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InCited2 secrete cardiogenic molecules

UNIVERSITY OF ALGARVE
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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(João André Pereira Lopes)

“We make versions, and true versions
make worlds’’

by Nelson Goodman

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First and foremost, I would like to start by thanking my family and friends that have been around for both best and worst times throughout all these years.

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Thank you all.
Sincerely,

João Lopes

ABSTRACT

The CBP/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2 (Cited2) interacts with many CBP/p300-dependent transcriptional factors. *Cited2* plays an important role in cardiac development in humans and mouse. Furthermore, *in vitro* studies using embryonic stem cells (ESC) have demonstrated its importance for their specification into cardiac cell lineages. Moreover, the WNT5a and WNT11 were identified and overexpressed in the conditioned medium of ESC overexpressing CITED2 (CM-CITED2) and showed to hold the potential to rescue cardiogenic defects caused by Cited2 depletion *in vitro* and in zebrafish embryos. We believe that more secreted proteins which may be influenced by CITED2 expression may also have the potential to rescue the cardiogenic defects caused by Cited2 depletion. Amongst these potential candidate proteins FGF10 was identified in a preliminary study but was not validated for its capacity to reverse cardiac defects in zebrafish triggered by Cited2 depletion.

Here, we have assessed the capacity of FGF10 to rescue defects caused by Cited2 depletion in zebrafish. Although, we were not able to definitively conclude about FGF10's ability to rescue defects caused by Cited2 depletion due to a possible degradation of the morpholinos (MO) used to inhibit Cited2 expression, we obtained some promising preliminary results.

In order to identify other candidate proteins that may be present in the secretome of CITED2 overexpressing cells, we have used Mass Spectrometry analysis to further determine the content of CM-CITED2 and control medium (CM-control). Interestingly, we identified a differential composition of proteins in the conditioned media, and amongst these proteins, some have been described to be associated with cardiomyocyte's development. The analysis of transcripts expression of genes encoding relevant proteins suggested that these proteins may be expressed at critical cardiogenic steps.

Overall, this work demonstrates that overexpression of CITED2 may promote the early onset expression of genes normally expressed in cardiac differentiated cells. And though we were not able to conclude about FGF10 ability to rescue Cited2 deficiency, we identified other secreted proteins that may as well hold a potential for the development of therapies.

Keywords: CITED2, FGF10, Secretome, Cardiovascular Defects, Embryonic Stem Cells, Zebrafish.

RESUMO

O coração apesar de ser o primeiro órgão a ser desenvolvido, é de extrema complexidade, incluindo cardiomiócitos, progenitores cardíacos, células endoteliais, fibroblastos, células vasculares do músculo liso, células neuronais, células do sistema imune, entre outras. Deste modo, a comunicação intracardíaca é essencial para manter a adequada função cardíaca, integridade e homeostasia. De facto, as células libertam para o meio extracelulares diversos fatores solúveis e vesículas extracelulares (EV) de forma a permitir a comunicação inter- e intracelular.

As doenças congénitas cardíacas (CHD) são caracterizadas por malformações no coração, provocando defeitos nas paredes, válvulas e/ou vasos sanguíneos. Aproximadamente 1% das crianças nascem com CHD sendo que uma grande percentagem irá ser submetida a cirurgia antes de completar 1 ano de vida. Para alem disso, os desenvolvimentos de tratamento e suporte médico ao longo dos anos, levaram ao aumento da sobrevivência e consequente prevalência das CHD. No entanto, apesar do aumento do conhecimento científico no que diz respeito ao desenvolvimento cardíaco, a sua complexidade dificulta a identificação de alvos e mecanismos moleculares inerentes às CHD.

O desenvolvimento cardíaco é controlado por uma complexa rede de vias metabólicas, desde a via NODAL/ACTIVINA, WNTS, NOTCH, FGFs, entre outras, que, por sua vez irão regular a cardiogénese a partir de fatores de transcrição como o MESP1, NKX2.5, ISL1 e TBX. Tendo em conta o impacto destas vias, já foram estudados o impacto de mutações pontuais em genes das vias mencionadas. De facto, essas mesmas mutações pontuais são responsáveis, de forma independente, de cerca de 10% das CHD, no entanto, na maioria dos casos ainda não se conhece a causa da doença. Neste sentido, de forma a melhorar o conhecimento de melhores métodos de diagnóstico e tratamento das CHD, é crucial descobrir fatores extra-genómicos críticos para o desenvolvimento cardíaco.

CITED2 é um fator de transcrição com alta afinidade para o domínio 1 rico em Cisteínas-Histidinas (domínio CH1) da p300/CBP. No entanto, não se liga diretamente ao DNA, sendo que atua como co-ativador ou co-inibidor de outros fatores de transcrição dependentes de p300/CBP. O domínio do terminal carboxil de proteínas da família CITED (CR2) é um domínio que tem alta afinidade com o domínio CH1 da p300/CBP. Desde 2005, que foram descobertas diversas mutações relevantes no gene *Cited2* em pacientes com CHD. Deste modo, os defeitos cardíacos mais associados ao CITED2 são: VSD, ASD, TOF e a TGA. No entanto, a maioria das variantes mutantes de CITED2 identificadas em pacientes com CHD

afetavam apenas marginalmente a sua atividade biológica mais conhecida como repressor do HIF-1α e co ativador de TFAP2C *ex vivo.*

Para além do papel crucial de CITED2 como regulador de fatores de transcrição dependentes de p300/CBP, foi mostrado no nosso laboratório que também induz a secreção de diversas proteínas que facultam a comunicação intra- e intercelular. De facto, foi mostrado que o meio condicionado obtido de células com sobre expressão de *Cited2* conseguem reverter os defeitos da depleção de *Cited2 in vitro* num modelo de mESC. Para além disso, com recurso à tecnologia de microarrays, foi possível identificar diversos genes cujas proteínas estavam descritas como proteínas que são secretadas e cuja expressão estava alterada em células com a sub expressão de *Cited2*. Desta forma foram identificados diversos genes que poderiam estar dependentes da expressão de *Cited2*, sendo WNT11 e WNT5a foram as proteínas que para além da sua expressão génica acompar a expressão de *Cited2*, foram suficientes para recuperar os defeitos cardíacos induzidos pela depleção de *Cited2* tanto *in vitro* (com mESC) como *in vivo* (com um modelo de Danio rerio). No entanto, tal como referido, mais candidatos foram identificados. De facto, a expressão de *Fgf10* também acompanhava a expressão de *Cited2,* tanto na análise de microarrays como posteriormente por qPCR em mESC sobre expressando ou depletando *Cited2*. Para além disso, quando FGF10 foi imunodepletado do meio condicionado obtido a partir de mESC sobre expressando *Cited2*, o mesmo meio condicionado deixou de conseguir reverter os defeitos cardíacos induzidos pela depleção de *Cited2*, exercendo um fenótipo pior do que o controlo. Desta forma, para efeitos da presente tese, foi estudado se FGF10 consegue reverter os defeitos cardíacos induzidos pela depleção de *Cited2*, num modelo *in vivo*. Para este efeito, foi usado um modelo *in vivo* préviamente validado no nosso laboratório com recurso ao peixe-zebra e a uma solução de morpholinos (MO) para depletar *Cited2*. No entanto, não conseguimos obter a mesma uma percentagem tão alta de defeitos cardíacos induzidos e morte com os Mos como previamente. Como tal, foi indagada a possibilidade de os MOs estarem degradados sendo que quando se repetiu o ensaio *in vivo*, mas com uma concentração 3x superior de MOs, foi observado um aumento dos defeitos e morte. No entanto, aquando da combinação de CITED2 com os MOs, apenas houve uma redução dos defeitos e morde induzidas. Desta forma, poder-se-á deduzir que haverá uma elevada probabilidade de os oligos dos MOs estarem degradados e de os defeitos e morte induzidos serem também de off-targets devido à presença de fragmentos dos oligos dos MOs. No entanto, foi possível observar que FGF10 teve um efeito parcial de reverter alguns defeitos e morte sendo que poderá ter um potencial para reverter os defeitos cardíacos induzidos pela depleção de *Cited2*.

De forma a identificar outros candidatos que estão presentes no secretoma, recorremos ao LC-MS do meio condicionado de mESC sobre expressando *Cited2* e do respetivo controlo. Desta forma foi possível identificar proteínas envolvidas no desenvolvimento cardíaco, mais precisamente em cardiomiócitos. No entanto, essas proteínas correlacionadas com os cardiomiócitos estão descritas como responsáveis pela remodelação da matriz extracelular (ECM). No entanto, a meta-análise dos respetivos candidatos diferencialmente representados no secretoma analisado, sugeriu a presença de proteínas expressas em momentos críticos para a cardiogénese.

Em suma, o presente trabalho demonstrou que a sobre expressão de Cited2 pode promover a expressão precoce de genes expressos em células cardíacas diferenciadas. E, apesar de não termos dados suficientes para concluir que FGF10 consegue reverter os efeitos da depleção de *Cited2*, fomos capazes de identificar outras proteínas secretadas com potencial para desenvolver novas abordagens terapêuticas.

Palavras-chave: CITED2, FGF10, Secretoma, Defeitos Cardíacos, Células estaminais embrionárias, Zebrafish.

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ABBREVIATIONS

Δneo	Neomycin Resistant Gene
4HT	4-Hydroxytamoxifen
Ao	Aorta
APC	Adenomatosis polyposis coli
AS	Aortic Stenosis
ASD	Atrial Septal Defects
AV	Atrioventricular
BMP	Bone Morphogenic Protein
bpm	Beating per Minute
BSA	Bovine Serum Albumin
Cer1	Cerberus
cDNA	Complementary DNA
CH1	Cysteine-Histidine rich domain I
CHD	Congenital Heart Disease
CITED2	CBP/p300 Interacting Transactivator with ED tail rich family member 2
CM	Conditioned Medium
CNCC	Cardiac Neural Crest Cell
CoA	Coarctation of Aorta
CPC	Cardiac Progenitor Cells
CR	Conserved Regions
CRE-ERT	Cre fused to a domain of the Estrogen Receptor
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DVL	Dishevelled
EB	Embryoid Body
eGFP	Enhanced Green Fluorescent Protein
ESC	Embryonic Stem Cells
EtOH	Ethanol
FBS	Fetal Bovine Serum
FC2	Flag-tagged *CITED2*
FGF	Fibroblast Growth Factor
FHF	First Heart Field
FLK	Fetal Liver Kinase
FZD	Frizzled
GATA4	GATA binding protein 4
GMEM	Glasgow Minimum Essential Medium
GSK	Glycogen Synthase Kinase
hESC	human Embryonic Stem Cells
Hh	Hedgehog
HIF	Hypoxia-Inducible Factor

ICM	Inner Cell Mass
IT	Inflow Tract
iPSC	induced Pluripotent Stem Cell
ISL1	Islet 1
JNK	c-Jun N-terminal Kinases
LA	Left Auricula
lacZ	β-Galactosidase
LC-MS	Liquid chromatography–mass spectrometry
LIF	Leukemia Inhibitory Factor
LV	Left Ventricle
MAPK	Mitogen-Activated Protein Kinase
MEM	Minimum Essential Medium
NEAA	Non-Essential Amino Acids
MHC	Myosin Heavy Chain II
mESC	mouse Embryonic Stem Cells
MO	Morpholino
n/s	non-significant
NKX2.5	Nk2-related homeobox 5
OT	Outflow Tract
PBS	Phosphate Buffered Saline
PBS-T	PBS with 0.1% Tween 20
PCP	Planar Cell Polarity
P/S	Penicillin-Streptomycin
PMSF	Phenylmethylsulfonyl Fluoride
PS	Primitive Streak
PSC	Pluripotent Stem Cell
PT	Pulmonary Trunk
qPCR	Quantitative Polymerase Chain Reaction
RA	Right Auricula
RNA	Ribonucleic Acid
RT	Room Temperature
RV	Right Ventricle
SHF	Second Heart Field
SRJ	Serine-Rich Junction
T	Brachyury
TAD	Transactivation Domain
TBX	T-box
TGA	Transposition of the Great Arteries
TGF	Transforming Growth Factor
TOF	Tetralogy of Fallot
TRE	Tetracycline Response Elements
VSD	Ventricular Septal Defect

CHAPTER 1

GENERAL INTRODUCTION

"Science means constantly walking a tightrope between blind faith and curiosity; between expertise and creativity; between bias and openness; between experience and epiphany; between ambition and passion; and between arrogance and conviction – in short, between an old today and a new tomorrow''

by Heinrich Rohrer

1.1 The early Heart development

The heart is the first functional organ to be developed during embryogenesis. This early development is of upmost importance for subsequent embryonic and prenatal development. The mammalian heart is a specialized organ that is composed by a large number of cell types that arise from distinct embryonic progenitor populations through the entire cardiogenic process. The correct cell fate decision of these progenitor populations also depends on specific spatial and temporal stimuli. This crucial pumping organ, responsible for the blood flow, is composed of muscle and non-muscle cells, amongst which atrial and ventricular cardiomyocytes, endocardial cells, valvular components, connective tissues, conduction system cells, smooth muscle cells as well as endothelial cells of the coronary arteries and veins. Understanding the mechanism by which all these complex cell systems build up the heart is key to unravel spatial and temporal settings for the cardiac development. Indeed, the knowledge behind the specifications of each cell lineages and progenitor origins, into terminally differentiated cell types within the four-chambered embryonic heart will undeniably open a wide range of therapeutic applications in the cardiovascular field.

