*, transplanted into the diseased heart and then differentiated into cardiovascular cells. Furthermore, these CPC/CSC are considered to stimulate the regenerative capacity of the heart through secretion of growth factors involved in signaling pathways, activating the endogenous cardiac cells and/or paracrine mechanisms. The SCIPIO clinical trial, which was the pioneer in the treatment of ischemic cardiomyopathy using CSCs demonstrated an increased recovery of left ventricular (LV) function and decreased infarct size [23, 24]. A second clinical trial, CADUCEUS, also showed a reduction in infarct size, but failed to show an improvement in LV function [25]. However, the low engraftment rates shown in the preclinical trials and the questionable capacity to form functional CMs were still a major concern. Moreover, the recent CAREMI clinical trial demonstrated that allogenic CSCs can be safely administrated in patients with MI, with no deaths or adverse cardiac events reported. The absence of immune rejection events was also described in this study, with no differences found in terms of LV remodeling and infarct size reduction, between CSCs and placebo-treated groups [26].

Pluripotent stem cells, including embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) are non-specialized cells with the capacity to proliferate continuously and give rise to differentiated cells, under the presence or absence of specific signals [27], making them an extremely attractive cell source for cellular therapy, drug discovery and disease modeling [28]. A major breakthrough in the stem cell research field arose when mouse ESC were isolated from the inner cell mass of the blastocysts in 1981 [29], followed by isolation of their human counterparts in 1998 [30]. Despite the pluripotent potential of these cells (i.e. ability to differentiate into cells derived from the 3 germ layers: ectoderm, endoderm and mesoderm) and their high self-renewal capacity (i.e. they can proliferate continuously and give rise to undifferentiated cells), there are still major issues preventing the fulfilment of their great potential, namely the ethical issues due to the manipulation of embryos, immunological incompatibility and propension to teratoma formation [28, 31]. These drawbacks in ESCs urged the discovery of new cell alternatives with similar pluripotent phenotype. Thus, in 2006, Takashi and Yamanaka showed that reprogramming adult mouse fibroblasts, forcing the expression of four recombinant factors (*OCT4*, *SOX2*, *KLF4* and *C-MYC*), was enough to convert these cells into embryonic-like state and named them iPSCs [32]. A year later they were able to reproduce this accomplishment with human somatic cells [33].

The first reprogramming protocols relied on the use of retroviral vectors to efficiently produce iPSCs. However, random transgene insertion could lead to insertional mutations, interrupting important genes which might result in tumorigenesis and disturb the pluripotent state of the cells. To overcome these risks, safer methods for reprogramming somatic cells have emerged, using non-integrative virus (e.g. adenoviruses, Sendai virus) or virus-free approaches (e.g. piggyBac transposon, microRNAs, plasmids, episomal and minicircle vectors) [34]. Nonetheless, a lower reprogramming efficiency is still observed when compared to the use of integrative vectors. Thus, new and more efficient approaches are still needed to promote the use of these cells in a safer and more efficient way in regenerative medicine.

iPSCs are remarkably like ESC in many key aspects critical for their application in regenerative medicine. Nevertheless, differences in gene expression profile can be found, like distinct microRNA (miRNA) expression and epigenetic markers [35]. When reprogramming happens, a global epigenetic remodeling occurs which is necessary for the successfulness of the reprogramming, but genetic aberrations can also arise during this process [27]. Both of these PSCs are easily expandable and can be differentiated into functional CMs *in vitro* offering the opportunity to obtain sufficient number of CMs for transplantation to the damaged heart. However, preclinical trials were not able to draw a conclusion yet about the efficacy of PSCs-derived CM for heart regeneration showing mixed results depending on the animal model [36–38]. Even though in some of these cases there was an improvement in LV function and remuscularization of the heart, their ability to form teratomas and induce arrhythmias remains a major problem [39]. The recent ESCORT trial, testing hESC-derived CPC is an attempt to set aside these concerns with promising preliminary outcomes [40]. In the end iPSC still hold great potential not only for clinical applications and personalized medicine but for cell biology research too. Being ethically less controversial, avoiding the need of embryo use and presenting a lower probability of immune rejection when compared with ESCs, since iPSCs can carry the genome of the patient from whom it was derived [31, 41]. Therefore, they can turn out to be the “Gold Standard” for regenerative medicine in the future.

To date the disappointing results obtained in the cell therapy clinical trials are assumed to be due to the poor engraftment an inadequate dosage of the cells regardless of the cell type used [42]. An emerging alternative approach for cardiac treatment is the repeated dose cell therapy, which already showed promising outcomes in rodent models, where one single dose of CPC was less efficient when giving the same number of cells divided into three smaller doses a few weeks apart [42, 43]. Nevertheless, new studies and clinical trials that incorporate these repeated treatments are still essential to test their truly safety and efficacy. Another emerging strategy is to develop genetically engineered cells to optimize their quality, functionality and performance, to serve as enhanced therapeutic agents for heart regeneration and repair [44]. However, the heart is one of the most challenging organs to repair, so an in-depth comprehension of how the heart develops and a better understanding of heart regeneration processes could improve the efficiency of therapies targeting cardiovascular regeneration after MI.

1.2.1.1.Understanding the human Cardiogenesis

While human cardiogenesis is still not fully understood, cardiac development in animal models (e.g. mouse) has provided sufficient insights that allowed the improvement of CMs differentiation protocols from hiPSCs. To efficiently differentiate hiPSC into CMs, the most common and reproducible strategies involve replicating in culture the key steps along their natural development path *in vivo* (**Figure 1.2**), which require stage-specific activation and inhibition of different signaling pathways, such as Wingless/INT (WNTs), Nodal, bone morphogenic protein (BMP) and fibroblast growth factor (FGF) .Therefore, an in-depth knowledge of these pathways is crucial, as different differentiation protocols rely on their modulation by exposing hiPSC to several growth factors at specific times points and in precise doses to guide them towards cardiac fate. [45].

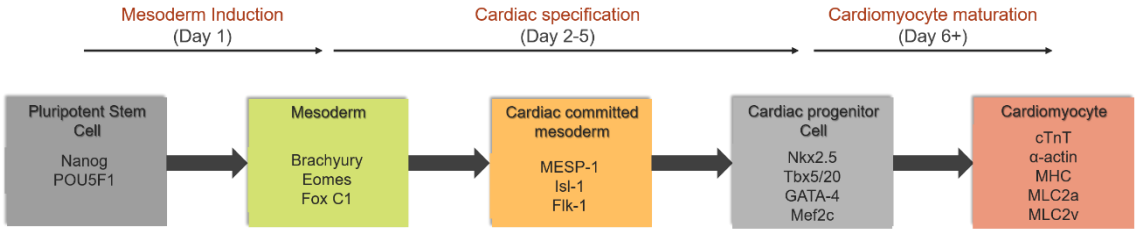

Figure 1.2 – Schematic representation of the expressed transcriptional factors along iPSCs cardiac differentiation. The five major stages in the differentiation of iPSC to cardiomyocytes: pluripotent stem cell, mesoderm, cardiac committed mesoderm, cardiac progenitor cells and cardiomyocyte, are characterized by the distinct expression of different transcription factors. Adapted from. [46].

Cardiac specification begins with Nodal signaling and gastrulation that prompt mesoderm formation. The Nodal signal also upregulates *BMP4* expression, which in turn induces *WNT3* expression. Then WNT promotes the expression of mesoderm markers such as *T* (brachyury) and *Eomes* (eomesodermin) with the consequent activation of *MESP1*, the “master regulator” of cardiac progenitors specification [47, 48]. In this phase, cardiac differentiation will proceed with the inhibition of WNT/ β-catenin signaling. This pathway has a biphasic role during cardiogenesis, being important at the beginning to induce the primitive streak formation and after this stage, inhibition of this pathway is crucial to direct the progenitor cells into cardiac fate. At this point the cardiac mesoderm is formed and several transcription factors begin to be expressed, such as *ISL1, GATA4, TBX5* and *NKX2-5*. This cardiac mesoderm gives rise to the endocardium, the first heart field (FHF) and the second heart field (SHF). While the FHF forms the left ventricle, the majority of the atria and part of the right ventricle, the SHF forms the majority of the right ventricle, outflow tract and part of the atria [49]. The three major cellular lineages that compose the heart tissue, CMs, endothelial cell (EC) and vascular smooth muscle cells (SMC) are all derived from the mesoderm phase [50].

To obtain functional CMs from hPSCs, the cardiac progenitors still need to differentiate into beating CMs, which are identified by the expression of certain proteins involved in

morphogenic events leading to the formation of the heart, such as α-actinin, α-myosin or the cardiac isoform of Troponin-T (cTnT) [51]. However, generated CMs are still immature, to the point where their phenotype resembles more the fetal CMs *in vivo* than the adult CMs [52]. This lack of maturity in hPSC-derived CMs can be assessed by analyzing the structure, energy related characteristics and gene expression of cardiac specific markers (**Table 1.1**), like the myosin heavy (MYH6, MYH7) and light chains (MYL2, MYL7). For example, while the MYH7 and MYL2 are predominant in adult CMs, their isoforms, MYH6 and MYL7 respectively, predominate in immature related-CMs [53].

Table 1.1 – Structural, gene expression and energy related characteristics in adult and immature-like CMs. Adapted from [53].

	Adult-CM	**Immature-CM**
Structure	Rod-Shaped	Round or polygonal
Alignment	Longitudinally aligned	Poorly organized
Nucleation	~30% cells bi- or poly-nuclear	Very limited bi-nucleation
Sarcomere organization	Highly organized	Disorganized
Aspect ratio	5–9.5:1	2–3:1
Banding	Z-discs, I-, H-, A- and M-bands	Mainly Z-discs and I-bands
Sarcomere length	2.2 µm	1.6 µm
Gene expression	MYH7 (βMHC) > MYH6 (αMHC) TNNI3 > TNNI1 MYL2 (MLC2v) > MYL7 (MLC2a) Titin isoform N2B predominates ADRA1A (α-adrenoceptor) expressed	MYH6 (αMHC) > MYH7 (βMHC) TNNI1 > TNNI3 MYL2:MYL7 ratio not determined Titin isoform N2BA predominates ADRA1A (α-adrenoceptor) not expressed
Metabolism	Mostly fatty acids	Glucose and lactate but can use fatty acids
Energy production	Mainly oxidative phosphorylation	Mainly oxidative phosphorylation
Mitochondria	Throughout cell; occupies 20–40% of cell volume	Near nuclei; numbers increase during differentiation
Beating	Quiescent	Spontaneous

Additionally, CMs populations derived from hiPSC are still heterogeneous. These populations include myocytes with nodal, atrial and ventricular properties [54]. This variability poses a challenge for the potential application of these cells in transplantation therapies, as it potentiate the risk of arrhythmia [55]. New approaches to generate homogenous populations of CMs from hiPSC in a reproducible way are still necessary. Alternatively, investigating how to efficiently purify a mixture of cells to select the desirable cell type could also be a promising approach. The uncertain outcome in cell-based therapies urged the search for new methods to improve cell differentiation, retention, survival and coupling, by using miRNAs [56], biomaterials [57], 3D cell constructs [58], bispecific antibodies [59],and cytokines [60].

1.2.2.Cell-Free Approaches for Myocardial Regeneration

Based on the hypothesis that the small improvements observed in cell therapy were due to paracrine mechanisms, new strategies bypassing the use of cells as the transplanted agents have emerged. For example, the direct administration of paracrine factors, such as growth factors, non-coding RNAs and extracellular vesicles or direct reprogramming of fibroblast into CMs (**Table 1.2**) [61, 62].

Table 1.2 – Preclinical results from cardiac regeneration therapies for ischemic heart diseases. Adapted from [63, 64]

Therapy	Mechanism	Disease Model	Delivery method	Outcome	Comments	Ref
Allogenic iPSC-CMs hECS-CMs	Direct replacement of CMs	IHF	Surgical intramyocardial injection	LVEF improvement (≈ 10%) at 12 weeks No significative changes in LVEF	Ventricular arrhythmias in all cell transplanted animals	[65] [37]
Microparticles loaded with FGF-1/NRG1	Angiogenesis and Reversal of fibrosis	IHF	Intramyocardial injection	LVEF improvement (≈ 9%) at 3 months	Reduction in ventricular remodeling and increase in vascularization	[66]
miR-199a-3p miR-590-3p	Promote endogenous CMs proliferation	IHF MI	Intramyocardial or with cationic lipid formulations	LVEF improvement (≈ 10-20%) at 8 weeks	Higher number of positive CMs for the DNA synthesis marker EdU	[67]
MR-409 (GHRH agonist)	Pleiotropic effects and activation of GHRH	IHF	Subcutaneous injection	Reduction in the scar size observed after 4 weeks	Failed to improve the cardiac function	[68]
Recombinant FSTL1 in patch	Pleiotropic effects Stimulation of CMs proliferation and arteriogenesis	IHF	Surgical implantation of a patch	LVEF improvement (≈ 10%)		[69]
Retroviral GHMT	Direct reprogramming of human fibroblasts towards the cardiac fate	IHF MI	Intramyocardial	LVEF improvement (≈ 25%) after 12 weeks	Reduction in the scar size	[70]

IHF, ischemic heart failure; FGF-1, fibroblast growth factor 1; FSTL1, follistatin-related protein 1; GHRH, growth hormone-releasing hormone; LVEF, left ventricular ejection fraction; MI, myocardial infarction; NRG1, neuregulin 1.

Although these preclinical studies showed some level of LVEF enhancement, they are still far away from complete regeneration of the infarcted heart. These marginal improvements (9%-25%), in **Table 1.2**, help to establish the therapeutic value of these approaches, paving the way for other therapeutic targets. Namely, restauration of coronary vasculature after MI, to

improve heart repair and increase the chance of survival. Nevertheless, this field is still recent and major issues and optimizations still need to be addressed to draw a concrete conclusion about their efficacy in the clinics.

1.2.2.1.Neovascularization and Lymphangiogenesis to augment heart repair

Treatment of ischaemic heart disease has focused on protecting the heart from progression to HF. Even though coronary intervention can restore coronary blood flow after MI, microvasculature obstruction still persists, due to endothelial cell death, inflammation and thrombotic and plaque debris. This leads to poor wound healing and ventricular remodeling as well as increase HF events, diminishing the patients' chances of survival [71]. Therefore, the regeneration of coronary microcirculation is essential for effective heart repair. To achieve this goal, a better understanding of how coronary vasculature is formed during the heart development is crucial. These vessels are essentially composed by vascular endothelium, smooth muscle and fibroblasts, while endothelial cells arise primarily from the sinus venosus (SV) and endocardium (Endo), the epicardium (Epi) acts as a source of trophic factors and progenitors' cells, which ultimately give rise to the smooth muscle cells and fibroblast [72, 73]. This different coronary progenitors' populations can compensate for each other if one exhibits defects, providing robustness in heart development. This compensatory mechanism was recently reported demonstrating the regulation of distinct coronary progenitor pools by both genetic timing (ELABELA-APJ signaling) and the microenvironment (hypoxia) to ensure the establishment of the proper vasculature needed for heart physiology [74].

A crosstalk between the Epi and myocardium is vital for coronary vessel formation during heart development. Epicardial cells secrete essential growth factors that support the developing myocardium, which in turn secretes angiogenic factors to promote vasculogenesis in the developing heart. For example, through fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) signaling pathways [75]. FGF, secreted by epicardium, is essential for normal formation of the coronary vasculature and stimulates the secretion of VEGF-A and VEGF-B by the myocardium. Moreover, myocardial factor thymosin β4 promotes EC migration, proliferation and initiates the epicardial progenitors cells activation [75]. After MI, quiescent epicardial cells reactivate to support heart repair and neovascularization [76]. One way to improve this neovascularization, is priming the epicardium before the injury with thymosin β4 [77]. Still, the precise mechanisms of coronary revascularization upon injury are uncertain and a better understanding of how epicardial response is modulated along the regeneration process would lead to more effective therapeutic approaches.

Besides CM replacement discussed above (in section 1.2.1), other therapies are being studied to promote heart regeneration, namely the stimulation of CM proliferation, the activation of lymphangiogenesis and angiogenesis, immunomodulation and reversal and/or inhibition of fibrosis [64]. For example, the delivery of specific recombinant proteins, such as VEGF-A was shown to improve neovascularization in animal models, although it failed to show beneficial

effects in clinical trials (EUROINJECT-ONE and NORTHERN) [78]. However, other growth factors, such as follistatin-related protein 1 (FSTL1), were shown to improve myocardium regeneration after MI [69]. Nevertheless, by changing the strategy of delivery, resorting to intramyocardial injection of synthetic modified RNA encoding human VEGF-A in a mouse MI model, led to an improvement of heart function [79]. Thus, the poor outcomes observed in the growth factor-based approaches in clinical trials might be due to inappropriate dosages, inadequate delivery strategy and/or lack of organ selectivity.

Another emerging strategy is the stimulation of lymphangiogenesis, which is required for clearance of edema and to reduce inflammation. One way to do this is through the stimulation of VEGF-C signaling [80], that is the key mediator of lymphangiogenesis during development and required for SV sprouting through its binding to VEGF receptor 3 (VEGFR3) following maturation process [81]. The vascular system consists of blood and lymphatic vessels; these lymphatic vessels are essential for tissue and body fluid homeostasis with vital role for the transport of macromolecules, immune system cells and absorption of lipids from the digestive system. During the differentiation process, lymphatic endothelial cells differ from the others due to the collective action of different transcriptional factors, thereby any defective mutation of these proteins may cause primary lymphedema [82, 83].

Genetic studies in zebrafish and mice models lacking lymphatic vessels development, as well as in rare individuals with Hennekam syndrome, have contributed to the identification of a secreted protein collagen and calcium-binding EGF domain 1 (CCBE1), that is required for proper lymphatic vascular development (**Figure 1.3 A**) [84, 85]. Moreover, over the last years reports suggested the requirement of this particular protein and a metalloproteinase (ADAMTS3) for the activation of VEGF-C [86].

1.2.3.The Role of CCBE1 as a potential modulator of cardiac function

CCBE1 was identified to be expressed in heart precursors in mouse embryos from embryonic day (E)7.0 to (E)9.5 [87], particularly near the developing lymphatic vessels and in the developing heart [88]. Also, it was identified in early cardiac progenitors in chick embryos and in heart precursors of first and second heart field [87]. In humans, mutations on this protein were found to be associated with Hennekam syndrome, which is an autosomal recessive lymphatic disorder where about 25% of the patients exhibit mutations in CCBE1 [89]. This rare disease displays diverse pathological features like lymphedema, lymphangiectasias and intellectual disability [84]. Moreover, EGF protein family, which includes CCBE1 was identified in embryonic cardiac fibroblasts as a paracrine factor involved in the regulation of CMs proliferation [90]. Also, there are several reports suggesting the importance of this protein on cancer context, such as tumor suppressor gene in ovarian cancer and as a potential biomarker in the detection of lung [91] and gastrointestinal stromal tumors (**Figure 1.3 B**) [92].

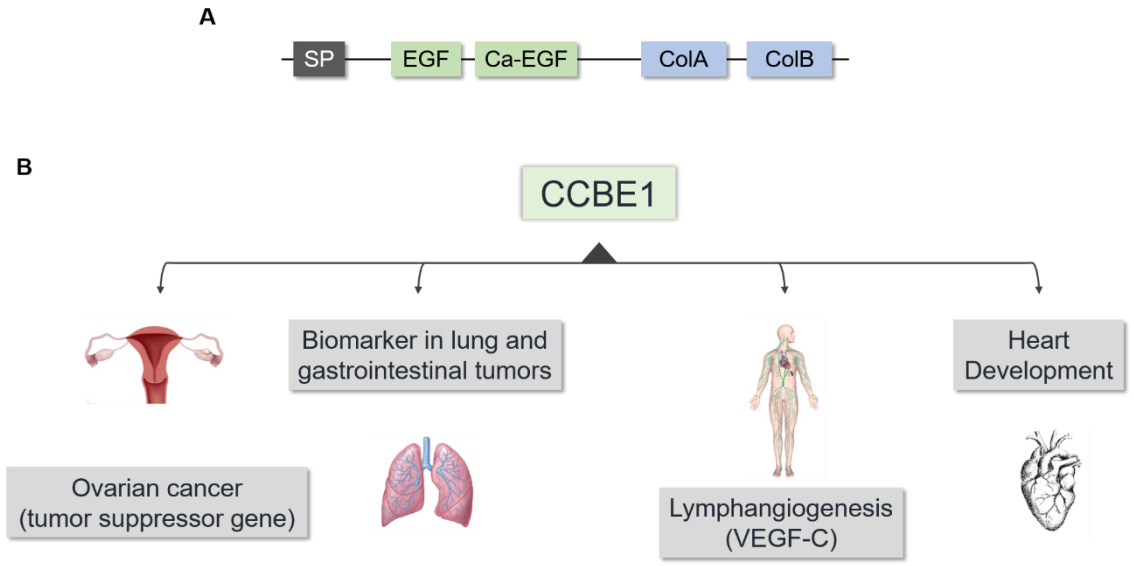

Figure 1.3 – **Schematic representation of CCBE1 and its key roles. (A)** CCBE1 protein domains. SP, signal peptide; EGF, epidermal growth factor domain; Ca-EGF, calcium binding EGF domain; ColA, collagen repeat A; ColB, collagen repeat B. **(B)** CCBE1 key roles suggested so far. Adapted from [93].

As previously mentioned, CCBE1 is involved in the activation of the major lymphangiogenic growth factor VEGF-C, which is crucial for lymphatic development in mouse embryos and for the major part of the lymphangiogenesis process in adults [93, 94]. However, VEGF-C is synthesized as a precursor molecule, the pro-VEGF-C, and needs to be further activated to play its key role [95]. For this purpose, the ADAMTS3 (A Disintegrin And Metalloproteinase with Thrombospondin Motifs-3) protease in a complex with CCBE1, is responsible for the cleavage of pro-VEGF-C to generate a mature and activated version of this factor, which binds to and further activates the VEGFR-3 receptor (**Figure 1.4 B**) [82, 86]. The C-terminal of CCBE1 is essential for an effective activation of VEGF-C, whereas the N-terminal is important for the colocalization of pro-VEGF-C with CCBE1 and ADAMTS3 on the endothelial cell surface which may be necessary for an efficient cleavage of this factor by ADAMTS3, contributing to the VEGFR-3 signaling increase [93, 96].

While mutations in CCBE1 C-terminal domain have shown to result in the absence of lymphatic structures in mice, mutations in the N-terminal resulted in incomplete and disorganized lymphatic vessels, suggesting a role in the organization and migration of lymphatic endothelial cells [93]. Recent studies also demonstrated the importance of CCBE1 in coronary vasculature development through the activation of VEGF-C during the embryonic development [74, 97]. In these studies, CCBE1 knockout in mouse models displayed similar heart defects as in VEGF-C mutants, exhibiting a stunted angiogenesis compared to the wildtype. Therefore, CCBE1 could also be used as a therapeutic factor to stimulate neovascularization after MI (**Figure 1.4 B**). However, obtaining sufficient amounts of stable recombinant full-length CCBE1 protein (49 kDa) remains a major hurdle, being an issue for functional and therapeutic studies using this protein [86, 98]. Alternatively, the use of precise gene editing tools (e.g. CRISPR/Cas9) in combination

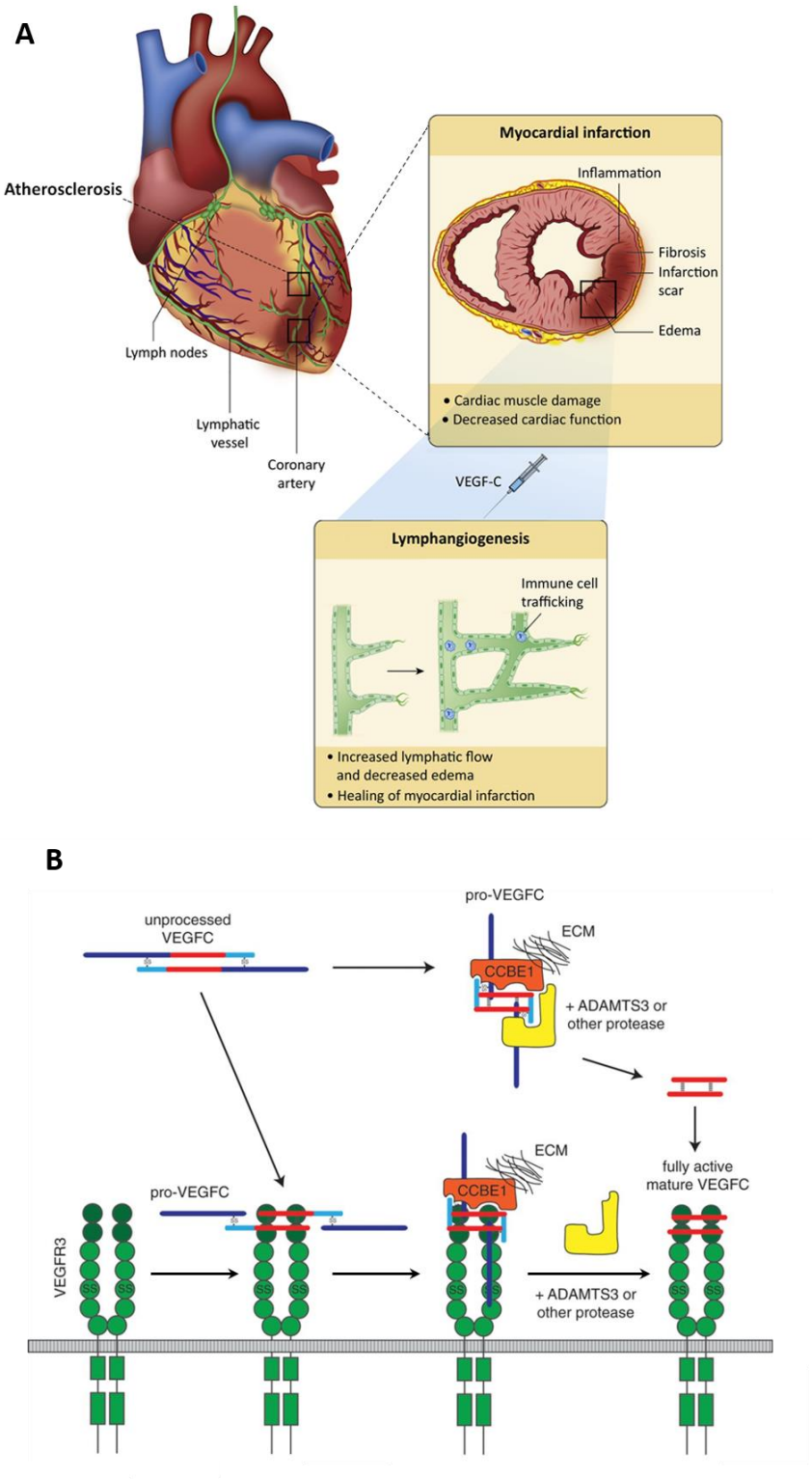

Figure 1.4 – Lymphangiogenesis: VEGF-C activation by CCBE1 and ADAMTS3 complex and its role in myocardial infarction (MI). (A) MI is followed by adverse remodeling of epicardial collector lymphatics, with subsequent edema, severe inflammation and fibrosis. A therapeutic approach is based on VEGF-C administration to increase lymph flow and resolves inflammation, improving the cardiac function. **(B)** CCBE1 secretion at sites of lymphatic vessel growth promotes the proteolytic cleavage of pro-VEGF-C form by the disintegrin/metalloprotease ADAMTS3. The mature form of VEGF-C can further activate VEGFR-3. Most of the VEGF-C cleavage may occur on lymphatic endothelial cell (LEC) surface mediated by CCBE1 and ADAMTS3. Adapted from [99, 100].

with hiPSCs would greatly contribute for *in vitro* studies helping to uncover CCBE1 role in human cardiac repair.