The heart development occurs with a tight orchestration where the pre-cardiac mesoderm cells migrate from the primitive strike (PS) to the splanchnic mesoderm. After ingression through the PS, cardiogenic progenitor cells migrate to an anterior lateral position caudal to the headfolds and form the so-called cardiac crescent. By this time, the first (FHF) and second heart fields (SHF) can be distinguished (1). FHF progenitors already exhibit a rather more differentiated phenotype, while cells within the SHF remain in a proliferative, an undifferentiated progenitor state until they enter the heart tube at a later time point (1). Thereafter the SHF cells will be recruited in order to contribute to the formation of the out track flow (OTF), right ventricle and atria (1–5), whereas the left ventricle will be composed by cells originating from the FHF (6).

1.1.1 Congenital Heart Disease

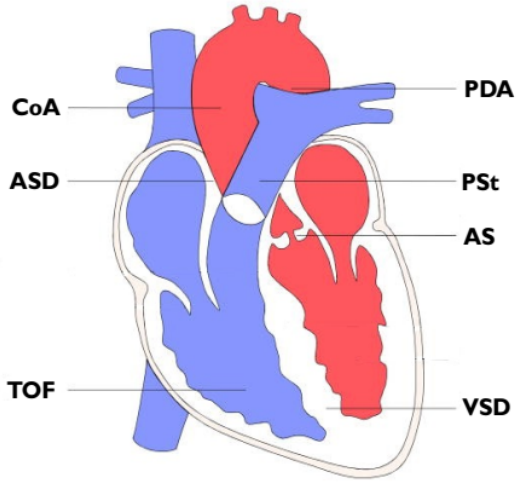
Congenital heart diseases (CHD) are structural anomalies of the heart and/or great vessels present at birth and that result from developmental defects during gestation. This disease affects 0.8-1 child per 100 live births, in this way being the most common type of congenital defect (7), representing one third of all major congenital anomalies (8).

Conotruncal defects, OFT defects, abnormal left-right (LR) patterning, defects affecting the inflow, and cardiomyopathies are amongst the most clinical relevant CHD (7). Dramatically, approximately one third of CHD patients have severe and sometimes lethal anomalies that require clinical or surgical intervention within the first year of life (7), resulting in a significant impact on morbidity, mortality, and health care costs in developed countries. Fortunately, improvements in surgical techniques and post-operative care have been made, with a consequent increase of the survival rate up to 95% of the patients with CHD (9). The surgical interventions do not correct the subjacent genetic defects causing CHD, thus the survival of patients results in an ever-increasing fertile population living with CHD that not only will be dependent of the health care system but also may spread this disease through the offspring (10).

Table 1 - Most common types of CHD worldwide (14).

Types	Description	Prevalence
Coarctation of Aorta (CoA)	Narrowing of the aorta, resulting in a reduced flow of blood throughout the body	5%
Atrial Septal Defects (ASD)	Anomaly in the wall between the left and right atriums.	13%
Tetralogy of Fallot (TOF)	Combined effects of PSt, VSD, RV hypertrophy and overriding aorta (aorta receives blood from both RV and LV)	5%
Patent Ductus Arteriosus (PDA)	Failure in closure of the ductus arteriosus. Ductus arteriosus is a blood vessel that connects the pulmonary artery to the aorta which necessary during fetal life but closes at birth.	10%
Pulmonary artery Stenosis (PSt)	Narrowing between the RV and the pulmonary artery which results in a reduced flow of blood to the pulmonary artery.	8%
Aortic Stenosis (AS)	Narrowing of the aortic valve, resulting in a reduced flow of blood throughout the body	4%
Ventricular Septal Defects (VSD)	The anomaly in the wall between the left and right ventricle.	34%

The complexity and broad range of the types of CHD (Table 1) have commonly been attributed to a multifactorial etiology with the combination of multiple genes and environmental factors (8,9). Regarding the genetic causes, the chromosomal aneuploidy is still the largest cause of CHD. Indeed, about half the patients with Trisomy 21 and Turner Syndrome have some form of CHD (11,12). More recently it has been shown that these cardiac defects are not necessarily due to multiple genes alterations but mostly to the change of dosage specific genes (Figure - **1**) (13). For instances, although more than 30 genes are deleted and involved in the DiGeorge Syndrome. However, it has been shown that the loss of the *T-box 1* (*TbxI*) gene is the main cause of the cardiac defects (14,15).



Gene	Phenotype
Cited2	ASD and VSD
Fog2	TOF
Gata4	ASD, PSt, VSD and TOF
Gata6	ASD, TOF, PSt, PDA and VSD
Hand2	TOF, VSD
Nkx2.5	ASD, VSD, TOF and COA
Tbx1	TOF
Tbx5	ASD and VSD
Tbx20	ASD and VSD
Zic3	PSt, ASD and VSD

Gene	Phenotype
ACVR2B	PSt
CFC1	TOF, ASD and VSD
FOXH1	TOF
GDF1	TOF
JAG1	TOF
LEFTY2	COA
NODAL	TOF
NOTCH1	AS and COA
SMAD6	AS and COA
VEGF	COA

Figure - 1 Common gene mutations in CHD patients and their phenotype. Blue Box Transcription Factors. Orange Box Signaling Pathways. Abbreviations: CoA, Coarctation of Aorta; ASD, Atrial Septal Defects; TOF, Tetralogy of Fallot; PDA, Patent Ductus Arteriosus; PSt, Pulmonary Stenosis; AS, Aortic Stenosis; VSD, Ventricular Septal Defects. Adapted from (13).

1.2 Pluripotent stem cells

Embryonic stem cells (ESC) are a useful *in vitro* model system to study cardiovascular development as they have the innate ability of self-renewal and to differentiate into virtually all cell types of organism (16). Apart from embryonic stems which are pluripotent stem cells (PSP), there is a second category of stem cells, termed adult stem cells (ASC). PSC are pluripotent as they can derive every cell type that is necessary to give rise to a full functional organism. On the other hand, ASC have a limit potential for differentiation and may only originate a subgroup of cells constituting the organ/tissue where they are resident. ASC have a variety of differential potency, ranging from several different cell types (multipotent) to one unique cell type (unipotent) (16).

Murine ESC (mESC) were first isolated in 1981 from the ICM of mouse blastocyst, from which one may be able to give rise to a fully functional embryo (17). They can be maintained indefinitely in culture through self-renewing division, as well as differentiating into all somatic cells (16).

1.2.1 Signaling pathways important for cardiac differentiation

Specific signaling pathways and a tight regulation of their activity, which is necessary for the specification of mesoderm, are of upmost importance for cardiac development as the mesoderm cells originate the majority of cardiac cells. Critical pathways involved in cardiac development include TGFβ/Activin/Nodal pathway, bone morphogenic protein (BMP), fibroblast growth factor (FGF) family , as well as canonical and non-canonical Wnt pathways (18,19).

During development, mesodermal progenitors have an initial wave of *Flk1* positive cells. *Flk1* is an early marker of lateral mesoderm, and important for cardiogenesis one of the lateral mesoderm early markers where cardiogenesis occurs (**Figure - 2**) (20–22). FLK1 activation triggers the downregulation of *Brachyury*. *Brachyury* acts as a marker of the early PS and activates. Afterwards, upon T-box transcription factor Eomesodermin action, there will be the expression of the *mesoderm posterior 1* (*Mesp1*) gene. *Mesp1* acts as a key regulator of cardiovascular progenitors and represents the earliest marker of cardiovascular progenitors (Figure - **2**), tracing almost all the cells of the heart including derivatives of the primary and second heart fields (23,24). By turning on the core cardiogenic transcriptional network, Mesp1 has been shown to further restrict these primitive mesodermal precursors toward a cardiovascular and anterior mesodermal fate (23,25,26).

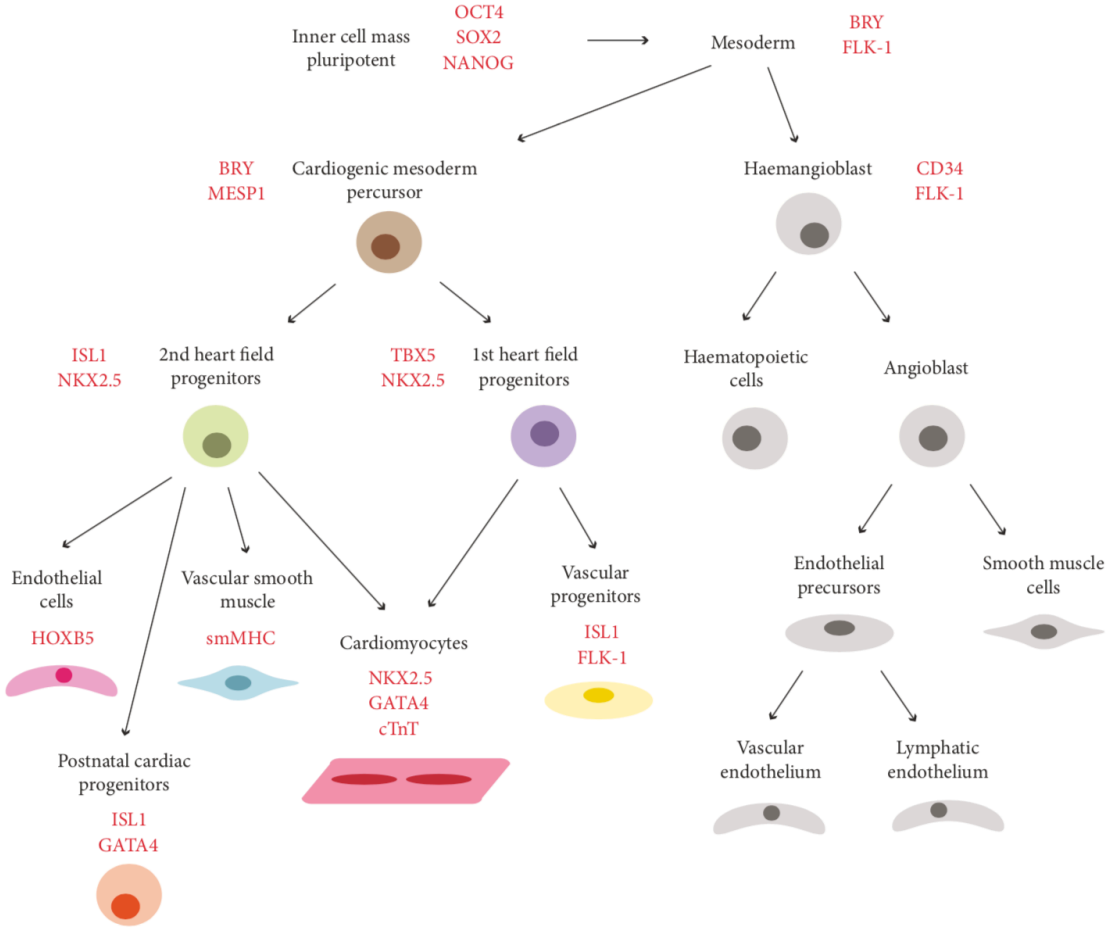


***Figure - 2 Cardiac differentiation schematic and some of the transcription factors characteristic for each cell stage throughout the embryonic development* (27)**

As FHF cells are more anterior and lateral with respect to SHF progenitors, they are more exposed to cytokines of the BMP (28) and FGF (29) families as well as to inhibitors of the Wnt pathway (19,30,31), resulting in the onset of cardiac differentiation marked by the expression of key regulators of the lineage, namely, Nkx2.5 (32), Gata-4 (33–36), and Tbx5 (37). Myocytic lineage commitment is associated with the expression of contractile proteins including myosin light chain-2a (MLC2a) and sarcomeric myosin heavy chain (MHC) first observed in the cardiac crescent and process of the formation of the heart (38).

There are no genes uniquely expressed either in early FHF or SHF progenitors. Nonetheless, SHF precursors, often express more the LIM-homeodomain transcription factor Isl-1, resulting in a typical SHF marker as its expression is almost absent in differentiated FHF derivatives (1,39). Isl-1 expression is dependent on canonical Wnt signaling (40,41), and its function is required for survival, proliferation, and migration of the SHF progenitors into the primitive heart tube. Isl-1 expression is extinguished as progenitor cells reach the developing

heart and differentiate (42). Several studies have shown that the molecular signature Isl-1^+/Nkx2.5^+/Flk-1^+ marks a specific pool of primitive embryonic SHF progenitors that are multipotent and give rise, by further lineage restriction, to downstream tissue-specific intermediates generating both myocytic and vascular cells (22,43–45). In particular, Isl-1^+/Nkx2.5^+ descendants (that have lost Flk-1 expression) are committed to forming cardiomyocytes and smooth muscle cells (SMCs), and extensively contributing to the proepicardium/epicardium (46,47), whereas the Isl-1^+/Flk-1^+ subset (in which Nkx2.5 expression has ceased) differentiates to form endothelial cells and SMCs (Figure - **2**) (43). The SHF progenitor's self-renewal and differentiation are controlled by many different signaling pathways. Among these pathways, FGF signaling promotes progenitor cell proliferation; Shh-mediated signals from the endoderm and canonical Wnt signaling from the midline (neural tube) are important for the maintenance of a proliferative state and inhibition of differentiation (1). On the contrary, BMPs secreted from lateral plate mesoderm as well as Notch and noncanonical Wnt signals promote cardiac differentiation of SHF progenitors (48).

1.2.1.1 TGFβ signaling pathway

TGFβ comprises an extensive number of proteins in which the well-known activins and bone morphogenic proteins (BMPs) are included. These proteins are not only important for their role in promoting cell growth, but also by stimulating epithelial-to-mesenchymal transition (EMT) (49). Remarkably, their role in inducing endothelial to mesenchymal transition (EndMT) has also been pointed out (50). For instances, EMT allows polarized epithelial cells to go through a wide range of changes that will make them acquire a mesenchymal cell phenotype. Whereas, EndMT will induce endothelial cells, that are an organized layer of cells in the vessel-lining, to undergo a phenotype change into mesenchymal cells state, acquiring enhanced migratory capacity, invasiveness and elevated resistance to apoptosis (50).