1.3.Gene editing tools

To better understand the regulatory networks that drive specific cellular activities, in healthy or disease conditions, one can use precise and effective tools for gene manipulation. The capacity to manipulate the expression of desirable genes either by repression or activation facilitates the understanding of pathophysiological mechanisms in cardiovascular diseases. This manipulation can now be achieved with the emergence of genomic-editing systems which are getting more advanced, efficient and simpler to use. Advantages and disadvantages of the currently major gene editing tools are summarized in **Table 1.3**.

Table 1.3 – Comparison of the major gene editing tools. Adapted from [101]

Gene editing tool	ZFN	TALENs	CRISPR/cas9
Source	Bacteria, Eukaryotes	Bacteria (Xanthamonas sp.)	Bacteria (Streptococcus sp).
Easy of design	Difficult	Moderate	Easy
Specificity	High	High	High
Efficiency	Low	High	High
Multiplexing	Low	Moderately High	High
Sequence limitations	Non-guanosine rich sequence hard to target	5′targeted base must be thymine for each TALEN monomer	PAM sequence must follow target site
Cost	High	Moderate	Cheap

Nowadays, different systems for genome manipulation have already been described, such as RNA-mediated interference (RNAi) and customized classes of DNA binding-chimeric proteins for instance zinc-finger proteins (ZFs), Transcription activator-like effector nucleases (TALENs) and more recently, the promising guide RNA (gRNA)-driven Cas9 (CRISPR) system. These tools offer a great prospect for the future of cardiovascular field and for in vivo genome-editing therapies. The RNAi was the first tool being explored [102]. In this system small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) bind to the target endogenous mRNAs transcripts promoting their cleavage [102]. However, its low reported efficacy and non-specificity has limited its wide application [103, 104]. For precise genetic modifications, custom engineered and site-specific endonucleases were successfully developed, namely the ZF and TALENs.

The ZFs consist of programable DNA-binding domains fused to a functional domain, which allows the manipulation of gene expression levels in a modular way by recruiting effectors into transcriptional sites of the target genes. For a better genomic specificity, a combination of at

least six ZFs in the DNA binding domain is recommended, as each one can recognize approximately only three bp of DNA [105]. This tool has already been applied for the genome-editing of several living organisms [106–109] and to different cell lines [110, 111] in a successful way.

TALENs system is similar to the ZFs in a way that both have a DNA-binding domain fused with a functional domain. However in this system the binding protein consists of highly conserved TALEN tandem repeats of 33-35 amino acids, that can be easily designed and with the potential to target any sequence with a high success rate [112].

An emerging alternative based on RNA-guided nuclease overcome the above limitations. The type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), derives from the adaptive immune system of Streptococcus pyogenes, which protects the bacteria from exogenous DNA-containing phages and plasmids, thanks to the combination of CRISPR loci and a Cas9 nuclease [113]. This loci together with short spacer sequences derived from past virus infections, are transcribed into long RNAs (crRNAs), which forms a complex with a small transactivating CRISPR RNA (tracrRNA), called guide RNA (gRNA) that will provide the capacity to search and guide the Cas9 nuclease to cleave the target viral DNA by creating a double strand break (DSB) [113]. The Cas9 has two nuclease like-domains, HNH and RuvC that will promote a DSB in the target sequence after binding to a short DNA sequence, named protospacer-adjacent motif (PAM) which flanks the RNA-binding site [114]. This DSB triggers the DNA repair mechanisms that sporadically introduces indel mutations in the target sequence, allowing the introduction, in a simple and easy manner, any desirable mutations in target genes [115, 116] One of the major drawbacks for the application of this technology is the off-target effects. It has already been reported that the Cas9 binds to off-target sites and although only a small subset of those are cleaved efficiently, they still represent a major concern, since other genes could be mutated with serious damage potential [117, 118].

In addition to gene editing, CRISPR technology can be used for regulation of gene expression, without cleaving the target site. For this purpose, a nuclease-deficient Cas9, labeled as dead Cas9 (dCas9) has been developed by inserting mutations into the two nuclease domains of Cas9, the HNH and RuvC. The CRISPR-dCa9, also called CRISPR interference (CRISPRi) is still capable to specifically bind to the target sequence when guided by the gRNA either in the promotor or regulatory sequences and manipulate its transcription process without changing the genomic sequence (**Figure 1.5**) [114, 119]. It can prevent the transcription initiation or elongation by blocking the binding of important transcription factors in the transcription site or the RNA polymerase II [114, 119, 120]. Furthermore, the genetic regulation using CRISPR-dCas9 is not permanent and can be easily reversed. Moreover, grouping of dCas9 with a transcriptional repressor, like the Krüppel-associated box (KRAB) [121] or four concatenated mSin3 interaction domains (SID4X) [122] can improve the repression of endogenous genes, this was already demonstrated in eukaryotic models [121] and more recently in hiPSC [123]. In addition, coupling

the dCas9 with a transcriptional activation domain, such as VP64 or p65, termed CRISPR activation (CRISPRa) can increase the expression of endogenous genes [124, 125].

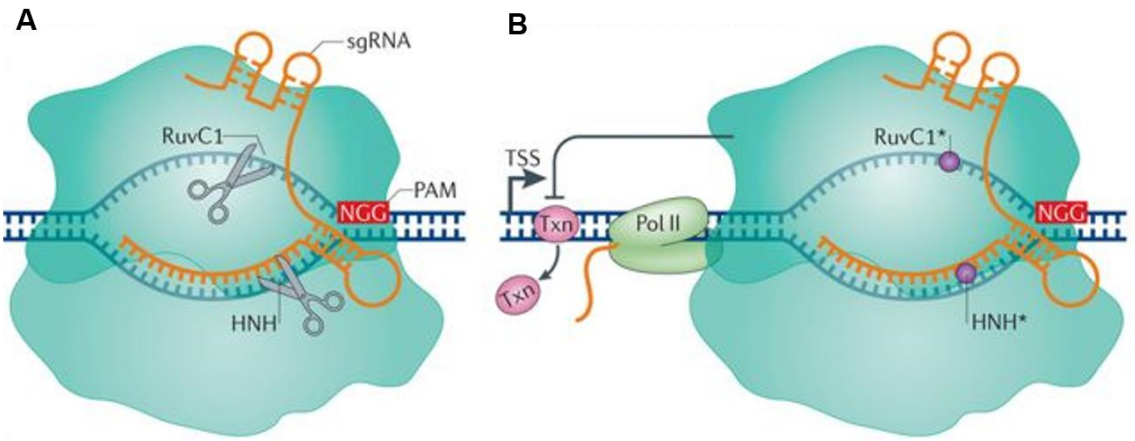

Figure 1.5 – Differences in action method of Streptococcus pyogenes Cas9 and dCas9. (A) The single guide RNA (sgRNA) leads Cas9 to the target DNA sequences. This targeting is dependent on the presence of a 5′ protospacer-adjacent motif (PAM) in the DNA, which in S. pyogenes is usually NGG. After binding the two nuclease domains (RuvC1 and HNH) cleave the target sequence. **(B)** Mutations in the two nuclease domains deactivate the Cas9 protein (dCas9), inactivating its nuclease function (circles), but still retains the capacity to target specific sequences through sgRNA guidance and PAM. dCas9 binds near the transcription start site (TSS) and blocks transcription elongation by obstructing RNA polymerase II (Poll II) or blocking the binding of important transcription factors (Txn). Adapted from [126]

In fact, genome editing has already been used to create more reliable cardiac disease models or correct genetic mutations in IPSC-derived CMs by introducing genetic alterations [127]. One example, was a disease model for Barth syndrome (mitochondrial disorder caused by mutation of the gene encoding tafazzin) developed by Wang and colleagues, which combined patient-derived IPSC and genome editing tools, like CRISPR/cas9 to mimic the pathophysiology of this specific disorder *in vitro* [128].

For loss-of function studies, Mandegar and colleagues developed a versatile CRISPRi-dCas9 inducible system for hiPSC lines. This repression system enables precise control of single or multiplexed gene expression upon doxycycline addition, making this CRISPRi-hiPSCs lines an attractive tool for identification of novel factors involved in cell differentiation and maturation. Moreover, this system was validated for multiple genes along cardiac differentiation, in hiPSC, CPC and CMs, demonstrating its efficacy and reliability (**Figure 1.6**) [123].

These current advances in gene editing tools and in iPSC technology offers a great opportunity to better understand the pathophysiological mechanism of cardiac diseases and to develop reliable differentiation protocols resulting in more homogenous cardiac populations and trustworthy cell models suitable to improve disease understanding and to propose novel cell therapies.

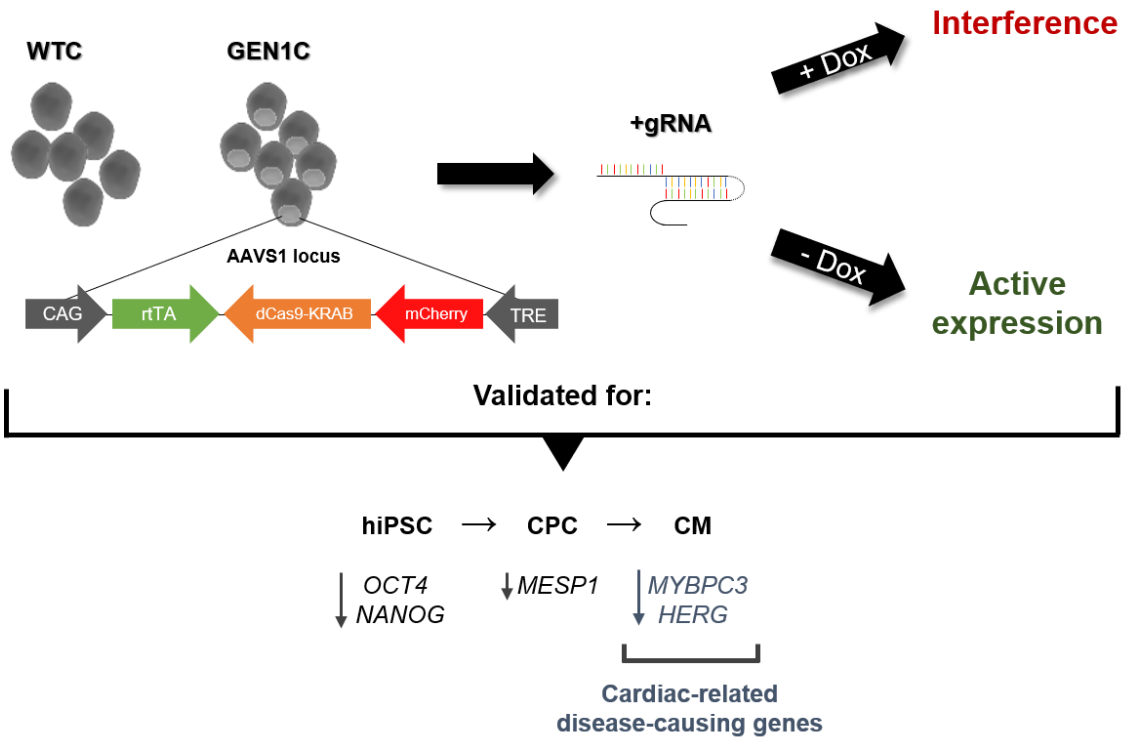

Figure 1.6 – Potential of CRISPRi hiPSC lines developed by Mandegar and colleagues. GEN1C cell line was generated by integration of CRISPRi construct into the AAVS1 locus of WTC. Posteriorly, a gRNA or gRNAs are selected and introduced in the cell line to specifically target a gene or genes of interest. After that, the targeted gene expression will be repressed upon Dox induction. This system is also validated for multiple genes specific in different stages of CM differentiation. Adapted from [123].

2.Aim of the thesis

CCBE1 has been studied as an important protein for lymphatic vessels development and despite the increasing evidence of a potential involvement in cardiac commitment, further investigation is still needed to validate this hypothesis. Therefore, the aim of this thesis was to unveil the role of CCBE1 on cardiovascular development exploring gene editing tools (**Figure 2.1**). In this context, we performed CCBE1 loss-of-function studies using the CRISPRi technology in hiPSC to knockdown CCBE1 gene expression during CM and EC differentiation process.

The first objective consisted on evaluating the CCBE1 knockdown efficiency and its impact on hiPSCs pluripotent phenotype. To accomplish this, CCBE1 knockdown hiPSC lines were generated. Additionally, their self-renewal capacity, differentiation potential and CCBE1 gene expression were assessed. The second objective was to evaluate CCBE1 knockdown impact along hiPSC differentiation into CM and EC. We performed a detailed characterization of hiPSC-derived CM/EC phenotype in CCBE1 knockdown cell line.

Overall this work provides new insights on CCBE1 role in cardiac development. The knowledge here described would help to identify CCBE1-modulatory pathways and explore CCBE1 as a therapeutic molecule for cardiovascular regenerative medicine.

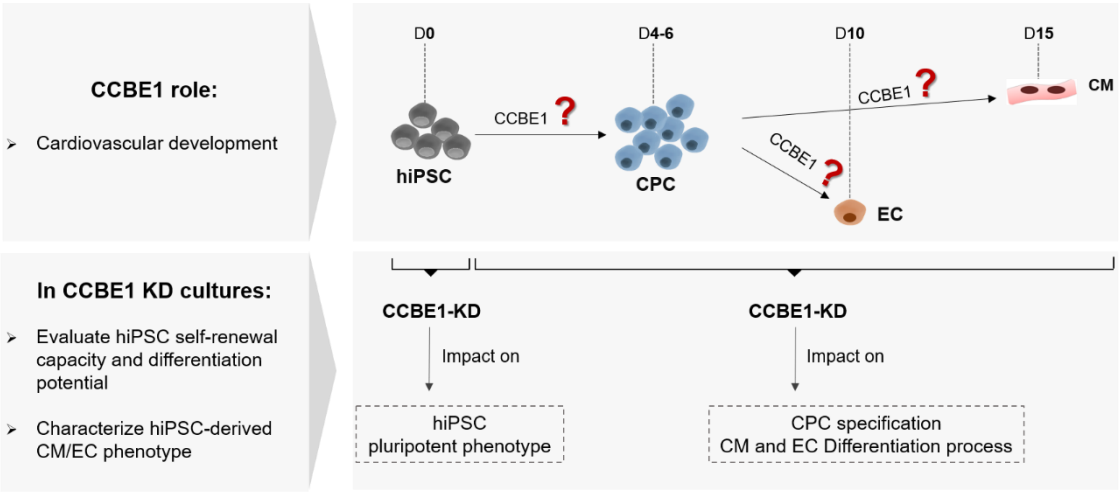

Figure 2.1 – Schematic representation of the major aims of this thesis and outlined strategy. The main aim of this thesis was to unveil the role of CCBE1 on cardiovascular commitment. hiPSC- human induced pluripotent stem cells; CCBE1- collagen and calcium-EGF biding domain; CPC- Cardiac Progenitor Cell; CM- Cardiomyocytes; EC- Endothelial Cells; KD- knockdown.