Briefly, TGFβ proteins are multifunctional cytokines that form heterotetrametric complexes with type I and II Ser/Thr kinase receptors and intracellular Smad transcription factors (51). TGFβ itself interacts with TGFβ type I receptor, also recognized as activin-like kinase 5 (ALK5), and int term, also interacts with TGFβ receptor II (TGFβ-RII). On the other hand, ACTIVIN interacts with activin type IB receptor (ActRIB or ALK4) and ActR-IIA/IIB. BMPs also have their specific receptors. Indeed, BMPs interact with ALK1/2 and BMP type

IA/IB receptor (also known as ALK3/6) thought it requires to form a complex with ActRIIA/IIB and BMP type II receptor (BMP-RII). Once either TGFβ or activivin type I receptor is activated, the receptor regulated (R-) Smad2 and -3 become phosphorylated, whereas activated BMP type I receptors induce R-Smad1, -5 and -8 phosphorylation. As if this was not complexed enough, the type II receptors are not only negatively regulated by INHIBIN A/B but also bind to NODAL (52).

Once the R-Smads are phosphorylated (for example the R-Smad2/3 or the R-Smad5/8), they form heteromeric complexes with Co-Smad (Smad4). These complexes can then translocate to the nucleus where they can interact with other transcription factors and consequently bind to Smad binding elements. Though, depending on which is the source of the activation of this pathway, different R-Smads with be phosphorylated and therefore different complexes will in turn bind to promotors of different genes. With this being said, TGFβ target genes include *Serpin family E member 1* (*SERPINE-1*), *inhibitory (I)-SMAD7, Collagen type I α 1 chain* (*COL1A1*), and *connective tissue growth factor* (*CTGF*). In its place, BMPs target genes include *ID1, ID3*, and *inhibitory (I)-SMAD6*. Though, as expected, this pathway is balanced in the way that BMP is antagonized by TGFβ (53).

Finally, but not least, TGFβ also cross-talks with other signaling pathways that are not Smad-dependentf. Out of these are included different members of the mitogen activated protein kinase (MAPK) (extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)) but also phosphoinositide 3 (PI3)-kinase/AKT and protein kinase C (PKC) signaling pathways, as well as a variety of Rho-GTPases (54).

1.2.1.1.1 TGFβ in the heart development

Of interest, the fact that either the BMPs or the Activin/Nodal, as well as the EndMT TGFβ dependent are involved in the heart development. For instances, *Bmp2* homozygous mutants is lethal and have abnormal heart development (55). Furthermore, *Bmp2* expression has been detected in mesoderm, cardiac progenitor cells (CPC) and myocardium (56). Likewise, BMP4 deficient mice lack of mesodermal structure required for the heart development (57). Instead, when overexpressed, BMP4 is able to induce PSC to differentiate into mesoderm by increasing the expression of Brachyury and CDX2, while inhibiting endoderm differentiation (58). Nonetheless, at later stages of differentiation, within cardiac mesoderm cells, BMP expression enables the specification into epicardial lineages instead of

cardiomyocytes (59). All in all, several BMPs are required during the embryogenesis due to their role in mesoderm specification and cardiac development *in vitro* but also *in vivo* (60,61).

The Activin/Nodal pathway is also noticeable for its importance in heart development. Actually, these proteins are crucial as early as the epiblast stage, during the implantation and they have been shown to maintain the cells pluripotency in both humans and mouse ESC (62,63). Indeed, their loss drives the loss of pluripotency markers and premature ectoderm specification (64). In addition, the Activin/Nodal signaling also regulates the mesoderm and endoderm commitment. In this regard, high levels of Activin/Nodal will promote cells specification into endoderm, whereas lower levels will induce the mesoderm specification (65,66).

TGFβ itself induces EndMT through its co-receptors (e.g., betaglycan and Endoglin). For example, the TGFβ receptor III (betaglycan) was shown to be upregulated in cardiac endothelial cells undergoing the EndMT whereas inhibition of the extracellular domain of betaglycan prevented EndMT. Furthermore, retroviral-mediated restoration of betaglycan expression restored the EndMT in response to $TGF\beta_2$ (67). Later on, Endoglin was shown to contribute to EndMT in the formation of endocardial cushions (68).

1.2.1.2 FGF10 signalling pathway

FGF protein family was first discovered in 1939 from embryo and brain extracts as a promoter for chicken periosteal fibroblast growth (69). Later on, this Fibroblast Growth Factor activity was found on a bovine pituitary extract as well (70). By 1983, one was able to purify these extracts, naming this protein as basic FGF (bFGF or FGF2) due to their basic composition of amino acids and high isoelectric point (71). Furthermore, when an extract from bovine brain that was purified free of myelin basic proteins, a second FGF was identified but this time with a low isoelectric point, beeing entitled as acidic FGF (aFGF or FGF1) (72). Up to now, many other FGF have been identified as growth factors for cell culture, as oncogenes, as genes responsible for hereditary diseases, by homology-based PCR/Databases (73,74).

Overall, the FGF family is responsible for many biological processes, such as cardiac development, homeostasis and disease. This large family regroups, to this point, as much as 22 members, which includes both secreted and intracellular FGFs. The best known are the secreted FGFs as they bind and activate cell surface tyrosine kinase receptors (FGF receptors or FGFRs)

(75). Whereas the intracellular FGFs work as cofactors for voltage gated sodium channels and other molecules (76).

The interaction between secreted FGFs and their respective receptors is regulated by extracellular binding proteins that include the heparan sulphates and the klotho family proteins. Though this interaction is tightly regulated, there is a redundancy on the activity of the receptors within members of the same subfamily. In this regard, FGF10 belongs to a subfamily that comprises FGF3, FGF7, FGF10, and FGF22. FGF3, 7, 10, and 22 have been shown to activate preferentially the IIIb splice variant of FGFR2. In addition, FGF3 and FGF10 also activate the IIIb splice variant of FGFR1 (75). Finally, the existence of heterodimer formation between FGFs and FGFRs may further increase receptor-ligand interaction possibilities (77) and thus the diversify the FGF signaling. Once activated, FGFRs mediate diverse intracellular signaling cascades including the RAS-MAPK, PI3K-AKT, PLCγ, and STAT signaling pathways (76).

1.2.1.2.1 FGF10 in the heart development

One of the most important characteristics that cells require for correct heart tube elongation, looping and arterial pole alignment is a proper communication within cardiac progenitors in order to promote a suitable homeostasis and consequently a fitting heart morphogenesis. For this purpose, FGFs are among the signaling pathways a critical one to promote this communication within early heart morphogenesis (1). As a growth factor, not only allows intercellular interchange of information as it allows a tight regulation between proliferation and specification. As proof of this concept, transgenic mouse models with conditional inactivation of *Fgfr1/2*, overexpression of a FGF antagonist (*Sprouty2*) within the SHF pool, revealed a disruption of the autocrine FGF signaling of the progenitor population, leading to an unregulated cell proliferation, reduction of the neural crest cells recruitment into the outflow tract cushions, outflow tract misalignment and overall compromised cardiac morphogenesis (78,79).

Multiple FGF ligands' expression has been detected in cardiac progenitors and tissues. Moreover, FGF10 has been identified as a endogenous marker for the SHF pool (80). Though, *Fgf8* is expressed in both adjacent pharyngeal ectoderm and endoderm (81,82), *Fgf15* has been detected in the pharyngeal endoderm (83) and *Fgf3* has shown to be expressed in both pharyngeal endoderm and ectoderm (84).

The canonical Wnt signaling pathway transcriptionally controls *Fgf10* expression within the SHF pool (85). Furthermore, there are other key transcriptional factors of the SHF

that also control the *Fgf10* expression. For instance, both ISL1 and NKX2.5 bind to regulatory elements in an intronic cardiac enhancer, thus, respectively, activating Fgf10 expression in progenitor cells and repressing it in differentiated myocytes (86). TBX1 also activates *Fgf10* through T-box binding sites in the same enhancer element (86).

Fgf10-null embryos exhibit altered heart morphology. Not only displaying absent pulmonary arteries and veins but also an abnormal positioning of the ventricular apex in the thoracic cavity (87,88). Nonetheless, SHF specification and heart tube elongation do not appear to be affected by the *Fgf10* deletion. Though, when the main FGF10 receptor (*Fgfr2b*) is deleted, major congenital heart defects are revealed, including ventricular septal defects, OFT alignment defects, and thin and poorly trabeculated ventricles (87). This may be a clue to consider the existence of a redundancy between FGF10 and others that may interact with FGFR2b during the heart development. As previously mentioned, FGF8 is also a regulator of the cardiac progenitors' cells, therefore is a plausible candidate for the redundancy stated. Moreover, a conditional loss of function demonstrated the *Fgf8* is not only required for the SHF expansion, thus, OFT elongation, septation and ventriculoarterial alignment (78,81), but also when combined with the *Fgf10* deletion, much severe phenotypes were observed (89). Also, these proteins are dose sensitive, as shown for FGF3 and FGF10 during heart tube elongation (84). All in all, SHF pool of cell proliferation, deployment and, consequently, normal cardiac morphogenesis if not only dependent of the FGF family of proteins, where FGF10 is included, but also dosage dependent.

Later, during the heart development (E10.5 onward), there will be both growth and remodeling of the myocardium without further addition of new cardiac progenitors. What happens is a regulated mechanism of proliferation and specification of cardiac myocytes that will drive the formation of the atrial and ventricular chambers. This process requires a tight spatial-temporal regulation of these cardiomyocytes proliferation as an impairment may lead to congenital heart diseases (90). Recently, FGF10 has been implicated in fetal cardiomyocyte proliferation regulation. In fact, it has been shown that, within the fetal stage, FGF10 through the interaction with its main receptor FGFR2b, is able to promote cardiomyocyte proliferation through FOXO3 phosphorylation and consequent downregulation of the cyclin dependent kinase inhibitor $p27^{kip1}$ expression. Moreover, FGF10 activity is specific for the formation of fetal right ventricular cardiomyocyte (88). Additionally, FGF10 is also able to interact with FGFR1 and FGFR2 in the epicardium, where it seems to be promoting epicardial-derived cells to migrate into the myocardial layer (91).

Another interesting point, is the fact that many FGF proteins and its downstream signals, including FGF1, 2 and 10, as well as p28 MAP kinase, are involved in the regulation of adult cardiomyocyte renewal (88,92–96). This feature is of particular interest as adult cardiomyocytes proliferation is quite limited and lean more to transitions from hyperplastic to hypertrophic (92). Although the heart in no longer considered as a post-mitotic organ, and cardiomyocytes are indeed generated throughout the life span, a healthy human and murine heart only have an estimated 0.5-2% of cardiomyocyte renewal per year (97).

1.2.2 Inducing Cardiac Differentiation in ESC

Two major approaches are undertaken to differentiate PSC into cardiomyocytes, which are Embryoid Bodies (EB) formation and directed differentiation.

The first hESC-derived cardiomyocytes were produced with the EB differentiation protocol, an *in vitro* 3D multilineage cell aggregate formation (98). EBs can recapitulate the embryogenesis (99) and the cardiomyocytes formation can be easily monitured by the spontaneous contraction of these cells (100). In EBs, the cell-cell communication and contact stimulates the expression of mesodermal and early cardiac markers, and, later on upon transfer of the EBs to a coated tissue plate, cardiac structures start to emerge (101). Notwithstanding, this protocol is rather inefficient in terms of forming spontaneous cardiomyocytes but, it stimulates the formation of all subtypes of cardiomyocytes, atrial, ventricular and pacemaker cardiomyocytes (102). However, the efficiency of the method can be enhanced to produce cardiomyocytes can be increased by adding factors that promote CPCs proliferation, such as WNT3A and Ascorbic Acid (44,103). Despite its inefficiency, EBs method is commonly used because of it is simple and low cost (104). Another plausible difficulty from the EBs differentiation comes with the sizes of the cell aggregates. Indeed, this method includes a hanging drop step of 20 µl drops with a defined number of cells in a petri dish. Interestingly, this method is widely applied for mESC, but for hPSC it induces too much variability. As such, instead of the hanging drop, hPSC can be forced to aggregate via spinning down in a round bottom V-shaped, low attachment 96-well plate (105).

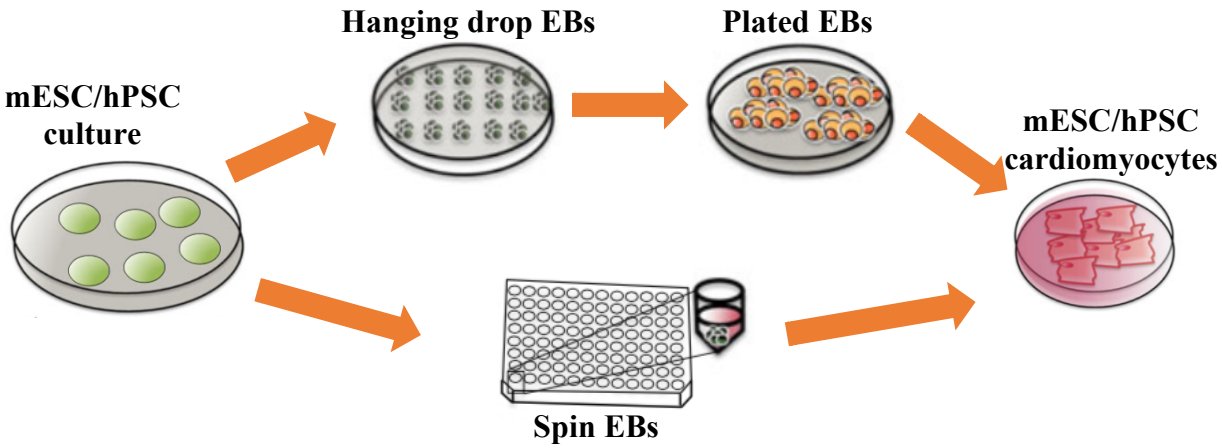


Figure 3 - EB differentiation of mESC and hPSC into CMs. On the top is presented the EB formation with the hanging drop method, where after the 20 µl drops are disposed on the petri dish which is after inverted and at the 5th day of the protocol the EBs are readily plated in a coated plate. On the bottom is represented the EB formation where the drops with the cells are disposed in a round bottom V-shaped, low attachment 96-well plate – spin EBs method.

To sum up, EB differentiation method requires the dissociation of ESCs into to single cell. Once detached, single cells are plated in suspended droplets in a lid of a Petri dish, which under gravity form aggregated of spheroid bodies that will undergo differentiation. Although this method mimics embryonic development in many aspects, EBs have as highly variable structure and composition, nevertheless, some derived cells of these aggregates invariably exhibit spontaneous contracting regions (100). The amount of serum and cell number used, influences EBs ability to differentiate into cardiomyocytes (106,107).

A directed differentiation is particularly useful to address specific issues regarding lineage commitment. Most of the methods involve the supplementation of recombinant growth factors or small molecules compounds to drive specific cell fate. In this regard, directed differentiation typically consists of activating the steps of differentiation by compounds that mimic the process of embryonic development to specific tissues. This method can increase the results robustness and yield of the desired cells. For cardiomyocytes, many different differentiation protocols have been optimized, and most of them have a cardiomyocyte yield higher than 90% (108–110).

1.2.3 Stem Cells in therapeutic approaches for cardiovascular disease

Stem cell research has showed that stem cells are a valuable tool for the understanding of the basic mechanism of human development as well as a promising solution for the treatment of several cardiovascular problems, including myocardial infarction. Nonetheless, all the ethics regarding the usage of a human embryo has hindered the development of hESC-based clinical therapies (111). Moreover, when talking about these kinds of cellular therapies, it should be taken into account safety issues as one of their characteristics being the ability for self-renewal and high proliferation rate. For this reason, the use of hESC in transplantation may often results in tumorigenicity, immunogenicity and genomic instability (112,113).

One may say that ASC would be an alternative solution to hESC. ASC appear to be safer, devoid of many of the ethical concerns and may be of autologous usage. This means that these cells can be removed from a person, stored and later on administered to the same person. Though, these cells are rather rare in mature tissues and most of them have low efficiency in generating cardiomyocytes (114,115). Nonetheless, different ASC were reported to be able to differentiate into cardiomyocytes such as the bone marrow, skeletal muscle, adipose tissue, peripheral blood and the heart stem cells (114–116).

CPCs may be a considered as an alternative to ASC which lack of efficiency as they have their differentiation potential restricted to cardiovascular cells (117,118). Nevertheless, it has been challenging to gather efficient methodologies to acquire and maintain CPCs in culture due to lack of information regarding their molecular and cellular identification. However, it has been recently reported that inducible expandable CPCs can robustly self-renew for several passages while maintaining their original morphology, gene expression pattern and potential to differentiate into cardiovascular lineages within the heart (119,120).

Lastly, induced pluripotent stem cells (iPSC) would unscramble the generation and maintenance problem with the CPCs. Moreover, reprogramming somatic cells from patients into a pluripotent state, followed by differentiation to disease-relevant cell types may ascertain new disease-relevant insights as well as an unlimited source of these cells to be used to set disease models able to be tested for a myriad of experiences (121). For instance, iPSC derived tissues can be used to understand the molecular disease mechanisms or even drug screening and set cytotoxic input for chemicals in drug development (122). Out of the many challenges for the clinical application of hESC, immune rejection is easier to overcome with iPSC due to their potential ability to generate autologous tissues that can be transplanted with reduced immune rejection (123).

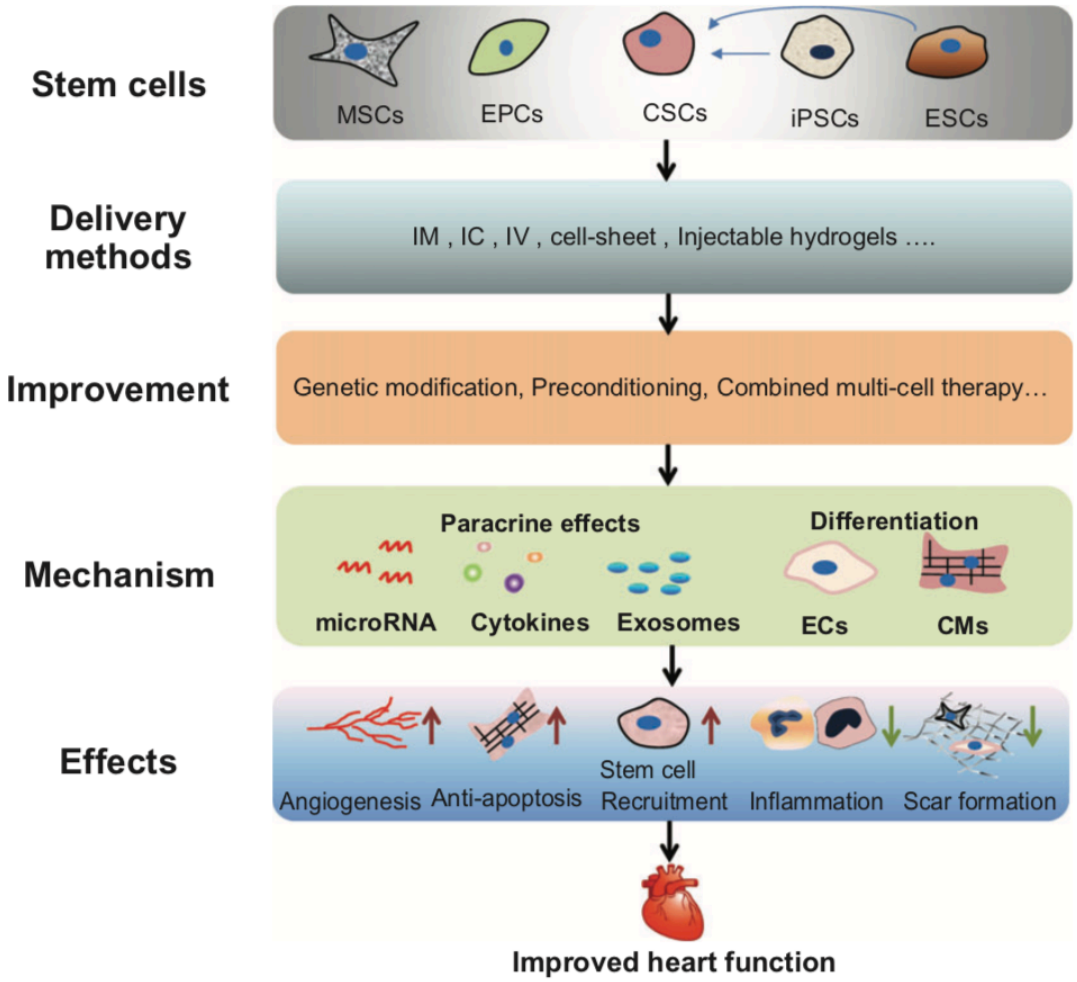


Figure 4 - Summary of the stem cells for treating ischemic heart disease. Different stem cells can be delivered through IM, IC, IV, cell-sheet and combined with injectable hydrogels. Their therapeutic effects can be enhanced by genetic modification, preconditioning and combined multi-cell therapy. Cytokines, miR and exosomes secreted from stem cells play a major role in the paracrine effects of stem cells. Stem cells can also differentiate into CMs and ECs although the number is minimal. ESCs and iPSCs are usually differentiated into CSCs or functional CMs before they are administrated into heart. The improved heart function may be through enhanced angiogenesis, less apoptosis of CMs, recruitment of more endogenous stem cells, and ameliorated inflammation and scar formation after stem cell transplantation (124).

Heart diseases are widespread throughout the general population. Indeed, as clarified at the beginning of this thesis, the improvement of health care is leading to an increase of the adult congenital heart disease (ACHD). Most cardiovascular diseases are treater with medication that will reduce the symptoms and decelerate the disease progression. Though, these drugs cannot restore or repair necrotic tissue. As such, pathologies like myocadiac infarction often lead to heart failure with high morbidity. This happens due to the fact that the

organism fails to replace the fibrotic tissue with new cardiomyocytes (125). This leads to a structural remodelling and subsequent limitation in the pumping capacity of the heart.

There are several therapeutic strategies that can be set up to promote cardiac regeneration in injured hearts. As such, there are many clinical trials on the go with potential to induce heart regeneration, either cell-based or cell-free (125–128). The development of cell-based therapies for the heart regeneration has emerged from many cell sources, including the bone marrow-derived cells (BMCs), hematopoietic stem cells (HSCs) and adult cardiac progenitors' cells (CPCs). Additionally, another cell-based strategy in use is the differentiation of PSC into cardiomyocytes (125–128). Nevertheless, most of these clinical trials have showed none or a modest therapeutic efficacy.

More recently, these experiments set designs started to change. As the myocardium is a rather complex multicellular tissue, the poor outcomes of previous trials may be due to the need of compiled multiple cell types coordinated in a fashion way, in order to induce cardiac tissue regeneration. In this regard, there has been under studies the combined transplantation of multiple cardiac cell types, such as the cardiomyocytes, vascular EC, SMC and cardiac fibroblasts (129). This way, it is thought that the multicellular complex populations will be capable to interacting and communicating with one another, enabling them to react to the needs of the damaged cardiac tissue. In line with this idea, cell transplantation of iPSC-cardiomyocytes, ECs and SMCs improved left-ventricular cardiac function and decreased the scar tissue following the myocardium infarction in a porcine model (130). Of interest, the hPSC-cardiamyocytes are able to integrate with the host myocardium and that both EC and SMCs are able to impact the host vasculature (131). This kind of studies may very well enable us to move towards an efficient cardiac cell therapy but also unravel the biological mechanism underlying the heart homeostasis.

1.3 Secretome

The secretome is the proteome that is secreted out of cells and cellular components. As such, it has been shown their important role in the crosstalk within the cell and between other cells (132,133). Moreover, of note, that secretory proteins are essential for signal transduction, mainly in regard to membrane receptor-dependent signal transduction, thus are broadly involved in biological cell regulation (134). These proteins are commonly processed through the endoplasmic reticulum (ER) and Golgi apparatus and afterwards will eventually be secreted either via classical or exosomal pathways.

The secretory pathway also undergoes major changes in response to the developmental programs. For instances, for both Drosophila melanogaster and Danio rerio, there are specific secreted proteins expressions that change throughout the development (135–137). These changes are due to the activation of the unfolded protein response (UPR) which will induce the expression of many genes that able the cell to secrete certain proteins such as enzymes involved in the synthesis of lipid bilayers (138). There is now a new trend for studying the secretory pathway induced cascades in response to exogenous stimuli such as hormones, nutrients or growth factors. Also, of notice, the fact that the membranes of the vesicles secreted hold signaling molecules such as kinases, that may this way, induce or propagate signaling cascades to other cells (134). Finally, but not least, the functional cellular outcomes will differ depending on the intracellular location these secreted proteins originate (ER or golgi versus plasma membrane).

One approach to study players on the cellular communication in the heart is through the analysis of the conditioned medium from *in vitro* cultures of cardiac structures such as cardiospheres (139), cardiac resident cells (140) and heart explant tissues (141). During an *in vitro* cardiomyocyte differentiation, it was found 1802 proteins significantly regulated, out of which 431 are secreted proteins. Numerous proteins detected regulate the Wnt, TGBβ, Activin A, Nodal, BMB and FGF signaling pathways (142). Further studies are required, but the identification of paracrine factors the regulate cardiomyocytes differentiation, proliferation and maturation may help to reveal more insights in the heart development process, as well as help to design new therapies for treatment of heart pathologies.

As mentioned, secreted proteins play a wide range of important roles in the biological stimuli response and homeostasis of the body. With this being said, there are two major proteomics workflows to study the secreted proteins: in-solution digestion coupled with LC-MS/MS, and SDS-gel fractionation/in-gel digestion/LC-MS/MS (143–145).

1.3.1 Stem Cell secretome

As previously mentioned, there is a wide range of applications for the use of stem cells. One of which is their application in cell-based therapies for treatment of degenerative diseases as myocardial infarction. Though, it has been shown that their ability to perform a beneficial impact on the cardiac regeneration is not due to their capacity to differentiate into the cardiac tissue itself, but rather resides on their capacity to secrete molecules that will induce endogenous cells to perform the regeneration of the damaged tissue (146). This comes with

high interest as this secretome can be analyzed and the secretions be quantified in a way that enables to manufacture a medium that can be freeze-dried, packaged and transported. Adding, the fact that as a cell-free therapy, there is no rejection reaction and, subsequently, there is no need to match a donor to the recipient. This way, stem cell secretome has a promise role in biomedical applications and health care improvement.