3.Material & 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. Wild-type C (hereafter designated as hiPSC-WT) and a modified cell line CRISPRi Gen1C (hereafter designated as hiPSC-CRISPRi), integrating a Tet-On inducible system that modulate the expression of a deactivated Cas9 (dCas9) fused with the repressor KRAB domain. mCherry reporter gene is under the control of the same inducible promoter (at downstream of dCas9-KRAB, separated by p2A). These cell lines were derived by Mandegar MA and colleagues [123] and provided by The J. David Gladstone Institutes under a Material Transfer Agreement.

3.1.2.hiPSC expansion

hiPSC lines were routinely propagated in static culture systems, 6-well plates (Falcon™), coated with growth factor reduced (GFR) Matrigel®, Phenol Red Free (BD Biosciences) using mTeSR1™ media (STEMCELL Technologies), according to the protocol described by Mandegar and colleagues [123]. Cells were maintained under humidified atmosphere with 5% CO_2 at 37ºC.

3.1.3.hiPSC cardiac differentiation

hiPSC were differentiated into CM in monolayer culture systems, according to the recently published protocol [129]. hiPSC single cell suspensions were prepared by incubation with Accutase (STEMCELL Technologies) for 3 min at 37ºC and seeded at a density of 7-9 × 10^4 cell/cm^2 in Matrigel coated 6-well plates (Falcon™) or µ-Slide 4 well formats (ibidi®). Two days after cell seeding, the differentiation was induced by replacing the expansion media with RPMI 1640 medium (Gibco®) supplemented with 2% (v/v) B27 minus insulin (Invitrogen), 12 µM CHIR99021 (Biogen Cientifica S.L), 80 ng/mL Activin A (PeproTech) and 50 µg/mL ascorbic acid (Sigma-Aldrich). Twenty-four hours later (day 1 of differentiation) the media was replaced by RPMI supplemented with 2% (v/v) B27 minus insulin, 5 µM IWR-1 (Sigma-Aldrich) and 50 µg/mL ascorbic acid. At day 3 of differentiation, cells were incubated with RPMI supplemented with 2% (v/v) B27 minus insulin, 5 µM IWR-1. From day 6 until day 15, the medium was changed 3 times per week with the RPMI supplemented with 2% (v/v) B27 minus insulin [129]. Cells were maintained under humified atmosphere with 5% CO_2 at 37ºC.

3.1.4.hiPSC endothelial differentiation

hiPSC were differentiated into endothelial cells according to the protocol described by Giacomelli and colleagues [130]. Cells were seeded at 1.25 × 10^4 cell/cm^2 in 6-well plates coated in Matrigel 24 hours prior initiation of differentiation process. At day 0 of differentiation, the media was replaced by APEL-Li (STEMCELL Technologies) supplemented with Activin A (20 ng/ mL), BMP4 (20 ng/mL) and CHIR99021 (1.5 µM). Then medium was replaced every three days by APEL-Li supplemented with VEGF (50 ng/mL) until day 10 of differentiation. Cells were

maintained under humified atmosphere with 5% CO_2 at 37ºC. All supplements were supplied by Peprotech.

3.2. CCBE1 knockdown: gRNA design, cell electroporation and selection

For CCBE1 knockdown, four gRNAs were designed to target near the transcription start site (TSS) of CCBE1 (between 150 bp upstream and 150 bp downstream). All gRNAs were phosphorylated, annealed and cloned into the pgRNA-CKB vector (kindly provided by Bruce Conklin; Addgene plasmid # 73501) at BsmBI restriction site. All the cloning steps were performed as described elsewhere [123]. gRNA oligo sequences are listed in **Table 3.1**.

The pgRNA-CKB expression vector, containing mKate2 as reporter gene and blasticidin as antibiotic selection marker (mKate2-T2A-Bsd), was transfected into CRISPRi cells using the Neon Transfection System (Thermo Fisher Scientific) according to manufacturer's instructions. Two conditions were tested (condition 1: 1400 V, 20 ms, 2 pulses and condition 2: 1100 V, 30ms, 1 pulse). CRISPRi cells (2×10^6 cells) were transfected with 5 µg of vector carrying a CCBE1-specific gRNA generating the CRISPRi-CCBE1 KD cell line or with empty pgRNA-CKB vector, without a gRNA to generate the CRISPRi-Ctrl cell line (control condition).

Table 3.1 – List of gRNA oligo sequences. Each gRNA indicates the binding relative to the transcription start site (TSS) of CCBE1 gene, and whether they target the template (T) and non-template (NT) strand. Forward and reverse primers for cloning into the pgRNACKB gRNA-expression vector are listed from 5' to 3'.

gRNA Name (Targeting Strand)	**Oligo Sequences** 5' – Forward Primer – 3' 5' – Reverse Primer – 3'
CCBE1 g-145 (NT)	TTGGAAGGGGGTACCTGCGGTGTC AAACGACACCGCAGGTACCCCCTT
CCBE1 g-82 (NT)	TTGGCAGGGGTCCGGAATATTATG AAACCATAATATTCCGGACCCCTG
CCBE1 g+22 (T)	TTGGAGCAGGACGCTTGGTCCGGA AAACTCCGGACCAAGCGTCCTGCT
CCBE1 g+37 (NT)	TTGGTCCCAGCGCCGAGCTCCGTC AAACGACGGAGCTCGGCGCTGGGA

Twenty-four hours post transfection, blasticidin selection was applied by culturing the cells in mTeSR1 supplemented with Y-27632 (10 µM) and blasticidin (10 µg/mL). Stable colonies were pooled and passaged five times to enrich for cells with integration into sites of active transcription. The percentage of nucleofected cells was evaluated by mKate2 expression using an inverted fluorescence microscope (Leica Microsystems GmbH).

CRISPRi mediated gene knockdown studies, using CCBE1 g+37 (**Table 3.1**), were performed by supplementing the media with doxycycline (Dox; 2 µM). To allow CCBE1 expression, cells were cultured in the absence of Dox. The gene knockdown efficiency was examined by RT-qPCR.

Both generated cells lines, CRISPRi-CCBE1 KD and control were also cloned by limiting dilution in 96-well plates (Falcon™) coated with Matrigel. Cells were seeded at a density of 0.5 cells/well and 7/6 clones were isolated from CRISPRi-CCBE1 KD/ control populations respectively.

3.3.hiPSC Characterization

3.3.1.Cell concentration and viability determination

Accutase (STEMCELL Technologies) and TrypLE™ Select (Gibco Life Technologies) were used to enzymatically dissociate hiPSC and differentiated cells respectively into single cell suspensions, during 3 minutes at 37ºC. Cell suspensions were then diluted in 0.1 % (v/v) Trypan Blue (Life Technologies) in DPBS (Gibco®). Trypan Blue cannot enter the viable cells membrane but enters in non-viable cells that exhibit damaged membranes [131], allowing the quantitative determination of cells viability and concentration after counting the cells in a hemocytometer counting chamber (Fuchs-Rosenthal).

3.3.2.Cell proliferation

The percentage of proliferating hiPSC cells was determined using Click-iT EdU (5-ethynyl-2´-deoxyuridine) Flow Cytometry (FC) Assay Kit (Life Technologies) following manufacturer's recommendations. Edu is a thymine analog that can be incorporated into DNA during cellular replication to detect cell proliferation. Cell cultures were incubated with Edu (10 μM) in culture media for 24 hours at 37ºC. After the incubation period, cells were fixed with the fixative solution (Life Technologies) provided in the Kit for 15 minutes and permeabilized with saponin-based working reagent for 15 minutes. Cells were then incubated with Click-iT AlexaFluor® 488 azide, for 30 minutes. All incubations periods were performed at RT and protected from the light. Samples were analyzed in a CyFlow® space instrument (Partec GmbH, Germany). At least 10,000 events were registered *per* sample.

3.3.3.Immunocytochemistry

The detection of CM markers in hiPSC-derived CM cultures (CRISPRi-Ctrl and CRISPRi-CCBE1 KD) was performed as described elsewhere [132]. Preparations were visualized in point scan confocal microscope (SP5, Leica). Cells were fixed in 4% (v/v) paraformaldehyde in DPBS for 15 min and then blocked and permeabilized (for intracellular epitopes) in 0.2% (v/v) in fish skin gelatin (FSG) and 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in DPBS for 30 min. Cells were incubated with primary antibodies diluted in 0.125% (v/v) in FSG and 0.1% (v/v) Triton X-100 at room temperature (RT) for 2 h. Then, cells were washed three times in DPBS and then incubated with secondary antibodies diluted in 0.125% (v/v) FSG and 0.1% (v/v) Triton X-100 in PBS at RT for 1 h. After three washing steps in PBS, cell nuclei were counterstained using DAPI (Life Technologies). Preparations were visualized in point scan confocal microscope (SP5, Leica). Primary and secondary antibodies used are listed in **Table 3.2**.

3.3.4.Flow Cytometry

Cells along cardiac and endothelial differentiation were collected and dissociated by incubation with Accutase for 3 minutes at 37ºC, for undifferentiated cells, or TrypLE™ Select (Gibco Life Technologies), for the differentiated cells for 5 minutes at 37ºC.

For membrane markers detection, cells were washed with 2% (v/v) FBS in DPBS (washing buffer), and then incubated for 1 hour at 4°C with the primary antibody. After the incubation period, cells were washed twice with washing buffer. For non-conjugated primary antibodies, cells were further incubated with the suitable secondary antibody for 30 minutes at 4ºC, followed by two washes with washing buffer.

For intracellular markers detection, cells were detached and washed with Intra Buffer (phosphate buffered saline, pH 7.2, supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA), after which, cells were fixed using Inside Stain Kit (Miltenyi Biotec), according to the manufacturer's instructions. Cells were incubated with primary antibodies for 30 minutes in the dark at room temperature (18-25ºC). After the incubation period cells were washed with Inside Perm reagent (Miltenyi Biotec), then re-suspended in Intra Buffer for analysis or incubated with the secondary antibody (30minutes in the dark at RT) if the primary antibody was non-conjugated. 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. Primary and secondary antibodies used are listed in **Table 3.2**.

3.3.5.mRNA Extraction and RT-qPCR

Cells were dissociated as previously described, collected and washed with DPBS. For storage these pellets were snap-freezed with liquid nitrogen and stored at -80ºC 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 Fisher). cDNA synthesis was carried out using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). RT-qPCR reactions were performed using the LightCycler 480 Instrument II 384-well block (Roche) and the program cycles as follow: pre-incubation for 10 minutes at 95ºC; 45 cycles of amplification with denaturation at 95ºC for 15 seconds and annealing at 60ºC for 1 minute; extension at 72ºC for 5 minutes. The primers and probes used in this work are listed in **Table 3.3**. The Cycle threshold (Ct) was determined using LightCycler 480 Software version 1.5 (Roche). The results were analyzed as described elsewhere (Livak & Schmittgen 2001), using the $2^{-\Delta\Delta C_T}$ 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).

Table 3.2 – List of all antibodies and dilutions used for immunocytochemistry and flow cytometry analysis.

	Antibody	Origin	Supplier	Catalog No.	Dilution (Application)
Primary	Col IV	Rabbit	Abcam	ab6586	1:100 (IC)
	TroponinT	Mouse	Thermoscientific	MS-295-P1	1:200 (FC, IC)
	TRA-1-60	Mouse	Santa Cruz	sc-21705	1:10 (FC)
	TRA-1-81	Mouse	Santa Cruz	sc-21706	1:10 (FC)
Secondary	Alexa 488, anti-mouse IgG1	Goat	Life Technologies	A-21121	1:200 (FC)
	Alexa 488, anti-mouse IgM	Goat	Life Technologies	A-21042	1:200 (FC)
	Alexa 488, anti-rabbit IgG1	Goat	Life Technologies	A-11008	1:200 (FC)
	Alexa 594, anti-mouse IgG1	Goat	Life Technologies	A-11005	1:200 (FC, IC)
Conjugate	SSEA1-FITC	Mouse	BD Biosciences	560127	1:10 (FC)
	SIRPα/β (CD172) PE	Mouse	BioLegend	323805/323806	1:5 (FC)
Isotype	FITC Mouse IgM		BD Biosciences	553474	1:400 (FC)
	Mouse IgG1		Santa Cruz	sc-2877	1:2.5 (FC)
	Mouse IgGκ1-PE		Santa Cruz	sc-2878	1:5 (FC)

Note: FC, flow cytometry; IC, immunocytochemistry.

3.3.6.Transmission electron microscopy (TEM)

Monolayers of differentiated CRISPRi-Ctrl and CRISPRi-CCBE1 KD cultures (day 15 of differentiation) were fixed in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hour and subsequently washed four times in 0.1 M phosphate buffer before fixation with osmium tetroxide (1% (v/v) in 0.1 M phosphate buffer) for 30 minutes on ice in the dark under agitation. After two washes with 0.1 M phosphate buffer and two washes with water, samples were incubated with tannic acid (1% (w/v) in water) for 20 minutes, on ice. After five washes with water, the samples were contrasted with aqueous uranyl acetate (0.5% (w/v), 1 hour, on ice, in the dark), washed three times in distilled water and dehydrated in a graded series of ethanol (30%, 50%, 75%, 90%, 100% (v/v)). Finally, samples were embedded in epon resin. Ultrathin sections of cell monolayers were cut on a Leica UC6 ultramicrotome using a diamond knife. Sections were collected on formvar-coated slot grids, stained with lead citrate, and analyzed on a FEI Morgagni 268 at 80 kV. Images were taken with an Olympus MegaView III using the iTEM software.