Stem cells are surrounded and modulated by a well-defined microenvironment, called the niche. This microenvironment is dynamic in a way that is able to induce cell maintenance but also the self-renewal and differentiation (147). The stem cells niche includes many components, the stem cells and stromal tissue, the ECM, blood vessels that carry systemic signals and other cells, the neural stimuli, oxygen saturation, as well as other physical conditions such as shear stress (147,148). As such, cell signaling in the niche should be seen as a highly dynamic process that results from the multiple interactions and stimuli involving interaction between cells, adherent and de-adherent ECM proteins, soluble trophic factors and EVs. For instances, proteoglycans and glycoproteins on the ECM support and give structure to the cell's niches. Moreover, the enzymatic degradation of the ECM releases bioactive fragments and proteins that will trigger cellular responses (149,150). Also, the ECM is a reservoir of growth factors and other bioactive elements and, these soluble factors may act locally or diffused throughout the niche generating concentration gradients that can activate or inhibit signaling pathways in the cells (147,151). Additionally, it is also important to consider the EVs, that may load a wide range of cargo, such as proteins, lipids, RNA, among others. As such, the EVs allow an intercellular communication (152,153). As stated before, the stem cells niche is dynamic, but it is also distinct and unique to each stem cell populations (154). Thus, stem cells behavior depends on the interactions between specific cells and niche elements.

Focusing in the heart, during the development, the cardiac niches have roles rather related to cell expansion and specification, allowing to control size and shape of all cardiac structures (155). As such, cell-cell communication comes with upmost importance. The secretome comprises a complex variety of secreted molecules and vesicles that impact cellular biological processes including cell fate an proliferation (140). Through the analysis of the ECM composition, soluble factors and EVs, out of cell and tissue cultures, we can manage to understand the human heart development. Regarding the adult heart, there is only a limited and somehow controversial understanding of the cardiac niches (156).

1.4 The endoplasmic reticulum and Ca^{2+}

Calcium (Ca^{2+}) is one the most ubiquitous and physiologically important signaling, as an intervenient (directly or indirectly) in every cellular function including secretion, contraction-relaxion cycles, cell motility, cytoplasmic and mitochondrial metabolism, synthesis, modification and folding of proteins, gene expression, cell cycle progression and apoptosis (157). The endoplasmic reticulum (ER) plays a key role in maintaining Ca^{2+} homeostasis within the cell. Ca^{2+} is released from the ER via the inositol 1,4,5-triphosphate ($InsP_3$) receptor and is transported back to the ER through the sarcomeric/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (157). On one hand, Ca^{2+} release from the ER activates store operated Ca^{2+} channels in the plasma membrane causing cytoplasmic concentration to increase. On the other hand, cytoplasmic Ca^{2+} binds to Ca^{2+}-regulated proteins, which includes protein kinases and phosphatases that will, thereafter, regulate the many biological functions previously stated (158). As such, the Ca^{2+} release from the ER and entry via plasma membrane must be cautiously regulated.

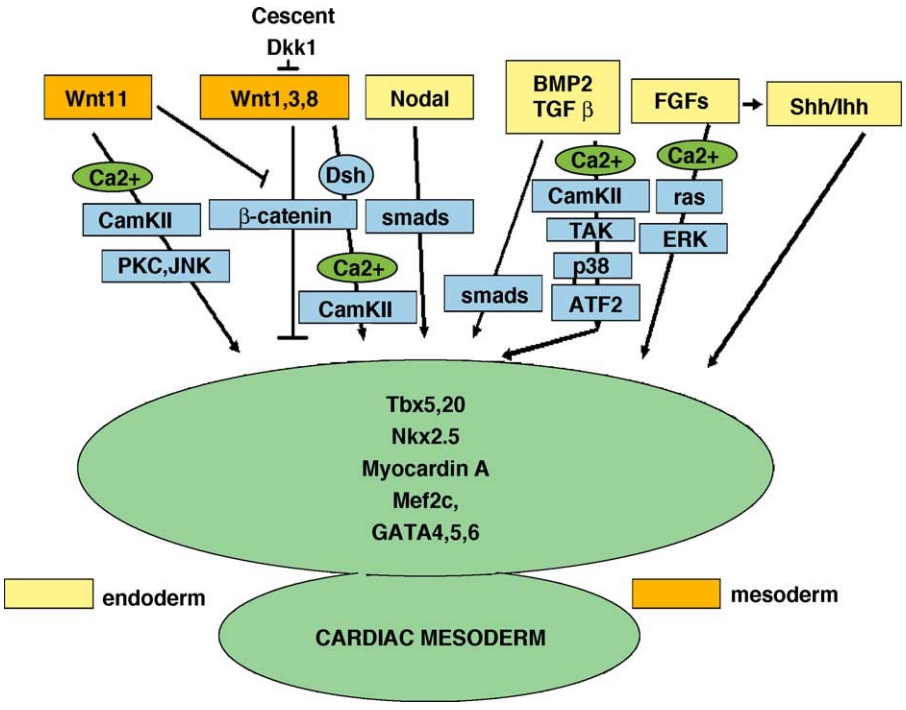


Figure 5 - Cardiac transcriptional cascades and Ca^{2+}-dependent signalling pathways. Mesoderm-secreted cardiogenic factors (Wnts), or endoderm-released factors of the TGFβ, or FGF families turn on Ca^{2+}-dependent signalling pathways. This leads to transactivation of cardiac transcription factors and in turn induction of the mesodermal cardiac field (159).

Regarding the intracellular Ca^{2+} biological function, there are several Ca^{2+}-dependent signaling pathways (Figure 5) that lead to the transactivation of cardiac transcription factors and induction of the cardiac mesoderm during the embryogenesis (159). Though, these responses are dependent on cardiogenic morphogens present on the extracellular matrix. The cardiac morphogens include the TGF superfamily (TGF, nodal, activin and BMPs), the FGF family and the WNT family (159). For instance, agonist binding TGF or BMP ALK (Activin-Like Kinase) receptors, lead to the translocation of the Smads proteins into the nucleus. Smads are activators of important cardiac transcription factors such as the Nkx2.5. On the other hand, the TGF receptors can activate a Mitogen Activated Protein Kinase Kinase Kinase (MAPKKK) – TAK. In turn, TAK kinase activity is dependent on Ca^{2+} and is important for the CREB and ATF2 transcription factor action. The ATF2 can, in turn, bind to the Smads, which will promote the expression of other cardiac transcription factors. Another morphogen, the FGF family can activate MAP Kinases p42/p44, the ERK, via monomeric G coupled protein, RAS. Regarding the Wnt family morphogens, these can be inhibited by antagonists such as Crescent and Dikkopf (DKK1), in a way to promote the cardiac differentiation. Notwithstanding, Dishevelled (Dsh), as a mediator of the Wnt canonical catenin-dependent pathway has been shown to activate CamK through Plana Cell Polarity (PCP) in a Ca^{2+} dependent way. Indeed, Dsh mutants or deletions lead to deficient cardiogenesis (160). In contrast, Wnt11, a non-canonical Wnt family member, has been shown to be a cardiogenic morphogen (160,161). Some of Wnt11 biological activities are the activation of Ca^{2+}-dependent calmodulin kinase 2 (CamKII), the Protein Kinase C (PKC) and the c-Jun Kinase (JNK). The cardiac cell specification is dependent on a widespread range of transcription factors. Moreover, these transcription factors are integrated in a network dependent on Ca^{2+}, among which are *Nkx2.5, Mef2c, GATA4,5,6, Tbx5* and *20* (Figure 5)

The ER is present in all nucleated cells. The ER membrane is involved in the correct folding and post-translational modifications of newly synthesized proteins, storage and regulation of Ca^{2+} release, as well as synthesis of steroids and phospholipids (162,163). The sarcoplasmic reticulum (SR), though it's similar to the ER, this organelle is present in striated, cardiac and smooth muscle tissues, having as a function the control of Ca2+ for muscle contraction (164). Nevertheless, both reticulum membranes are important to the heart development. For instances, the ER is involved in the synthesis of proteins needed for the cardiogenesis whereas the SR plays an important role by regulating the cardiac muscular contraction cycles (165,166). As previously stated, cardiovascular diseases are the leading

cause of death in the western world and the common congenital birth defects in humans are heart related. Moreover, many of the heart pathologies are associated to impaired function of the ER or SR (165,167,168).

The ER stress refers to the accumulation of misfolded or un-folded proteins within the lumen of the ER in response to a stimulus. There are three main signaling pathways as part of the unfolded protein response (UPR), the activation of the activating transcription factor 6 (ATF6), inositol-requiring kinase 1 (IRE1) and protein endoplasmic reticulum kinase (PERK) (169,170). Upon stress, ATF6 translocates to the Golgi complex where it is cleaved, releasing its cytoplasmic basic leucine zipper (bZIP) domain. Consequently, this bZIP domain translocates to the nucleus to promote the transcription of ER stress elements genes (171). IRE1 and PERK, also dissociate in response to ER stress. The IRE1 homodimer auto-phosphorylates, enabling its endonuclease activity, resulting in the splicing of XBP1 mRNA (171). Thereafter, the protein encoded by the spliced mRNA will induce the expression of ER stress elements genes. Once PERK is activates, upon ER stress, it forms homodimers that are able to phosphorylate eukaruotic translation initiation factor 2, α subunit (eIF2α), diminishing the translational process (169,170).

Heart failure, either in humans or mice, can be associated with an increase expression of ER chaperones such as BiP, Grp94 and calreticulin (172). It has also been shown that rat cardiomyocytes *in vitro* proteasome inhibition leads to ER stress and apoptotic cell death (173).

Calreticulin is a major Ca^{2+} buffering protein of the ER and it plays a vital role in the heart. The roles of this ubiquitously expressed protein of the ER range from Ca^{2+} homeostasis regulation, to chaperone activity and cell adhesion regulation (174). Moreover, though its low protein expression in adult tissues, it is highly expressed in the cardiovascular system during the embryogenesis. Its role throughout the development is essential as calreticulin deficiency in mice is lethal due to impaired cardiogenesis (174). Interestingly, calreticulin deficient mice show abnormal thin left ventricular walls (174), increased glycogen deposition (175) and overall impaired myofibrillogenesis (176). The molecular mechanisms responsible for the myofibrillogenesis induced by calreticulin deficiency are not clear, though they have been associated to Ca^{2+} dependent transcriptional pathways (165). Indeed, Calreticulin deficient cells exhibit an impaired Ca^{2+} release from the ER in response to stimulation. Thus, calcineurin activity becomes impaired due to its Ca^{2+}-dependent phosphatase (174). This comes with high importance as one of the roles of calcineurin is to phosphorylate the NFAT3 enabling its nuclear translocation (177). Additionally, constitutively expression of calcineurin in calreticulin null cells restores the nuclear localization of the NFAT3 and reverses embryonic

lethality in cardiac tissue (178). Moreover, these calreticulin null mice embryos with calcineurin constitutively expressed no longer exhibit ventricular wall thinning but, can only survive for 3 to 5 weeks. Interestingly, these transgenic mice had a metabolic defective regulation, indicating that calreticulin may play a role in energy metabolism upon birth (178). Although high levels of calreticulin expression are required for the proper heart development, its downregulation is equally important postnatally. Indeed, mice overexpressing calreticulin in the heart suffered sudden death (179). These transgenic mice lethality was found to be due to cardiac conduction problems, more precisely, sinus and atrioventricular node disfunctions, both caused impaired Ca^{2+} current in cardiomyocytes (179). It has also been used a model with ES cells with overexpression of calreticulin, but this time in pacemaker cells (180). Upon the embryoid bodies protocol of differentiation, these cells had reduced levels of beating colonies (180). Moreover, this phenotype was dependent on impaired Ca^{2+} flux, indicating that this calreticulin overexpression also had impaired the ER Ca^{2+} homeostasis with consequent impaired beating (180,181). It is also of important notice the fact that genes encoding ER resident proteins that are activated during the cardiogenesis may as well hold an important impact in adult cardiac pathologies. For instances, calreticulin expression requires a fine-tuned regulation since either overexpression or downregulation level could impact the cardiogenesis. In this regard, calreticulin induced expression is carried out by many factors known to cardiogenesis, such as the Nkx2.5, myocyte enhance factors, 2c (MEF2C) and GATA6. Also, calreticulin is suppressed by chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) and ecotropic viral integration site 1 (Evi1) (178,182,183).

1.5 Mass Spectrometry

The identification of proteins provides a window into complex cellular regulatory networks. In the past, polypeptides were sequenced by stepwise chemical degradation from N terminus to C terminus (Edman degradation) (184), with subsequent identification of the released amino acids by UV absorbance spectroscopy. As time has gone by, mass spectrometer was developed and improved their resolution to provide as much information as possible from simple samples to a rather more complex one. With the increased performance and versatility, new protein analytical strategies have emerged in which mass spectrometry is the central element.

Mass spectrometry (MS)-based proteomics is now a current method and indispensable mean of unbiased analyses of a large number of proteins sample as it is the secretome. This method allows to study the composition and dynamics of subcellular organelles, protein complexes, interactions, modifications, and the mechanisms of cell signaling (185–189). Most interesting is the fact that this method not only is applied to the detection and quantification of single proteins but also a wide range of proteins of complex samples such as the secreted proteasome. Indeed, there is a trend in trying to understand the whole proteomic expression patterns for different cellular states rather than just pursuing single protein expression (190).

Once the proteolytic digestion has been done, there will be a peptide mixture which will be separated by a liquid chromatography and afterwards electro sprayed for the MS and tandem mass spectrometric analysis (MS/MS). In this regard, one of the difficulties of this technique is the ability to form enough fragments that able to identify a protein through the MS/MS when comparing the database (191,192). Due to the fact that this mixture will have thousands of peptides, there has been a further development on improving the sensitivity, sequencing speed and resolution (193,194). Nowadays there is in use of a shotgun proteomics with four main mass spectrometric separation principles: quadrupole mass filters, time of flight (TOF) mass analyzers, linear ion traps, and Orbitrap™ analyzers.