Table 3.3 – List of all Primers used in RT-qPCR.

Gene	Supplier Reference
CCBE1	Hs99999905_m1
Nanog	Hs02387400_g1
POU5F1	Hs00999632_g1
T	Hs00610080_m1
KDR	Hs00911700_m1
MESP1	Hs00251489_m1
GATA4	Hs00171403_m1
Nkx2.5	Hs00231763_m1
VCAM-1	Hs01003372_m1
TNNT2	Hs00165960_m1
MYL2	Hs00166405_m1
MYL7	Hs00221909_m1
MYH6	Hs01101425_m1
MYH7	Hs01110632_m1
TNNI1	Hs00913333_m1
TNNI3	Hs00165957_m1
PECAM-1	Hs01065279_m1
CDH5 (VE-cadherin)	Hs00901463_m1
RPLP0	Hs99999902_m1
GAPDH	Hs99999905_m1

Note: Primers and Probe Mix were purchased from Life Technologies

3.4.Statistical Analysis

Statistical parameters including the exact value of n, precision measures (mean ± SEM) and statistical significance are reported in the Figures and the Figure Legends. Statistical analysis was performed by unpaired Multiple t tests using 0.5 % False Discovery Rate (FDR) approach to compare CRISPRi-CCBE1 KD with CRISPRi-Ctrl at different time points of differentiation. For all graphs, data are represented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; were considered significant. PRISM software was used for analysis, graphing, and statistical analyses (www.graphpad.com/scientific-software/prism/).

4.Results and Discussion

4.1.CCBE1 expression is transiently upregulated at early stages of cardiomyocyte differentiation

We aimed to assess CCBE1 expression and explore its functional role on cardiac commitment/differentiation of hiPSC. For this purpose, a modified and inducible hiPSC line with CRISPRi system, that harbors a deactivated Cas9 (dCas9) fused with a repressor domain (KRAB) was used. This approach enabled us to take advantage of this new genetic tool system (CRISPR) and avoid the complications related with mutated alleles, created by double-strand breaks which are induced by Cas9 in CRISPRn technology, that could cause unwanted partial loss-of-function or gain-of-function phenotypes.

These cells, recently developed by Mandegar and colleagues [123], contain a Tet-On inducible system to control the expression of deactivated cas9 (dCas9) and a mCherry fluorescence marker, induced by the presence of doxycycline (Dox). These characteristics let us control the expression level of a target gene (inducible and reversible system) along expansion and at different stages of cardiac differentiation in hiPSCs lines, carrying the gene-specific gRNA. Fluorescence microscopy was used to monitor Dox responsiveness based on the intensity signal of mCherry, that works as a surrogate for dCas9-KRAB expression, allowing an easy monitoring in hiPSC and along the cardiac differentiation process over time. So, with this approach, we can easily generate loss-of-function phenotypes in this hiPSCs and their derivatives in a rapid and efficient manner.

Initially, the modified hiPSC line (CRISPRi) and its wild type (WT), both with the same genetic background, were characterized according to their pluripotent phenotype and ability to differentiate into CMs. Before initiating the differentiation process, undifferentiated state and pluripotency of hiPSC were assessed at gene and protein expression levels, by RT-qPCR and flow cytometry analysis, respectively (**Figure 4.1**).

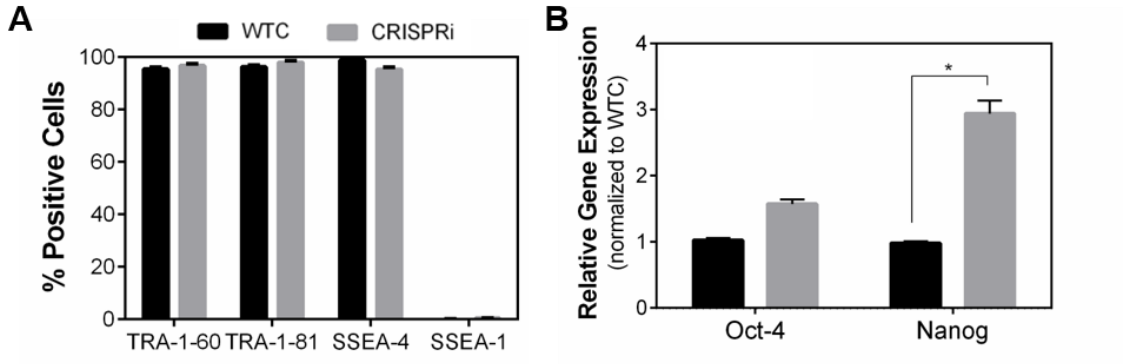

Figure 4.1 – Pluripotency of WTC and CRISPRi hiPSC lines. (A) Flow cytometry analysis of stemness markers (TRA-1-60, TRA-1-80, SSEA-4) and SSEA-1 in both CRISPRi-hiPSC and WTC-hiPSC lines along the expansion process. **(B)** Gene expression of stemness markers Oct-4 and Nanog relative to WTC line. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method relatively to day 0 of differentiation of each cell line (housekeeping: RPLP0). Data are presented as mean ± SEM of 3 independent experiments. * $p< 0.05$, Unpaired t-test with Welch's correction.

High percentage of cells (≥ 95%) expressing stemness markers, such as TRA-1-60, TRA-1-81, SSEA-4, and very low percentage of positive cells (≤ 0,5%) for SSEA-1 (early differentiation marker) were detected in both cell lines by flow cytometry (**Figure 4.1 A**). However, although both cells lines show gene expression of pluripotency marker Oct-4 (*POU5F1*) in a similar level, the modified CRISPRi cell line expressed higher levels of Nanog (3-fold increase), another pluripotency marker, when compared with WT line (**Figure 4.1 B**).

We then differentiated CRISPRi and WT cell lines into CMs by modulation of stage-specific pathways (Activin and Wnt/β-catenin-signaling) through sequential addition of growth factor and small molecules (CHIR99021, ascorbic Acid and IWR-1) essential for CM specification, following a protocol previously published by our group [132] (**Figure 4.2 A**). Both cultures were able to differentiate into beating CMs, showing a monolayer of cells with CM-like cell morphology at day 15 (**Figure 4.2 B**). The generated CMs were characterized based on their structural features using immunocytochemistry tools, where it was observed the presence of Col IV and cTnT markers (**Figure 4.2 C**). Moreover, differentiation progression was confirmed by the expression of stage-specific genes, namely of mesoderm formation at day 1, (expression of Brachyury T (*T)),* cardiac mesoderm differentiation at day 2-3 (expression of *MESP1*), cardiac progenitors cells (CPC) specification after day 4 (expression of *GATA4*), and CMs differentiation from day 6 onwards by expression of *TNNT2* (**Figure 4.2 D**). Additionally, comparable percentage of differentiated cells expressing cardiac markers (SIRPα/β and cTnT) suggests that both cell lines have similar differentiation capacities (**Figure 4.2 E**). At day 15 of differentiation WTC and CRISPRi cell cultures were already 84.2 ± 0.5% and 88.2 ± 6.8% positive for cTnT and 61.3 ± 1.1% and 77.8 ± 7.7% positive for SIRPα/β, respectively, indicating their commitment towards CM lineage. In summary, CMs obtained from both cell lines present morphologic and phenotypic properties typical of CMs, as showed by phase contrast microscopy, immunocytochemistry, flow cytometry and gene expression analysis (**Figure 4.2**).

CCBE1 expression profile was also identical in both cultures, showing a downregulation at day 1/2 of differentiation and from day 3 expression start to increase, with a peak of expression in the cardiac progenitors' phase (day 4), being downregulated again at day 15 (**Figure 4.2 F**). This indicates that genetic alteration of the WT line, did not affect CCBE1 expression pattern and that this protein is primarily expressed during CPC specification along the cardiac commitment. This result is consistent with what is described in the literature, namely, in chickens, CCBE1 was reported to be expressed in the bilateral cardiogenic mesoderm, in the early cardiac progenitors [133]. In mice, CCBE1 was confirmed to be expressed in FHF, SHF and proepicardium [87], corresponding to the cardiac progenitors phase during heart organogenesis.

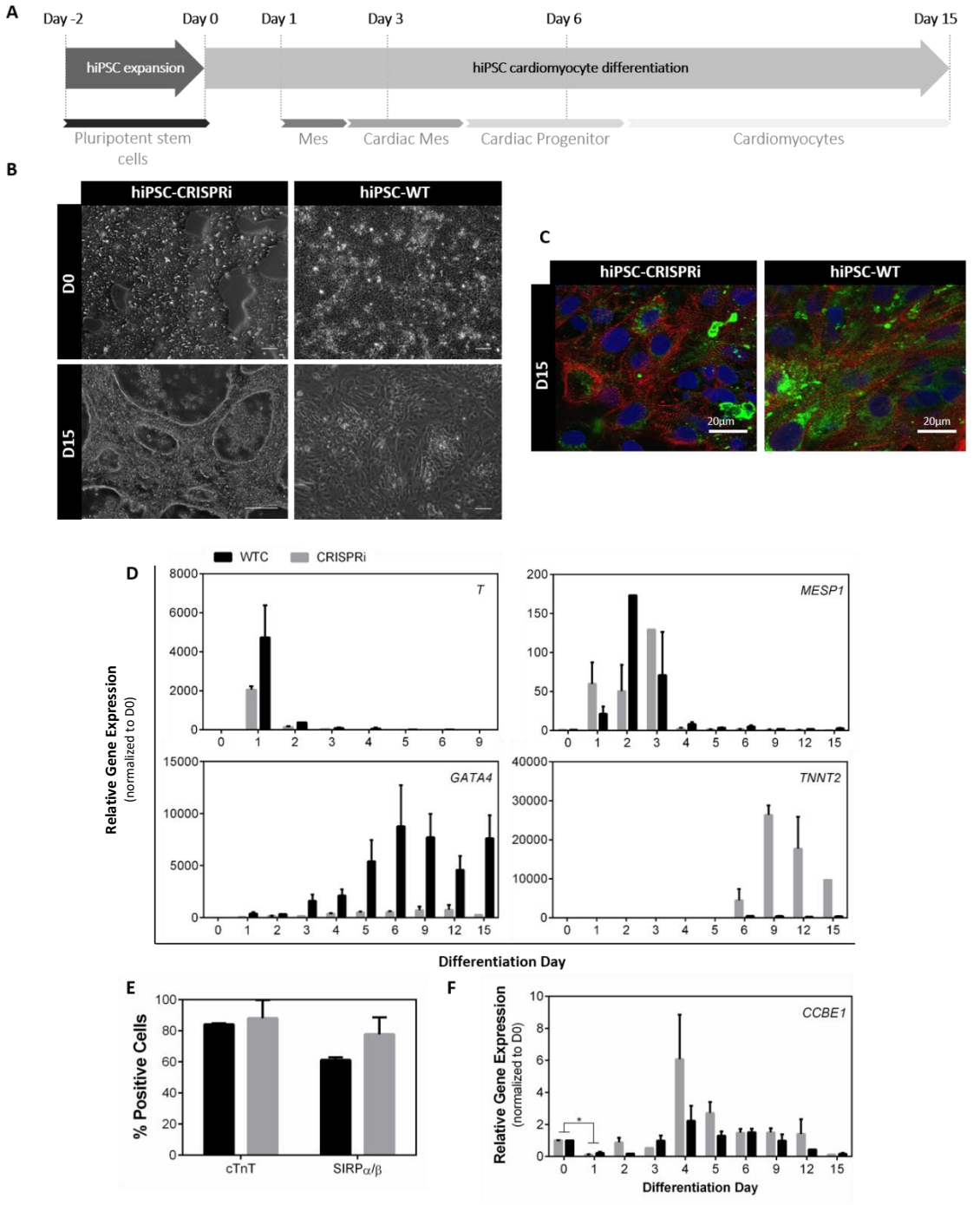

Figure 4.2 – human induced pluripotent stem cells efficiently differentiate into cardiomyocytes. (A) Schematic differentiation protocol using growth factors and small molecules for Wnt signaling modulation using hiPSC lines, as recently published [129]. Legend: Mes - mesoderm; Cardiac Mes - cardiac mesoderm. **(B)** Morphology of hiPSC-CRISPRi and hiPSC-WTC cells cultures at the beginning of differentiation and at day 15. Scale bar, 100 µm. **(C)** Detection of cardiomyocyte markers ColV (green) and cTnT (red) at day 15 Nuclei were counterstained with DAPI (blue). Scale bar represent 20 µm. **(D)** Relative expression mesendoderm (*T*), cardiac mesoderm (*MESP1*), cardiac progenitor (*GATA4*) and cardiomyocyte (*TNNT2*) genes in hiPSC (CRISPRi & WT) lines along cardiac differentiation. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method relatively to day 0 of differentiation (housekeeping: *RPLP0* and *GAPDH*). **(E)** Flow cytometry analysis of cardiac markers, SIRPα/β and cardiac troponin T (cTnT) in both hiPSC-CRISPRi and hiPSC-WTC- lines at the last day of differentiation. **(F)** CCBE1 gene expression along cardiomyocyte differentiation in both WTC and CRISPRi hiPSC lines. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method relatively to day 0 of differentiation of each cell line (housekeeping: *RPLP0* and *GAPDH*). Data are presented as mean ± SEM of 3 independent experiments. * $p < 0.05$, Unpaired t-test with Welch's correction.

4.2.Generation of CCBE1 knockdown in iPSC cell line

To accomplish CCBE1 knockdown (KD), for loss-of-function studies, a new cell line was generated by electroporation of the previously used hiPSC-CRISPRi with the selected CCBE1-targeting gRNA, called CRISPRi-CCBE1 KD. Although CRISPRi technology is unquestionably effective, it only knocks down gene expression efficiently when gRNAs are targeted near the transcription site (TSS) of the gene of interest (between -250 to +250 bp) [123, 134]. Hence, we constructed four gRNAs to target near the TSS, yet only the gRNA targeting 37 bp downstream of CCBE1 TSS was selected for further experiments, since it was the only one without unwanted insertional mutations during the cloning process. For the control culture CRISPRi cell line was electroporated with an empty pgRNA vector, without a gRNA, named CRISPRi-Ctrl. Therefore, in the control condition, dCas9 expression is induced by Dox presence, however without the aptitude for CCBE1 KD since the gene-specific gRNA is missing. Both plasmids used, with or without gRNA contain mKate2 fluorescence marker, that allows an easy monitoring of cells that successfully incorporated the plasmid (**Figure 4.3 A**). Higher electroporation efficiencies (60 ± 4% using the pgRNA containing the gRNA4 and 52 ± 2% using the empty pgRNA) were attained using higher voltage condition (1400 V, **Figure 4.3 B**).

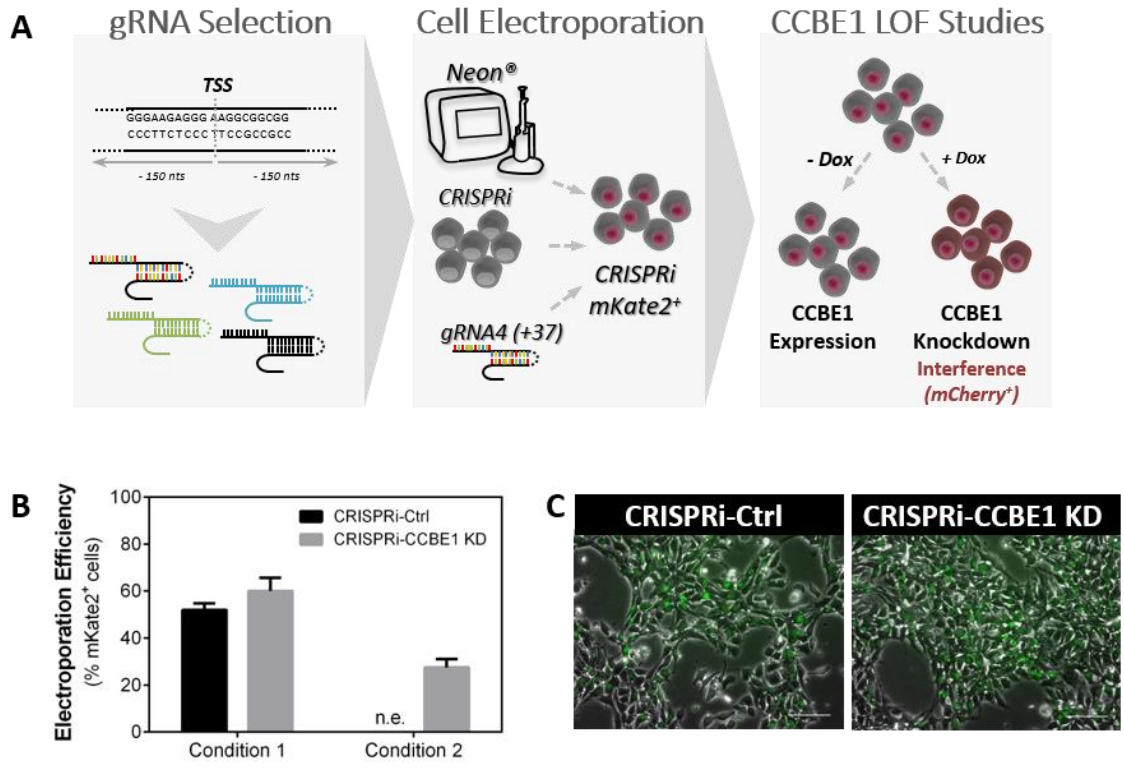

Figure 4.3 – CRISPRi technology for CCBE1 knockdown. (A) Schematic representation of guideRNAs design (+/-150 bp from the transcription start site (TSS)) and selection (detailed information in **Table 3.1**); hiPSC-CRISPRi electroporation with the selected gRNA; and CCBE1 loss-of-function (LOF) studies by doxycycline (Dox) induction. **(B)** Electroporation efficiency of hiPSC-CRISPRi cell line with CCBE1-targeting gRNA (CRISPRi-CCBE1 KD) and empty pgRNA plasmid as control condition (CRISPRi-Ctrl) assessed by mKate2 expression. n.e., not evaluated. Condition 1: 1400 V, 20 ms, 2 pulses and Condition 2: 1100 V, 30ms, 1 pulse **(C)** Detection of mKate2 reporter on cell nuclei of efficiently electroporated hiPSCs after 15 days post-blasticidin selection. Scale bar, 100 µm.

Two different electroporation conditions were tested for this purpose (detailed in Materials and Methods section). Condition 1 showed superior efficiency compared to Condition 2 (≈60% vs ≈30%, **Figure 4.3 B**). The plasmid transfection efficiency obtained for these hiPSCs is comparable with reported literature using an electroporator. These efficiencies are hiPSC line and electroporation condition (voltage and pulse number) dependent, ranging from 17% to 64% [135]. Afterwards, both newly generated cell lines went through a blasticidin selection step for a period of 15 days to increase the percentage of cells containing the pgRNA-CKB vector. Since the transfected plasmid allows constitutive expression of blasticidin resistance marker (bsd), after this step both polyclonal populations displayed higher than 90% of mKate2^{+} cells (**Figure 4.3 C**)

To test the efficiency of the selected gRNA inserted in hiPSCs, we cultured CRISPRi-CCBE1 KD polyclonal population with (+Dox) and without (-Dox) Dox addition for 8 days. Cells were counted daily, and the results obtained showed no significative differences between the two conditions; Both +Dox and -Dox cultures demonstrated a similar cell specific growth rate (0.038 h^{-1} vs 0.042 h^{-1} respectively) (**Figure 4.4 A**) and corresponding doubling time of (≈ 18h vs ≈17h), which is close to the 18-20h described in the literature for iPSC [136]. Moreover, hiPSCs presented high proliferative capacity as demonstrated by the incorporation of Edu in approximately 99% of cells from both cultures (**Figure 4.4 B**). Cell pellets were also collected for gene expression analysis. Results showed approximately 80% downregulation of CCBE1 expression after 6 days of Dox induction without changes in pluripotency markers such as Nanog (**Figure 4.4 C-D**). The knockdown level shows an efficient CCBE1 KD (80%) with the selected gRNA, consistent with the levels reported by Mandegar and colleagues for different genes that varied between 60%-99% of knockdown [123].

In summary, we showed an efficient CCBE1 KD with no impact on hiPSC growth and stemness (**Figure 4.4**). In addition, with this experiment we can observe that to obtain a significant CCBE1 KD with the selected gRNA, these hiPSCs need to be cultured with Dox for at least 6 days (**Figure 4.4 D**).

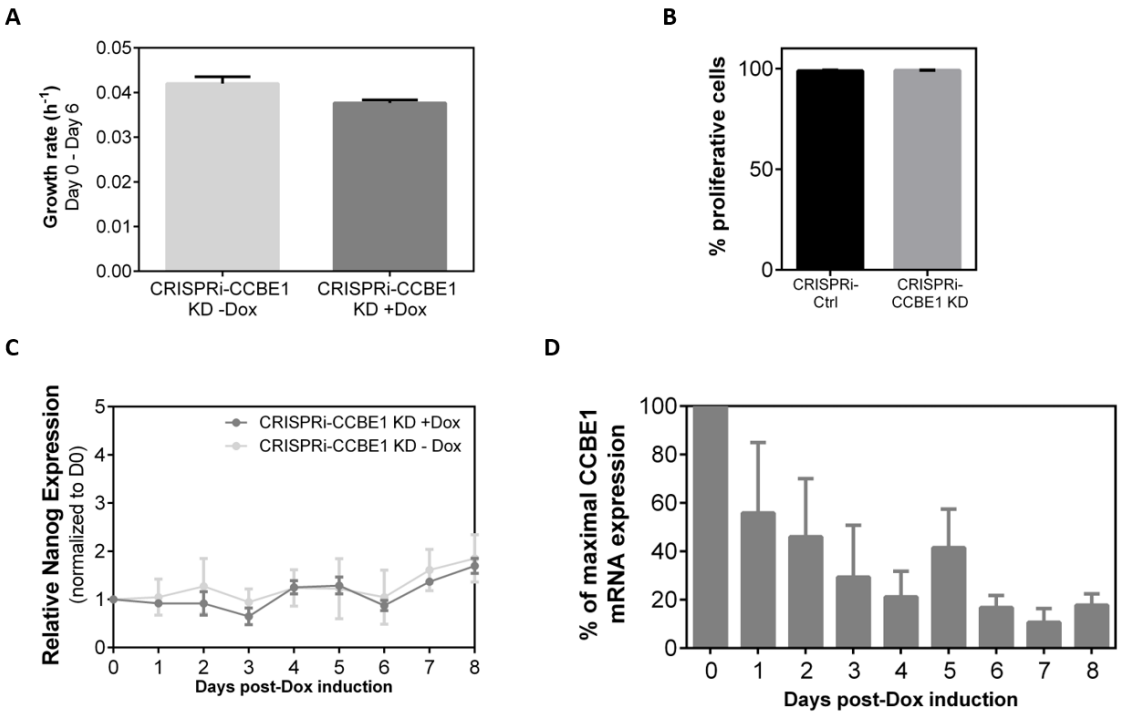

Figure 4.4 – Efficient CCBE1 knockdown with the selected gRNA in hiPSCs. (A) Determined cell specific growth rate (h^{-1}) of CRISPRi-CCBE1 KD cell line with and without Dox addition. **(B)** Percentage of proliferative cells (CRISPRi-Ctrl and CRISPRi-CCBE1 KD) after 8 days under doxycycline induction. **(C)** Pluripotency marker (Nanog) gene expression was not affected along 8 days of doxycycline induction. **(D)** CCBE1 was knocked down by 80 % in polyclonal CRISPRi-CCBE1 KD after 8 days of Dox induction. Data are presented as mean ± SEM of three independent experiments.

4.3.CCBE1 knockdown impacts cardiomyocytes differentiation

The next step consisted on evaluating if CCBE1 is crucial for cardiac progenitors' generation, proliferation and the impact along CM differentiation. Both CRISPRi-Ctrl and CRISPRi-CCBE1 KD generated cell lines were differentiated under Dox induction. Before the beginning of differentiation, these hiPSC were expanded for 7 days with Dox to ensure that CCBE1 was efficiently knocked down (at least 80 %), as previously shown by our results (**Figure 4.4 D**). As demonstrated above in the gene expression profile of CCBE1 along the CM differentiation (**Figure 4.2 F**), the peak of its expression was at day 4 (Cardiac mesoderm), suggesting that it is at this stage that CCBE1 would play its major role in cardiac specification. Therefore, we differentiated these hiPSCs into CMs for a period of 15 days, where the Dox was present until day 3 of differentiation (cardiac mesoderm stage), aiming to induce CCBE1 KD only during this phase, after that, the differentiation protocol continued without Dox induction (**Figure 4.5 A**). In parallel, daily Dox addition was also performed along the whole differentiation process with similar results obtained (data not shown).

The gene expression analysis, of three independent experiments, revealed a lower expression of CCBE1 until day 3 in CRISPRi-CCBE1 KD, demonstrating an efficient CCBE1 KD

along differentiation. However, the expression of mesoderm and cardiac mesoderm genes (*T* and *MESP1*, respectively) was unaffected compared to the control condition, which did not have a gRNA and so, no CCBE1 knockdown (CRISPRi-Ctrl). Moreover, no significant differences were detected in cardiac progenitors' gene expression levels (*NKX2-5* and *GATA4*).

Still, when we analyzed the CMs specific marker expression (*TNN2*), a significant reduction in the CRISPRi-CCBE1 KD culture can be observed (gene expression results) in comparison to the control condition (**Figure 4.5 B**), suggesting a potential impact of CCBE1 on cardiac differentiation. However, it did not affect the percentage of cardiac troponin T-positive cells at day 15, were we attained more than 95 % cTnT positive cells in both conditions (**Figure 4.5 D**). Mutations in *TNNT2* can alter cTnT function and lead to hypertrophic (HCM) and dilated (DCM) cardiomyopathies, where sarcomeres organization are severely affected [137, 138]. Similar spontaneous beating rates were also observed, 28 bpm vs 22 bpm for the CRISPRi-Ctrl and CRISPRI-CCBE1 KD cultures, respectively (**Figure 4.5 E**). The spontaneous beating rates were within the range of reported values for hPSC-CMs *in vitro* (21–84 bpm) [139]. Moreover, similar number of CMs (data not shown) and low percentage of proliferative cells (EdU^+ cells) were also detected at day 15 in both cell lines (**Figure 4.5 C**). Besides that, no impact on the proliferative capacity of CPC at day 6 or CMs at day 15 was also observed (**Figure 4.5 C**). These results suggest that CCBE1 role may be more relevant for CM specification rather than cardiac progenitors' generation and proliferation.

To further investigate the effect of CCBE1 KD on CMs differentiation, we also evaluated the gene expression of other cardiac specific markers, such as *TNN1*, *TNN3*, *MYL2* (MLC2v), *MYL7* (MLC2a), *MYH6* (αMHC) and *MYH7* (βMHC), in both CRISPRi-CCBE1 KD and control cell lines. The ratio of cardiac troponin I isoforms gene expression (*TNNI3*:*TNNI1*) and α- and β-cardiac myosin heavy chain genes (*MYH7*:*MYH6*) were lower in CCBE1 KD compared to the control condition at day 15 of differentiation (**Figure 4.6 A-B**). In addition, a lower expression ratio of light chain genes (*MYL2:MYL7*) was detected in CRISPRi-CCBE1 KD at day 6 although no significant impact of CCBE1 KD relative to this ratio was observed at day 15 (**Figure 4.6 C**). These results are in accordance with the literature and suggest a more immature phenotype in CRISPRi-CCBE1 KD cell line. It is described that gene expression profile of immature CMs express predominantly the *MYH6* and *TNNI1* cardiac markers. Whereas, in adult CMs these cardiac genes isoforms (*MYH7* and *TNNI3*) are expressed at higher levels and *MYL2* expression is also more predominant than its isoform (*MYL7*) [53].