The Orbitrap mass analyzer consists in a small electrostatic device where ion packets are injected at high energy to orbit around a central, spindle-shaped electrode (195–197). The image current of the axial motion of the ions will thereafter be detected and this signal is Fourier transformed to yield to high resolution mass spectra. Within the field of study of this thesis, the proteomics of the secretome, the combination of a low-resolution linear ion trap with the high resolution Orbitrap analyzer, has become a standard protocol (Figure 6) (198,199).

This instrument, combines a linear ion trap with radial ejection and an orbitrap mass analyzer Figure 6 (199). Furthermore, the orbitrap mass analyzer is an electrostatic trap where injected ions rotate around a central electrode, thus being confined an appropriate voltage between the outer and central nodes. The mass analysis is based on image detection of frequencies of axial oscillations.

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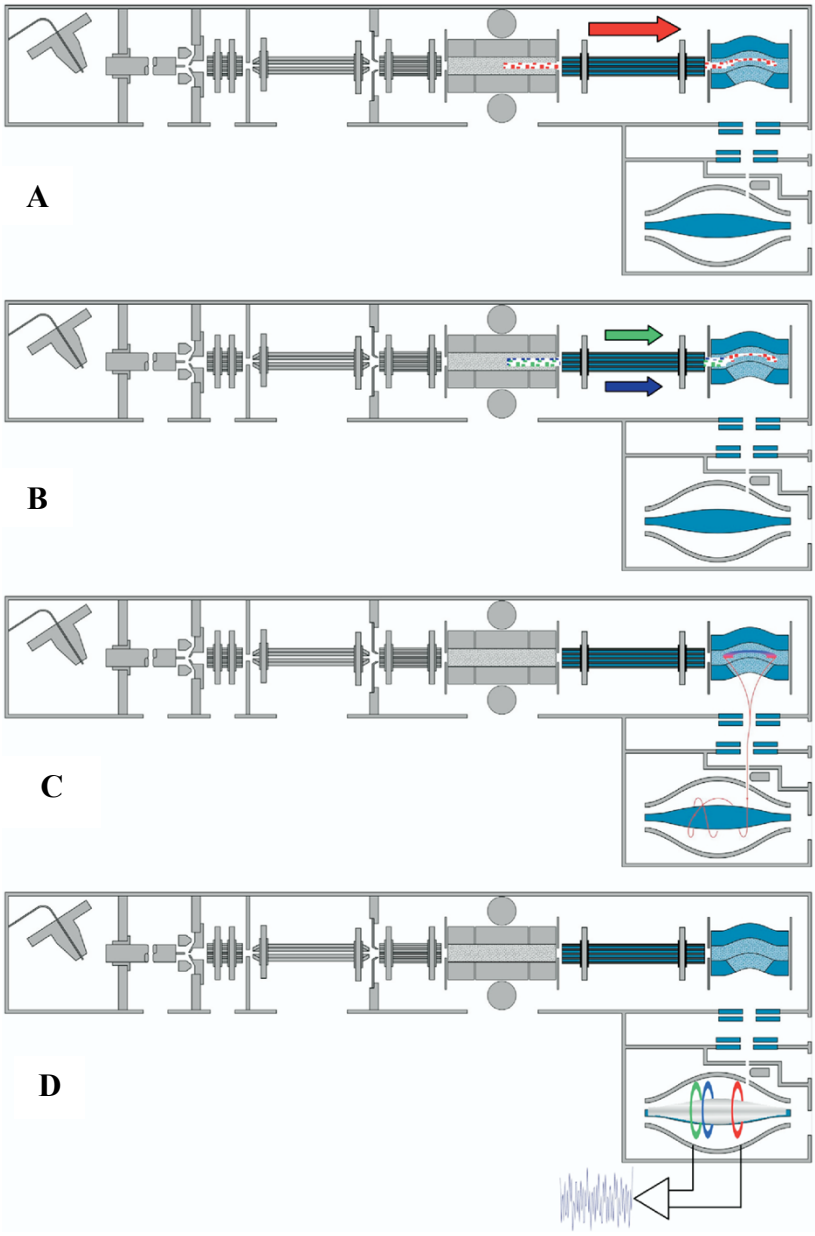


Figure 6 - Schematic sequence for measurements of the extent of mass accuracy the orbitrap mass analyser. A) Injection of the first set of ions and trapping in the C-trap; B) injection of the second set of ions and trapping in the C-trap; C) pulsed injection of mixed ion population into the orbitrap; D) ion detection in the orbitrap.

1.6 Animal models to study cardiovascular development

In recent years, great progress has been made in determining many of the factors that regulate cardiovascular development. This has been possible through both *in vivo* and *in vitro* model systems. The mouse is a very interesting model not only because it has a cardiovascular system like the human but also because it provides the advantages of a highly tractable organism for genetic studies. The ability to introduce or remove DNA sequences of interest in the germline genome has rendered the mouse a powerful and indispensable experimental model in fundamental and medical research (200,201). Furthermore, conditional gene activation or inactivation through, for example, Cre recombinase and loxP system, allows the precise temporal and spatial assessment of gene function (202). Even so, like any other mammal system, mice embryos are difficult to access and/or observe as they develop inside their mothers' uteri which is inconvenient for studying cardiac development and CHD.

The chick model is also widely used to study cardiac morphogenesis. The embryos are large and develop externally to the mother. The avian heart is also four-chambered but, the major advantage of chick embryos is their accessibility for surgical manipulation and functional interference approaches, through both gain and loss of function. Furthermore, chick embryos can be cultured for time-lapse imaging, which enables tracking of fluorescently labelled cells and detailed analysis of tissue morphogenesis (203).

Other vertebrate models include the frog and the fish. *Xenopus laevis,* the clawed frog, offers very similar advantages to the chick model (204). Zebrafish have two muscular cardiac chambers and is highly sought as model for questions concerning the development and looping of the heart tube, atrial and ventricular patterning, and myocardial differentiation. The zebrafish is also widely used as a powerful genetic and drug screening system. Finally, zebrafish is a very interesting model to study cardiac regeneration due their regeneration capability (205).

The fruit fly, *Drosophila melanogaster,* has emerged as a useful model for cardiac development and diseases. The fly is a unique and valuable system as it is the only invertebrate genetic model with a working heart developmentally homologous to the vertebrate heart. Thus, the fly model combines the advantages of invertebrate such as large populations, easy genetic manipulation and short lifespan with physiological measurement techniques that allow meaningful comparisons with data from vertebrate model systems. As such, the fly has been mostly contributing to the understanding of complicated interactions between environmental factors and genetics in the long-term regulation of cardiac development (206,207).

Although *in vivo* models are critically important, their intrinsic complexity can bring several disadvantages. While their application can be limited, *in vitro* models can offer unique advantages. The major advantages of *in vitro* model systems are the precise control of experimental conditions and access to a large pool of sample. This results in an ability to efficiently conduct studies regarding signaling pathways, cell-specific mechanisms studies, and high-throughput drug screening.

Neonatal mouse cardiomyocytes are easily isolated and are able to maintain proliferative *in vitro* for a few passages (208,209). They are mainly sought as drug screening and response to stimulus because these cellular responses closely represent the changes found in cardiomyocytes *in vivo* (210). To overcome the limited cultured ability of primary cardiomyocytes, efforts have been made to develop cardiac cell lines (210). Nowadays, immortalized cell lines exist that retain phenotypical and contractible characteristics of cardiomyocytes (211,212).

1.7 Cited2

The CBP/p300 interacting transactivator with ED tail rich family member 2 (CITED2) is a transcription factor which binds with high affinity to the Cysteine-Histidine rich domain 1 (CH1) of the transcriptional co-activators p300/CBP (213,214). Although CITED2 is a transcription factor, it does not bind the DNA directly but acts as a co-activator or co-inhibitor of other transcription factors dependent on the p300/CBP for their optimal activity (215). The C-terminal domain of CITED proteins (CR2) is a transactivation domain unique to this protein family, which exhibits a high binding affinity to the CH1 domain of CBP/p300. Cited2 is conserved in all vertebrates and absent in invertebrates such as *Drosophila melanogaster*. Mouse *Cited2* KO embryos die *in utero* with multiple organs affected (216–218). In humans, CITED2 gene is located in the 6q23 region and mutations in CITED2 have been previously associated with CHD (219–221).

1.7.1 Cited2 regulatory network/Stem Cells

The best-described role of *Cited2* is in the regulation of the cellular responses to hypoxia. Indeed, Cited2 compete out other transcription factors from the CH1 domain, such as the Hypoxia-Induced Factor-1 α (HIF -1α), and consequently inhibit HIF/hypoxia-mediated

responses (213). In fact, part of the cardiac defects observed in CITED2 null embryos are likely due to an enhanced activity of HIF1α (222). Indeed, the persistent hypoxia found in *Cited2* null hearts is rescued by HIF1α haploinsufficiency (223). In terms of molecular mechanisms, CITED2 transactivation domain (TAD/CR2) disrupts the complex between the CH1 domain of p300/CBP by binding to the CH1 with higher affinity than HIF1α. CITED2 activates a highly responsive negative feedback circuit, as HIF itself induce the expression of *CITED2*, that rapidly and efficiently attenuates hypoxic response, even at modest CITED2 concentrations (214,224).

In addition to HIF-1α, CITED2 also negatively regulates the activity of other factors interacting with the CH1 domain of CbP/p300, such as RXRα, NF-κB, STAT2, p53 and ETS-1 (213,226–228). Notwithstanding, CITED2 co-activates many other transcription factors that require a cooperation with CBP/p300 to be transcriptionally efficient such as, the TFAP2 members, LHX2/3, SMAD2/3, PPARα/β HNF4α, WT1, GCN5 and ISL1 (217,218,229–238) (

Figure **7**).

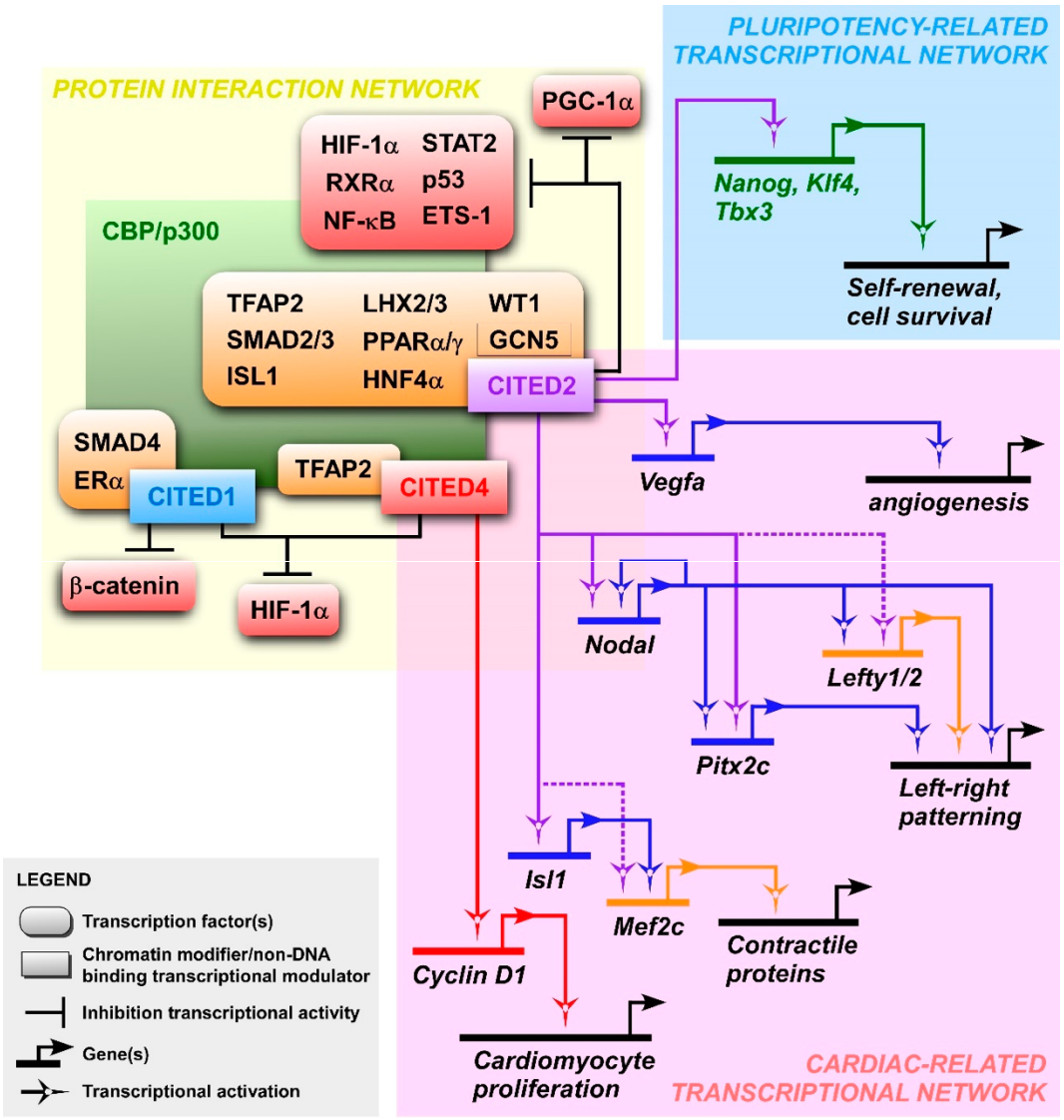


Figure 7 - CITED protein interactions and gene regulatory networks involved in the maintenance of pluripotency and cardiogenesis. The yellow panel presents interactions between CBP/p300, CITED proteins and known CITED-interactors. The inhibitory effect of CITED proteins on the transactivation of the indicated transcription factors is represented by inhibitory lines. The absence of inhibitory lines indicates that CITED proteins co-activate the interacting transcription factors indicated. The blue and pink panels show circuit diagrams representing respectively the pluripotency- and cardiac-related transcriptional networks in which Cited2 and Cited4 are involved. The solid lines indicate regulations that have been supported experimentally, while dashed lines represent connections between CITED proteins and the indicated gene regulatory regions suggested by genetic interactions (225).