At the last day of differentiation (day 15), both CRISPRi-CCBE1 KD and control cultures contained beating CMs, with no impact at the morphological level (**Figure 4.7 A**). Although, well defined and aligned fibers stained for cardiac troponin T (cTnT) could be seen in both conditions, in CRISPRi-CCBE1 KD more areas with poorly aligned fibers were found (**Figure 4.7 B**). These results were consistent with the lower *TNNT2* gene expression profiles in CCBE1 KD (**Figure 4.5 B**) and gene expression ratios of others cardiac markers (**Figure 4.6 A**), these data suggest a more immature cardiac phenotype in CRISPRi-CCBE1 KD cultures.

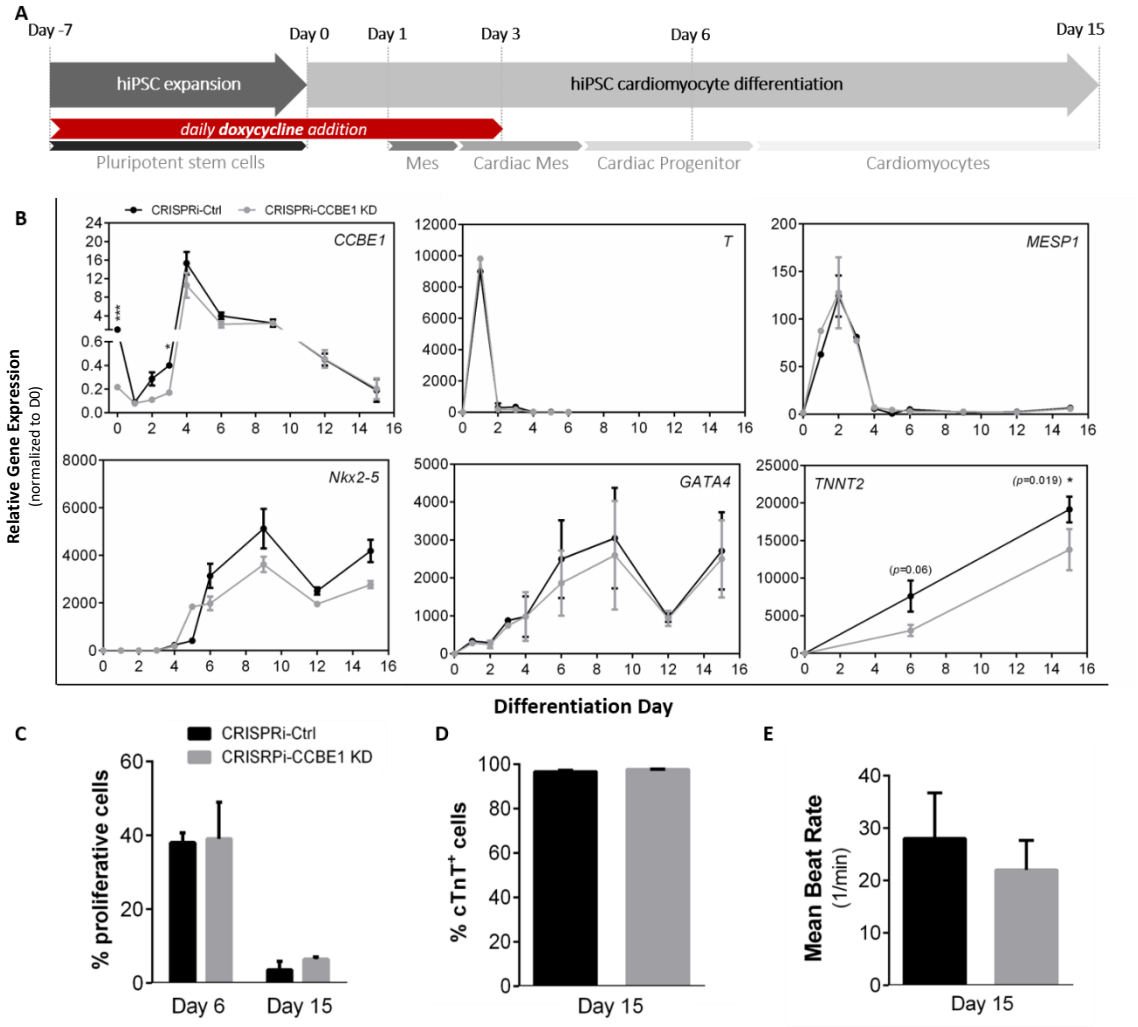

Figure 4.5 – CCBE1 knockdown during cardiomyocyte differentiation. (A) Schematic representation of doxycycline supplementation mode (2 µM from Day -7 until Day 3 of differentiation) along cardiomyocyte (CM) differentiation using both control (CRISPRi-Ctrl) and CRISPRi-CCBE1 KD lines. **(B)** RT-qPCR analyses of the following genes in control (CRISPRi-Ctrl) and CRISPRi-CCBE1 KD cultures along CM differentiation: *CCBE1*, *T*, *MESP1*, *NKX2-5*, *GATA4* and *TNNT2*. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method relatively to CRISPRi-Ctrl culture at day 0 (housekeeping: *RPLP0* and *GAPDH*). **(C)** Percentage of proliferative cells in CRISPRi-Ctrl and CRISPRi-CCBE1 KD cultures at day 6 and 15. **(D)** Percentages of cTnT positive cells at day 15 of differentiated cultures. **(E)** Mean Beat Rate (beats/minute) in both cultures at day 15. Data presented as mean ± SEM of three independent experiments. * p< 0.05, Multiple t-test (FDR 0.05).

In addition, using TEM analysis we observed that the CRISPRi-CCBE1 KD cultures displayed a poorly organized contractile machinery, characterized by low myofibril density and orientation, variable Z-disc alignment and few mitochondria close to the myofibrils (**Figure 4.7 D-E**). As well, a higher density of mitochondria with more defined and prominent cristae were observed close to the sarcomeres in the CRISPRi-Ctrl (**Figure 4.7 E**). Furthermore, in the control condition cells presented a higher density of aligned myofibrils composed by sarcomeres with organized Z-disks, A- and I-bands, with lower angle dispersion (4.29 ± 3.49 in CRISPRi-CCBE1

KD *versus* 2.68 ± 2.40 in CRISPRi-Ctrl) and higher sarcomere length (1.58 µm in CRISPRi-CCBE1 KD vs 1.72 µm in CRISPRi-Ctrl, **Figure 4.7 C**).

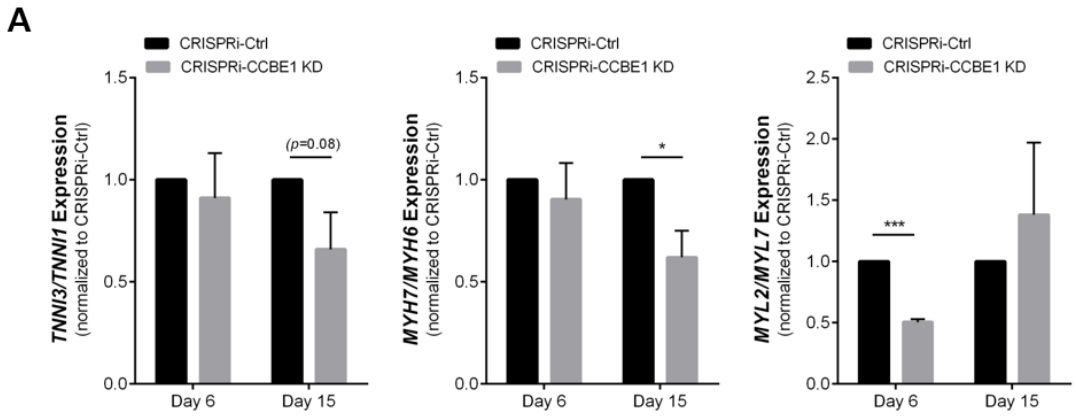

Figure 4.6 – CCBE1 knockdown affects other cardiac specific markers gene expression. (A) Ratio of relative expression of *TNNI3*:*TNNI1*, *MYH7*:*MYH6* and *MYL2*:*MYL7* genes in both cultures at day 6 and 15. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method relatively to CRISPRi-Ctrl culture at day 6 (housekeeping: *RPLP0* and *GAPDH*). Data are presented as mean ± SEM of three independent experiments. * p< 0.05, *** p< 0.001, **** p< 0.0001, Multiple t-test (FDR 0.05).

It has already been described that in adult cardiomyocytes sarcomeres are longer (2.2 µm), well organized (with defined Z-disks, I-, A-, M-bands), and show longitudinal alignment, while the more immature CMs have shorter (1.6 µm) and poorly aligned sarcomeres, mainly with Z-disks and I-bands [53]. Also, the number and volume occupied by mitochondria in cells increase along differentiation. Thus, we can assume that CCBE1 can modulate the CMs at maturation level by regulating key mediators of cardiac development, since we can observe a more immature phenotype both at structural (**Figure 4.7 A-E**), and gene expression levels (**Figure 4.6 A**), when CCBE1 gene expression is knock downed.

These results regarding CCBE1 role during CMs maturation might impact stem cell and cardiovascular research. It is known that one of the major problems delaying clinical progress in these areas is the lack of robustness and time-consuming *in vitro* methods that are used nowadays to obtain mature CMs, i.e cells able to mimic with reliability the behavior of CMs in adult hearts. The fact that hPSC-CMs experience structural and functional maturation process when transplanted into the functional myocardium of host species, shows that this cells can effectively mature, as long as they grow in the right environment [140]. This information led many scientists to investigate the physical, genetic and environmental cues that could facilitate the maturation process. Current protocols to differentiate hiPSCs into CMs can produce beating CMs by day 6 of differentiation. However, the maturation status of hPSC-CMs is still not well defined. It is also known that longer culture times improve the maturation process, which may take up to 1 year to show molecular signatures similar to those seen in mature cardiac tissues *in vivo* [141]. Moreover, changes in sarcomeres ultrastructure, calcium handling and ion channel expression also occur

over long periods of time [142, 143], which makes this protocols impractical for biomedical applications due to this extensive culture periods.

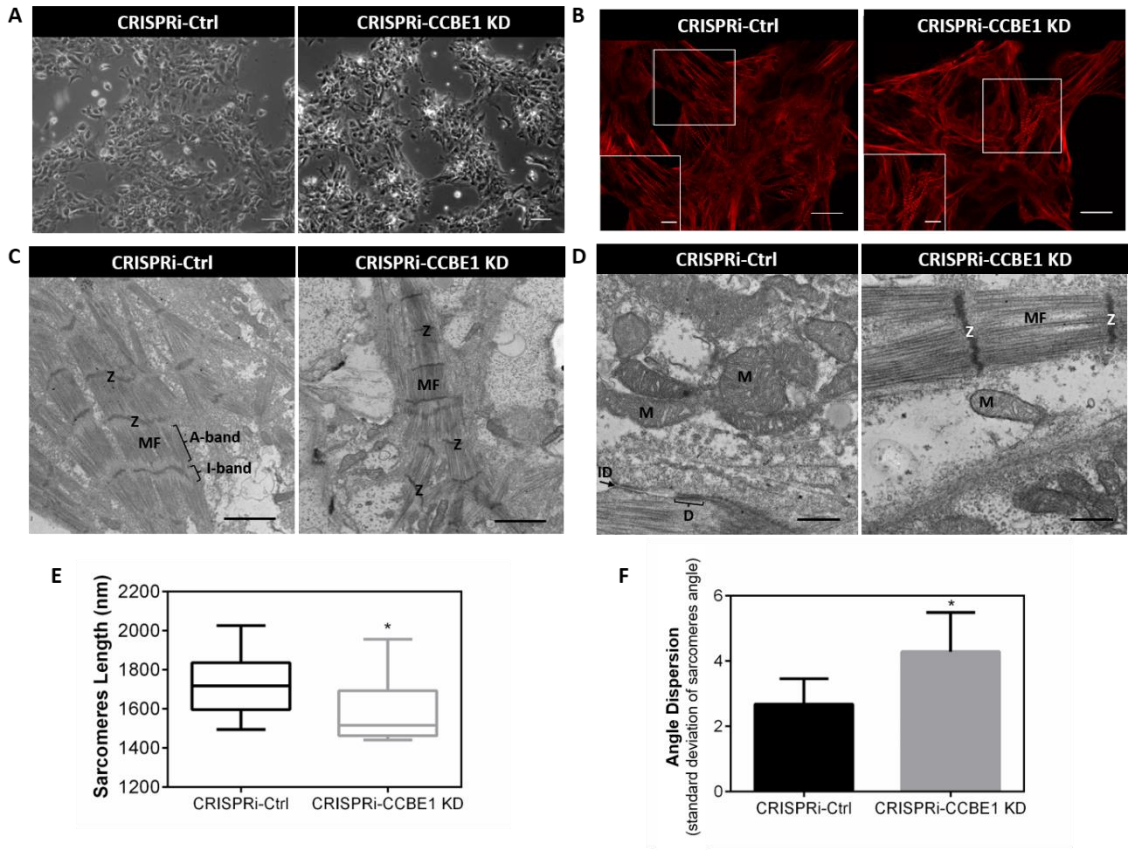

Figure 4.7 – Impact of CCBE1 knockdown on cardiomyocyte differentiation/maturation. (A) Morphology of CRISPRi cells from control and CCBE1 KD cultures at day 15. Scale bar, 100 µm. **(B)** Detection of cardiomyocyte marker cTnT at day 15. Scale bar, 50 µm and 10 µm in insight. **(C-D)** TEM images of CM from both cultures at day 15. Myofbrils (MF), Z-disks (Z), sarcomeric bands: A- and I-bands in panel D, intercalated disks (ID) and desmosomes (arrows) connecting adjacent CMs, and Mitochondria (M) in panel E are highlighted. Scale bars= 2µm (C), 500nm (D). **(E-F)** Sarcomeres length (nm) and sarcomeres alignment (determined by the standard deviation of the sarcomere angle in each taken image),in both conditions was assessed from TEM images using Fiji Image J software.