1.7.2 Cited2 and heart development

Outstandingly, *Cited2* expression can be found throughout the mesodermal and cardiac derived structures, during the mouse embryonic development (239). Indeed, when *Cited2* is depleted in the epiblast, there can be observed heart malformations. But, when *Cited2* is conditionally knocked out in a Brachyury/T-Cre and Mesp1-Cre models, there is not such harsh phenotype (218,240). This may be due to the fact that CITED2 requirement is only a concern to the development before Brachyury and Mesp1 expression. Indeed, Cited2 depletion at the onset of differentiation causes the most severe impact on cardiac differentiation and results also in the impairment of Brachyury and Mesp1 expression at day three *in vitro* differentiation (230). Therefore, there is a clear indication of the important role of Cited2 at early steps of differentiation, at least in part before the expression of Brachyury and Mesp1. Since Brachyury and Mesp1 are important genes on the mesoderm and cardiac progenitor specification, Cited2 may very much be important for early mesoderm commitment.

Furthermore, *Cited2* depletion at the onset of differentiation significantly impairs the expression of *Brachyury*, *Mesp1*, *Isl1*, *Gata4*, and *Tbx5*. Though, when overexpressed not only stimulates the same genes expression in ESC but also induces cardiac commitment and differentiation (230). It has also been shown that CITED2 not only is recruited to *Ils1* promoter but also interacts physically with ILS1 in a way that both proteins synergize to promote cardiogenesis in ESC (230). Furthermore, Cited2-null ESC have an impaired differentiation, affecting thereafter the cardiac commitment (241). All in all, CITED2 regulates the expression of important genes to cardiogenesis of which, involved in the mesoderm and cardiac progenitor specification.

Interestingly, Cited2 expression seems to be enriched in SHF cardiac progenitors. Moreover, Cited2-null embryos display a variety of cardiac developmental defects such as ventricular septal defects with overriding aorta or double-outlet right ventricle, outflow tract defects, and trans- position of the great arteries, which may result from anomalies of SHF and cardiac neural crest cell progenitors known to express ISL1 (218,242).

1.7.3 Cited2 mutations associated to CHD

Cited2 has three conserved regions (CR1, CR2 and CR3) that are present within the CITED family (243). Of which, CR2 is the one that participates in most known important

biological activities by binding to p300-CH1 (213,243). Moreover, it is of particular note the fact that also exists a Ser-Gly junction domain (SRJ) where most missense mutations cluster but, when this domain was withdrawn, no heart defect observed (220).

Since 2005, when functionally relevant mutations in *CITED2* were identified in patients with CHD, a growing number of mutations have been reported to be associated to CHDs (219). With this being said, the most frequent heart defects associated with CITED2 are the following: VSD, ASD, TOF and the transposition of the great arteries (TGA), a CHD where the aorta is connected to the RV, and the pulmonary artery is connected to the LV (

Figure **8**). Though, most of *CITED2* variants identified in patients with CHD, only marginally affected the ability of CITED2 to repress HIF-1α and/or co-activate TFAP2C transcription factor *ex vivo* (220,244–248).

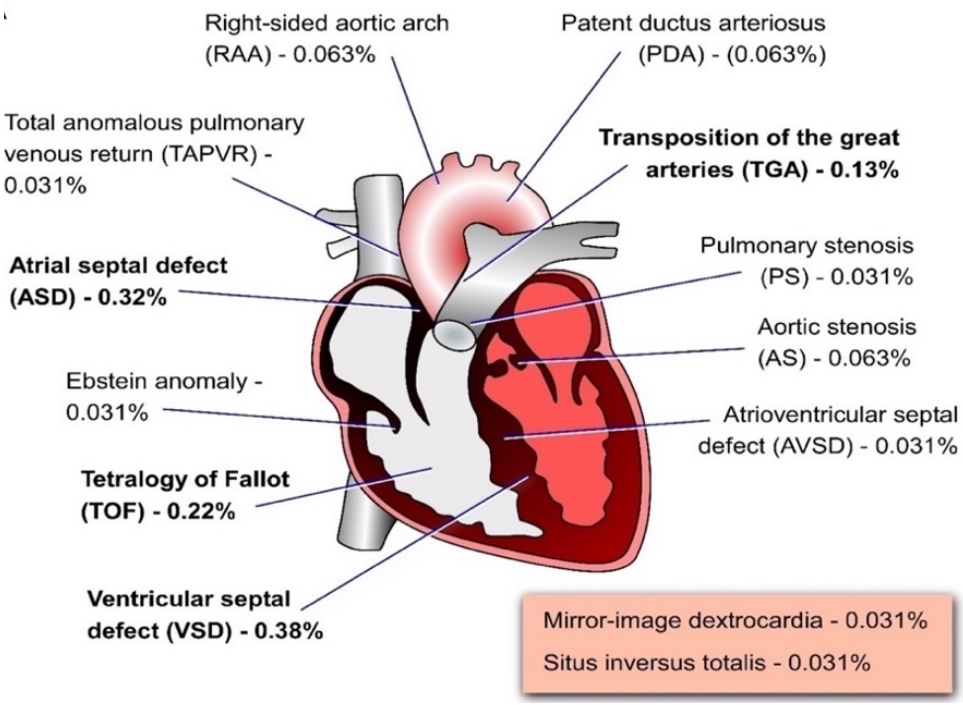


Figure 8 - Prevalence of CITED2 mutations in CHD patients. Schematic representation of the adult human heart indicating the heart defects detected in patients of CHD carrying CITED2 mutations. The percentages represent the proportion of each of the heart abnormalities associated with CITED2 mutation in a cohort of patients with CHD (184).

1.7.4 CITED2 induced conditioned medium

The crosstalk among cells are crucial during the all biological processes, including the cardiac development from the mesoderm specification and migration, heart tube formation and

looping, morphogenesis, maturation of the heart valves and chambers and even heart septation. With this being said, there are several secreted proteins that alter the development process. For instances, endocardium-derived NOTCH has been shown not only to promote EMT and cell migration, but also regulation of the cardiac valve formation (249).

Not only the proteins alone are present in the secretome. There are also extracellular vesicles (EVs) among the secretome. Indeed, these EVs have been also subject of studies as they mediate intercellular communication among numerous cell types (250). EVs are an heterogeneous population of very small biphospholipid membranous particles that carry may carry out important biological cargo. The content of the EVs scope many bioactive molecules such as proteins, lipids, RNA and even DNA (251,252). Of interest that CPCs exossomal miRNA transcriptional profile shown that they are abundant with key mRNAs such as antiapoptotic miR-210 (Inhibitor of proapoptotic ephrin A3 and protein tyrosine phosphatase 1B mRNAs) and proangiogenic miRNA- 132 (inhibits antiangiogenic RasGAP-p120 mRNA) (253). Moreover, it has also been shown that CPCs-derived exosomes exhibit an antiapoptotic and cardioprotective effect in a rat model of heart injury (254).

It has been recently shown on our lab (225,255) that CITED2 is not only present throughout the embryonic differentiation but also, its induced secretome (CM-CITED2) is able to rescue CITED2 ablation cardiac defects by restoring the beating foci (Figure 9B). With this being said, it is clear that CITED2 affects the crosstalk between cells through the induced release of certain cardiopoietic molecules (255). Indeed, through a microarray analysis, we identified several genes whose respective proteins are described as secreted proteins and also that their expression was impaired by *Cited2* knockdown. Moreover, it has also been shown that WNT11 and WNT5a, whose expression was increased on *Cited2* overexpressing cells (Figure 9D) and decreased on *Cited2* knockdown cells (Figure 9E), had the ability to rescue *Cited2* ablation cardiac defects both *in vitro* and *in vivo* (255). Through the search of WNT5a and WNT11, we also came across other possible candidates whose expression also followed the manipulated expression of *Cited2* (Figure 9D and E). Moreover, *Fgf10* was also one of the candidates that was further studied on our lab. It has been previously shown on our lab that the CM-CITED2 with FGF10 immunodepleted, barely induced any beating foci in the EBs. For this reason, we decided to pursue this candidate ability to rescue *Cited2* ablation cardiac defects.

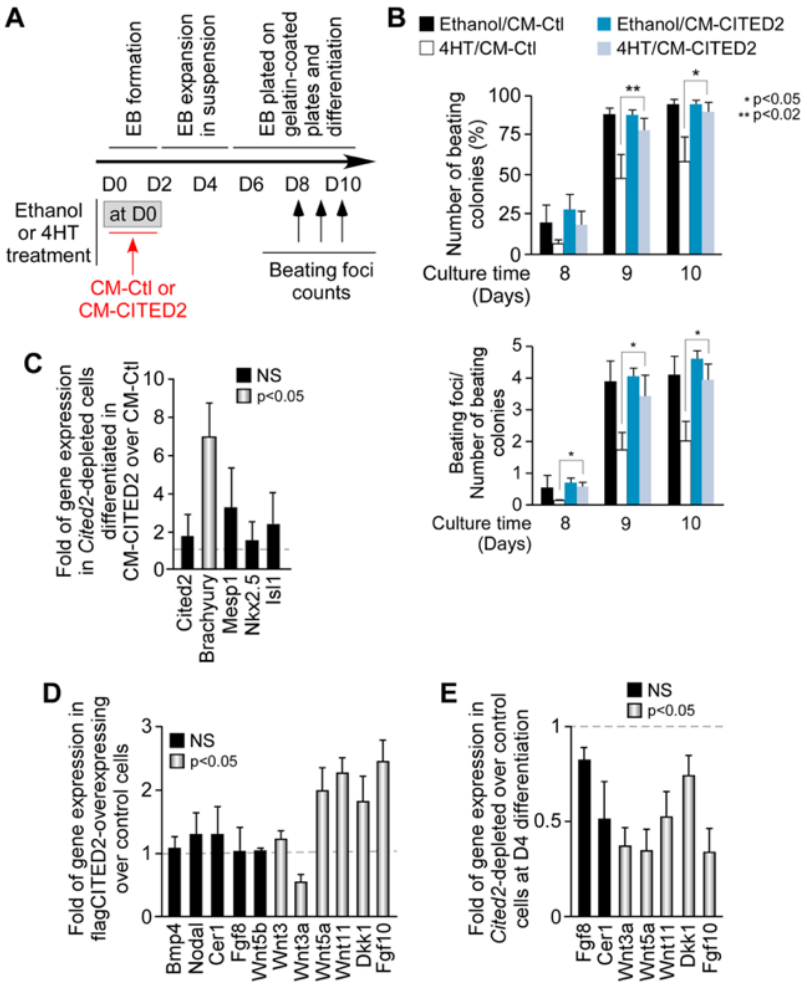


Figure 9 - The secretome of CITED2 overexpression embryonic stem cells rescues cardiac differentiation of Cited2-depleted cells. a Timeline depicting the protocol used for differentiation C2fl/fl[Cre]ESC from D0 onward. The time of ethanol or 4HT treatment, as well as the supplementation with the conditioned media, and the days of beating activity assessment are indicated. **b** Percentage of colonies with contractile foci (top panel) counted at 8, 9 and 10 days after the initiation of differentiation in cell cultures derived from $C2^{fl/fl}$[Cre] ESC treated with ethanol or 4HT at D0 of differentiation, and simultaneously supplemented with conditioned medium either from control cells (Ethanol/CM-Ctl and 4HT/CM-Ctl, respectively) or from cells overexpressing CITED2 (Ethanol/CM-CITED2 and 4HT/CM-CITED2, respectively). The number of beating foci per beating colony is also indicated (bottom panel). **c** Relative expression of Cited2, Brachyury, Mesp1, Nkx2.5, and Isl1 determined by qPCR at D4 of differentiation in cultures derived from C2fl/fl[Cre] ESC treated with 4HT either in the presence of and CM-Ctl or CM-CITED2 as described in **b**. The expression of the indicated genes is presented as the fold of expression in cells treated with CM-CITED2 over cells treated with CM-Ctl. The black bars indicate variations without reaching statistical significance, and gray bars indicate genes with statistical significance by Student's t-test. NS, not significant. **d** Relative expression of the indicated genes encoding secreted proteins involved in cardiogenesis, determined by qPCR in E14/T ESC transfected with a plasmid expressing flag-tagged CITED2 (flagCITED2) or the control empty plasmid (control cells). The results are presented as in **c**. **e** Expression of the indicated genes encoding secreted proteins involved in cardiogenesis, in cells treated as described in **c**. The results are presented as in c. Results are presented as the mean ± SEM of three independent biological experiments (255).

1.8 Objectives

The goal of this thesis is to better understand the function of CITED2 in cardiac development. Therefore, we will seek to identify secreted proteins that have their expression or activity dependent of CITED2. To start, as a first objective, we will investigate the impact of FGF10, as one of the candidates detected previously on our lab with the use of microarrays (255). For this we aim to investigate if FGF10 is able to rescue CITED2 defects *in vitro* and in an *in vivo* model using the Danio rerio. Following the same main objective, we further desired to study the secreted cardiopoietic proteins whose presence on the secretome may be dependent on CITED2. For this purpose, we will use the LC-MS to identify the proteins present in the secretome. Out of the candidates gathered we aim to confirm if their expression may be as well altered by CITED2.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Mouse embryonic stem cell lines

2.1.1.2 *Cited2*$^{fl/fl}$

Cited2$^{fl/fl}$ are mESC that permit the spatiotemporal conditional KO of *Cited2*. *Cited2*$^{fl/fl}$ mESC derive from the blastocyst of mice *Cited2*$^{fl/fl}$ and were successfully isolated and characterized *in vitro*. Excision of *Cited2* can be achieved by the activity of the Cre recombi nase enzyme. Lastly, the excised region of the exon2 is replaced by a cassette of LacZ reporter that allows the verification of *Cited2* depletion and track cells where it occurred (Figure 10).