Therefore, modulation of factors that are important during the maturation process can be useful to obtain consistent and mature CMs in a shorter period. One example already studied is the modulation of let-7 microRNA family, which was identified as the most highly up-regulated microRNA family in hESC-CMs culture [141]. In this study, gain- and loss-of-function analyses of let-7 in hESC-CMs demonstrate that these microRNAs are important for maturation, but not for early differentiation of CMs. Moreover, the overexpression of let-7 family members in hECS-CMs enhanced cell size, sarcomere length, force contraction and respiratory capacity of this cells [141]. This boosted maturation, was further suggested to be the result of down-regulation of the phosphoinositide 3 kinase (PI3K)/AKT protein kinase/insulin pathway and an up-regulation of fatty acid metabolism. Suggesting let-7 microRNA family as an important mediator in the maturation process of cardiomyocytes [141]. Given this, we may hypothesize that CCBE1 could play a similar

role during maturation process, where through the correct modulation of this protein, higher maturation levels of CMs *in vitro* could be obtained.

4.4.CCBE1 knockdown has no impact on endothelial expression markers

Next, we wanted to investigate the role of CCBE1 on other cardiac lineages. As CCBE1 is known as an important factor for efficient formation of coronary vasculature [144], we evaluated its impact on cardiac endothelial cell differentiation (**Figure 4.8**).

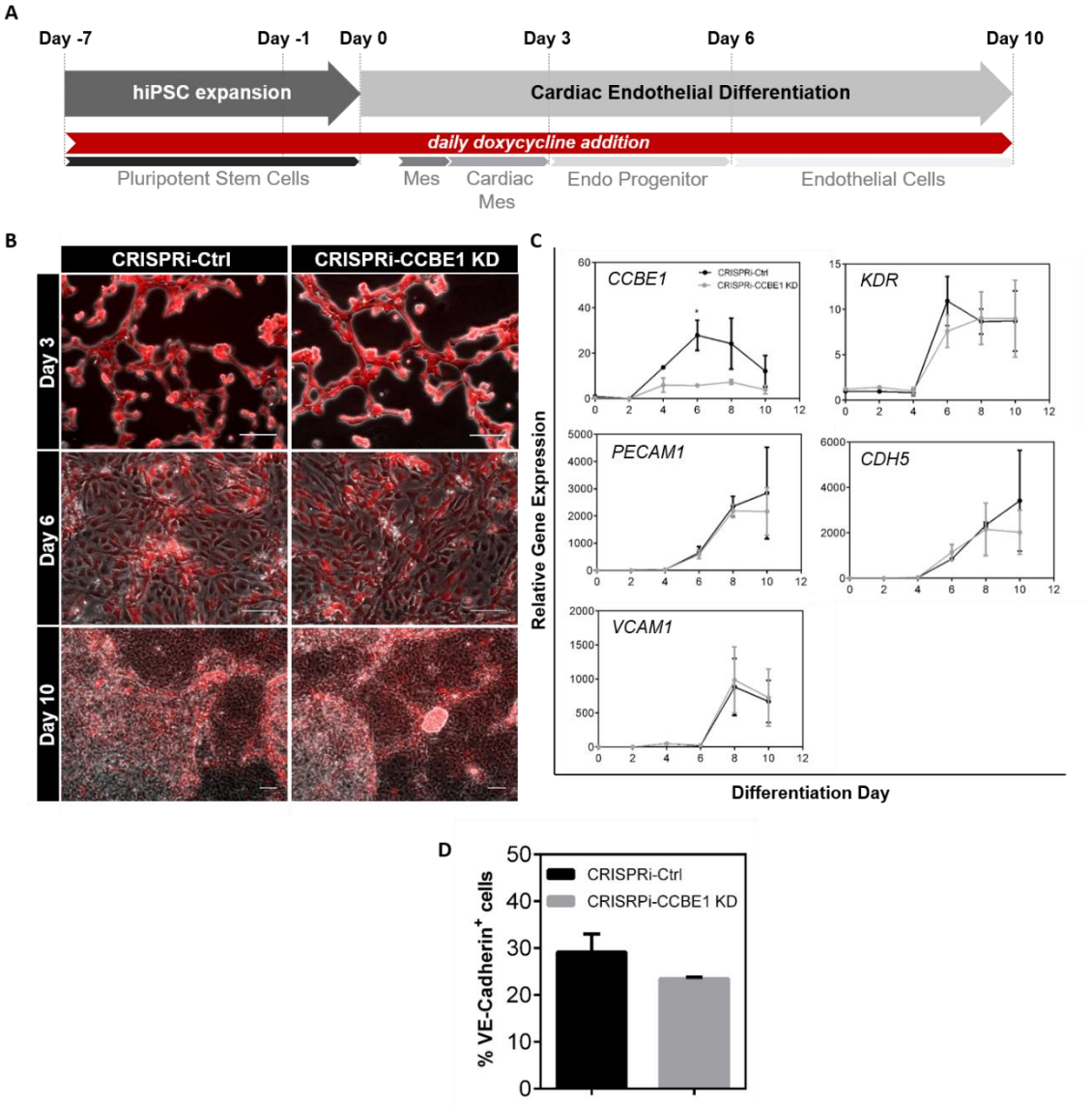

Figure 4.8 – Effect of CCBE1 knockdown on cardiac endothelial differentiation. (A) Schematic representation of cardiac endothelial differentiation with doxycycline induction (from day -7 until day 10). **(B)** Detection of mCherry^{+} cells (cytoplasmic staining) and mKate2^{+} cells (nuclear staining) in CRISPRi at day 3, 6 and 10. Scale bar, 100 µm. **(C)** RT-PCR analyses of CCBE1, PECAM1, VCAM1, KDR and CDH5 (VE-cadherin) genes in control (CRISPRi-Ctrl) and CRISPRi-CCBE1 KD cultures along cardiac endothelial differentiation. Gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method relatively to CRISPRi-Ctrl culture at day 0 (housekeeping: RPLP0 and GAPDH). **(D)** Percentages of VE-cadherin positive cells at day 10 of differentiation in both cultures. Data are presented as mean ± SEM of two independent experiments. * p< 0.05, *** p< 0.001, **** p< 0.0001, Multiple t-test (FDR 0.05).

For this purpose, we differentiated both control and CRISRPi-CCBE1 KD polyclonal populations into endothelial cells following initial cardiac mesoderm induction, as reported in [130, 145]. In this experiment we added Dox, to induce CCBE1 KD, every day until the last day of differentiation (day 10) (**Figure 4.8 A**). The normal CCBE1 gene expression profile along endothelial specification is different in comparison to CM differentiation (**Figure 4.5 B**). An upregulation is observed from day 4, with a peak of expression at day 6 and continues to be expressed until the last day of differentiation at day 10 **(Figure 4.8 C**).

Both cultures were able to differentiate into cardiac endothelial cells, displaying a monolayer of cells with endothelial-like cell morphology (**Figure 4.8 B**) and despite the effective CCBE1 KD along the endothelial differentiation process, no changes on endothelial-related genes expression such as, *PECAM1* and *VE-cadherin* (CDH5), were detected (**Figure 4.8 C**). In addition, no significative differences were observed on the percentage of VE-cadherin positive cells at the last day of differentiation in both populations (29.2 ± 2.8 % and 23.5 ± 0.3 % in CRISPRi-Ctrl and CRISPRi- CCBE1 KD differentiated cells, respectively). These results suggested no effect of CCBE1 KD on cardiac endothelial differentiation.

In the future, alternative and more efficient protocols for EC differentiation could be explored to detect any changes on endothelial phenotype on CCBE1 KD populations, since a low percentage of endothelial cells were obtained (≈ 30% of VE-cadherin positive cells) with the reported protocol herein used (**Figure 4.8 D**).

4.5.CCBE1 knockdown is more prominent in the selected clones compared to the polyclonal populations

To further increase the level of CCBE1 KD obtained (80%, **Figure 4.4 D**), we selected clonal populations for each condition (CRISPRi-Ctrl and CRISPRi-CCBE1 KD) according to their pluripotent phenotype, by choosing the clones with higher percentage of TRA-1-60 positive cells and lower SSEA-1. All clones were obtained by limited dilutions method (described in Material and Methods section). Since, in the polyclonal population used until now there is a higher heterogeneity, we also assessed CCBE1 KD with the selected clones, performing the same kind of experiments already carried out for the polyclonal populations (**Figure 4.9 A**).

In CCBE1 KD condition clone #25 was discarded due to lower TRA-1-60 positive cells, whereas clones #14 & #86 were selected for further analysis, since they expressed high levels of this pluripotency marker. In the control condition the same type of selection was performed, where clones #9 & #22 were chosen. After dox induction a higher percentage of CCBE1 KD was observed in both selected clones, #14 and #86, (>99%) when compared to the polyclonal population (80%), without significative changes in the expression of Nanog (**Figure 4.9 B**).

In the future, we could also evaluate the impact of higher CCBE1 KD level in clones along CM/EC differentiation and compare with the polyclonal population results.

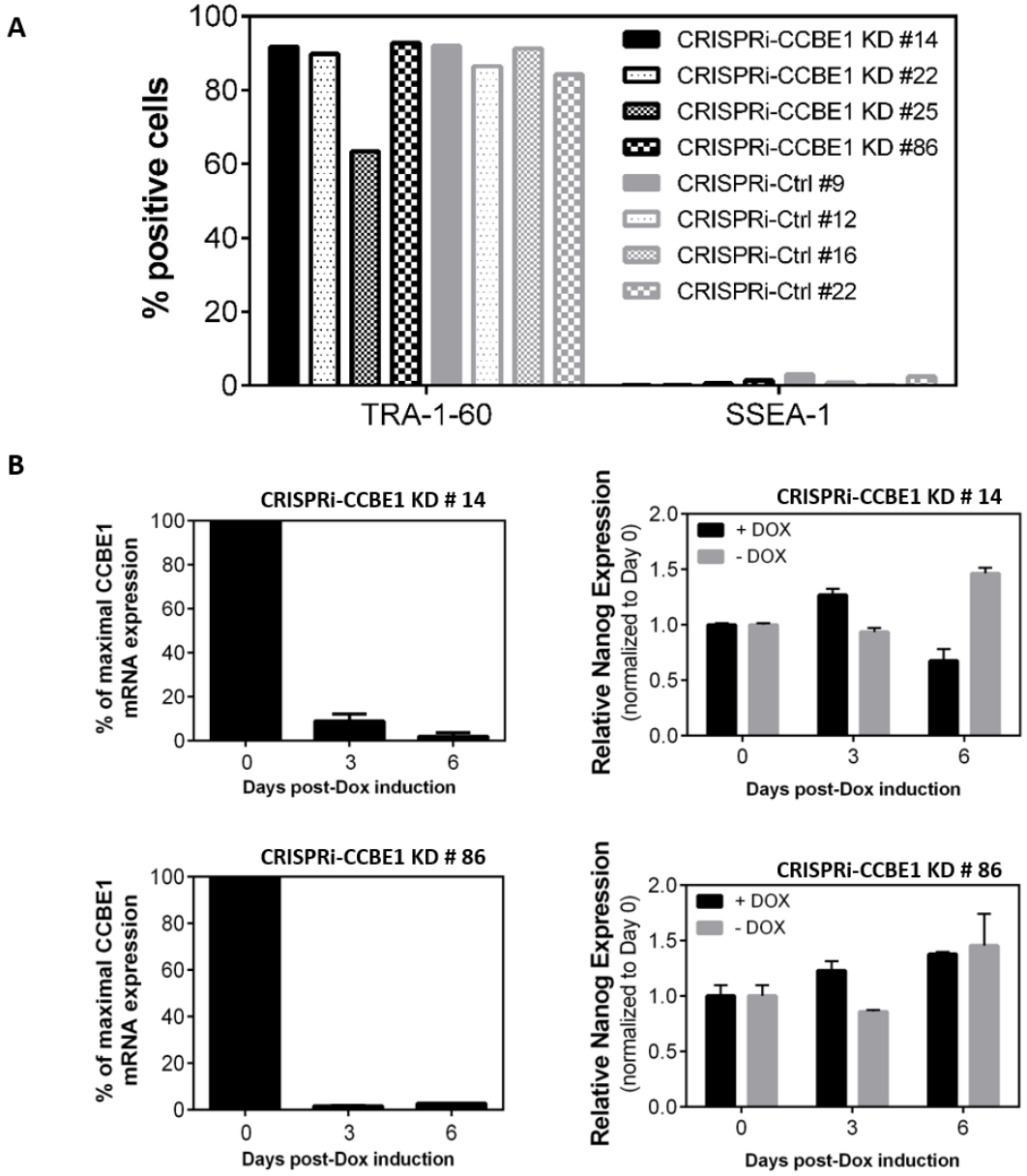

Figure 4.9 – Efficient CCBE1 knockdown with the selected CRISPRi-CCBE1 KD clones. (A) Percentages of TRA-1-60 and SSEA-1 positive cells in different clonal populations of both cultures by Flow cytometry analysis. **(B)** CCBE1 was knocked down by 100 % in the selected clonal populations without affecting and Nanog gene expression along 6 days of Dox induction.

5. Conclusions and Future Perspectives

In this work, a CCBE1 knockdown with the selected gRNA was successfully implemented on hiPSC cultures without affecting their growth rate, viability and stemness. A downregulation of CCBE1 gene expression resulted in sarcomeres with smaller length and poor alignment, that in combination with changes in gene expression of cardiac specific markers revealed a more immature phenotype in these differentiated CMs. Moreover, no differences in endothelial cells phenotype were found when CCBE1 gene was downregulated during the differentiation process. Additionally, we successfully selected hiPSC clonal populations that showed a significant higher level of CCBE1 knockdown when compared with the polyclonal populations. These clones could be used in the future to comprehend if different CCBE1 gene expression levels could modulate cardiogenesis in a different manner.

Here, we showed for the first time that CCBE1 may exert a modulatory effect on hiPSC-derived CMs phenotype. These new insights can contribute for the implementation of new knowledge-driven approaches for more efficient CMs differentiation and maturation protocols. Moreover, additional investigation on how CCBE1 modulates cardiogenesis may support the progress towards novel cardiac regenerative therapies.

These data can be further validated in gain-of-function studies and/or in a hiPSC line with a different genetic background. Also, transcriptomics studies can be performed to unveil the signaling pathways and identify key regulators involved on CCBE1 modulation of cardiac phenotype, by revealing the cellular transcriptome changes caused by CCBE1 KD along the CM/EC differentiation process. Nevertheless, the assessment of oxidative metabolism and electrophysiological characteristics in the differentiated cultures under study would also be valuable readouts to further delineate the CM maturity in our system.

6.References

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