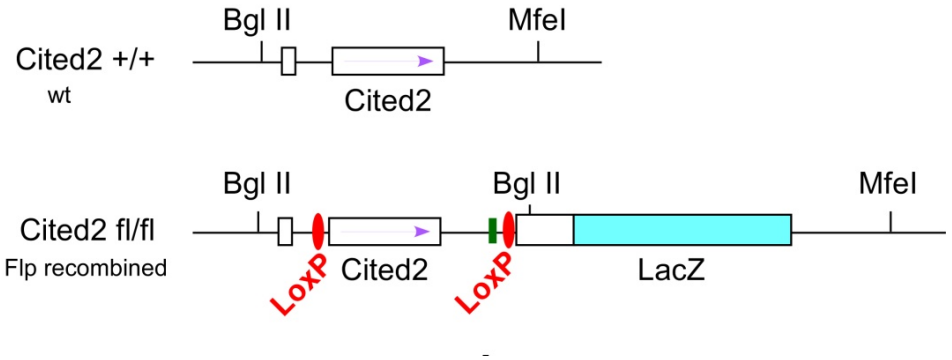


Figure 10 - Cited2 Conditional KO system. The exon2 of the Cited2 is flanked by two LoxP sites. Upon Cited2 excision, the exon is replaced by a LacZ cassette.

2.1.1.2 *Cited2*$^{fl/fl}$ [Cre]

Cited2$^{fl/fl}$ mESC were stably transfected with a plasmid expressing Cre fused to a domain of the Estrogen Receptor containing a ligand binding domain (Cre-ERt). Excision of Cited2 can be triggered by supplementation of 4-Hydroxytamoxifen (4HT), a potent Estrogen antagonist, that causes Cre-ERt to enter the nucleus and excise the exon 2 of Cited2 encompassed by loxP sites.

2.1.1.4 E14/T

E14/T is a mESC line derived from the blastocyst of a mouse strain 12910la that express polyomavirus large T antigen that can be transfected with plasmids carrying a polyomavirus

origin of replication (ORI) at three orders of magnitude greater than DNA transfection efficiencies than other ESC. Provided selection, the transfected plasmid with the polyomavirus ORI is maintained episomal and propagates without the risk of chromosomal the integration (256). These cells were gently given by Austin Smith (University of Cambridge, UK).

2.1.2 Plasmid Vectors

2.1.2.1 pPyCAGIP

The pPyCAGIP, hereafter referred as CAGIP, is an episomal expression vector that harbours a polyoma ORI with the F101 mutation allowing episomal replication in ESC. Complementary DNA (cDNA) may be cloned in place of the stuffer fragment linked to the puromycin resistance gene. The CAGIP backbone was used to generate two different vectors used in this study. A vector harbouring an enhanced green fluorescent protein (eGFP), hereafter termed CAGIP-eGFP and, a vector harbouring the human full-length CITED2 (FC2) fused to a flag peptide at its N-terminal domain, hereafter termed CAGIP-FC2. The pPyCAGIP vector was gently given by Austin Smith (University of Cambridge, UK).

2.2. Methods

2.2.1 Embryonic Stem Cell culture

All ESCs were cultured in Glasgow Minimum Essential Medium (GMEM) BHK-21 (Gibco®, 21710) supplemented with:

- 10% Fetal Bovine Serum (FBS) (Sigma®, F7524),
- 1x L-Glutamine 200mM (Gibco®, 25030),
- 1% Penicillin-Streptomycin (P/S) (Gibco®, 15140),
- 1x Sodium Pyruvate 100mM (Gibco®, 11360),
- 1x Minimum Essential Medium Non-Essential Amino Acids (MEM-NEAA) (Gibco®, 11140),
- 0.05mM 2-Mercaptoethanol (Gibco®, 31350).

Cells were also maintained on plates previously coated with 0.1% Gelatine (Sigma®, G1393), for at least 10min, and kept at 37ºC in a humidified incubator with 5% CO_2. To

maintain the cells under pluripotency conditions 10^3 U/ml LIF (Merck Millipore, ESG1107) was added to the medium.

The cell medium was changed every two days, and cells were split upon 60%-70% confluency. At such confluency, cells were dissociated as follow:

- Washed with phosphate buffered saline (PBS)
- Added Trypsin EDTA (0.25%) (Gibco®, 25200),
- Centrifugated at 800g for 4 min,
- Resuspended in 1mL of medium
- Seeded each plate with $200x10^3$ cells
- Added according (to each plate) amount of medium GMEM supplemented as previously stated

The day after seeding cells, each plate was washed once more with PBS in order to remove cells that were in suspension, and after added the according (to each plate) amount of medium GMEM supplemented. The follow up was done by observing the cells colonies formation and by changing the medium every two days (or whenever required by observing the color of the medium in culture as it changes depending on the pH).

2.2.2 Embryoid Bodies Formation and Cardiac Differentiation

To induce mESC differentiation, we used a hanging-drop method. The differentiation medium used was the same used for the mESC culture, but without LIF. We started by dissociating mESC as follow:

- Washed with phosphate buffered saline (PBS),
- Added Trypsin EDTA (0.25%) (Gibco®, 25200),
- Centrifugated at 800g for 4 min,
- Resuspended in 1mL of medium.

Out of the solution with the cells in suspension we withdrawn $5x10^4$ cells and resuspended in 1mL of differentiation medium. Thereafter we pursued the differentiation protocol as follow:

- **Day 0 of differentiation**
    - Approximately 50 droplets of 20µL were prepared in low adhesion plates, with approximately $1x10^3$ cells/drop
    - Plates were then inverted to ensure the formation of EBs, by gravity force.

- **Day 2 of differentiation**
    - The plates were inverted and supplemented with 5mL of differentiation medium.

- **Day 5 of differentiation**
    - The EBs were transferred to previously 0.1% gelatin-coated plates allowing their adhesion to the plate.

- **Day 7 of differentiation**
    - Observed he first beating foci.

- **Day 8 to 12 of differentiation**
    - Counted the number of EBs that had beating foci,
    - Assessed the average beating foci per EB.

The differentiation medium used on Cited2$^{fl/fl}$ [CRE] was supplemented at D0 with 4HT at a final concentration of 1µM (Sigma H7904) or treated with the same volume of Ethanol (EtOH) used as a vehicle. As referred for the Cited2$^{fl/fl}$ [CRE] cell line, the addition of 4HT induces the excision of *Cited2*, thus enabling to have a knockout condition.

2.2.3 pPyCAGIP-based vectors transfection

All transfections were performed with Lipofectamine® 2000 (Invitrogen, 11668). In brief, $2.5x10^5$ ESC were plated in 0.1% gelatine-coated 6-well plate. On the following day, 1µg of total DNA vectors were transfected per well according to the recommendation of the manufacturer. To sum up, Lipofectamine® 2000 and the DNA vectors were separately mixed with the the GMEM without FBS and, after an incubation period of 5 min at the room

temperature, both mixtures were merged (1:1 ratio). Subsequently, upon 5 min at room temperature, the mixture with both DNA vectors and Lipofectamine® 2000 was added to the cells in culture in the 6-well plate (250 µL per well). After 4 hours, the ESC medium was added to cultures. On the following day, selection antibiotic was supplemented to the medium.

2.2.4 RNA extraction and cDNA synthesis

Total RNA extraction and purification were performed according to the manufacturer's protocol of the RNeasy Mini Kit (Qiagen 74104). Total RNA concentration was measured with Nanodrop2000 (Thermo Scientific). To prepare cDNA from total RNA, 1µg of total RNA was used for reverse transcriptase according to the manufacturer's protocol, using the NZY First-Strand cDNA Synthesis Kit (Nzytech MB125).

2.2.5 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed using SsoFast Evagreen Supermix (BioRad), in a CFX96™ Real-Time PCR detection system (BioRad) and using the CFX Manager™ Software (BioRad). The master mix was prepared as follow:

- 10uL (for each plate well) of Evagreen,
- 0.4uL (for each plate well) of the Forward Primer,
- 0.4uL (for each plate well) of the Reverse Primer,
- 8.2uL (for each plate well) of H2O.

We have chosen to add first 1uL of the respective cDNA to each well of the PCR plate in order to make easier to confirm that all wells had the sample. Afterwards we added the respective master mix to each gene analyzed, in a smooth gentle move. The organization of the PCR plate was as on Figure 11. The respective control for the primers was considered by adding to the qPCR plate one well with only the master mix for each gene analyzed (without the cDNA sample). To make it easier on the plate organization each control was disposed on the last line of the plate on the same column as the gene in consideration (Figure 11). For the purpose of the polymerase reaction control, we took into consideration the respective melting curves for each gene and for each qPCR run. Whenever a melting curve was not sharp as expected (mainly due to primers dimerization), the respective result was not considered. Furthermore, we had

some genes that had a quantification cycle (Cq) that was higher than 38. Whenever that happened, the respective results were discarded.

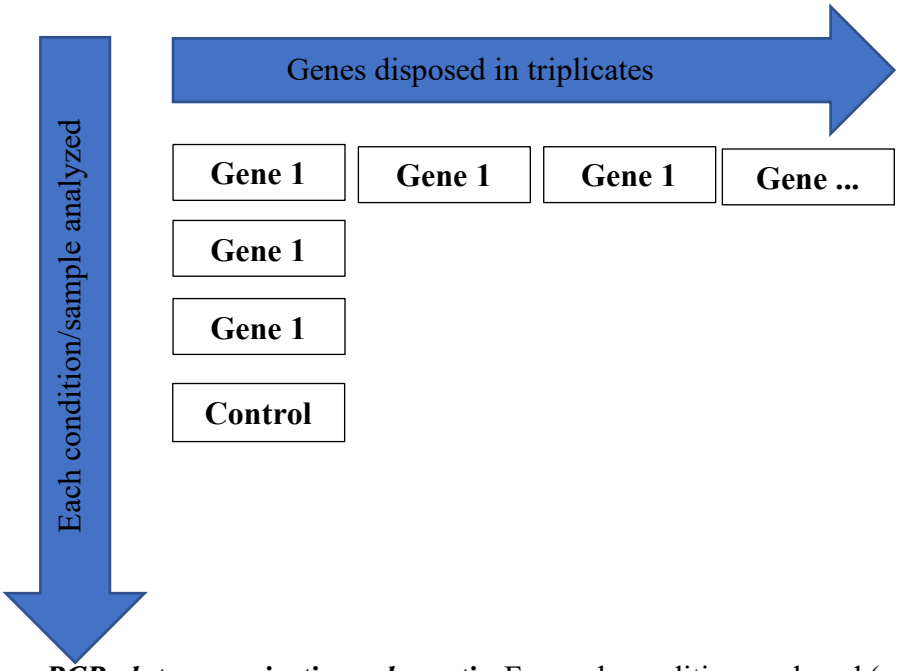


Figure 11 - qPCR plate organization schematic. For each condition analyzed (each line) there was a triplicate for each gene disposed through the columms for each line.

For all primers, we used an annealing temperature of 60ºC (**Figure 12**), and we normalized gene expression levels to the levels of *Gapdh* expression. The remaining qPCR conditions were as showed on **Figure 12**. The list of primers used for qRT-PCR is presented in Table 2.

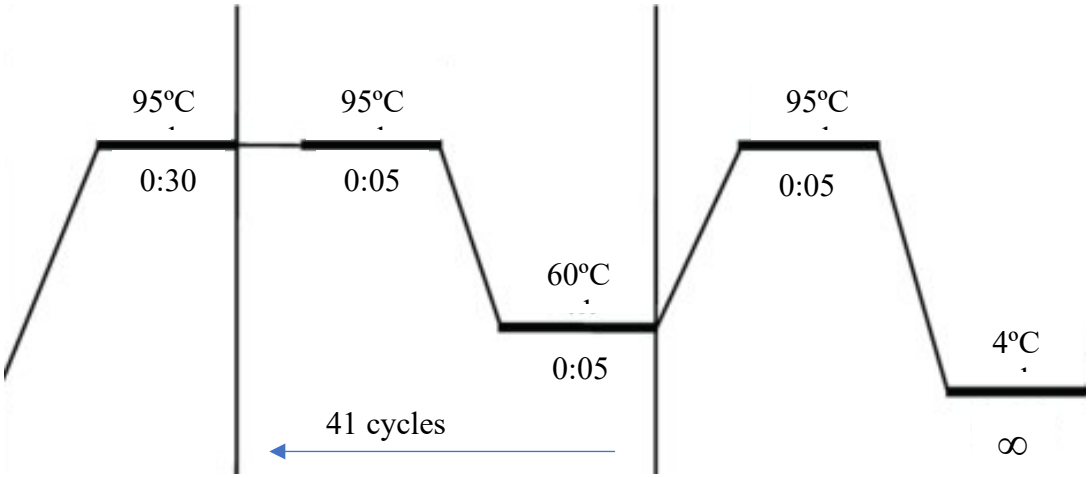


Figure 12 - qPCR conditions including time, temperature and number of cycles.

Table 2 - List of primers used for qPCR

Gene	**Forward Primer**	**Reverse**
Mmrn2	ACT TTC CTG AAC ATG GCT ACT TC	AAC TCA AAC CAC GCT CTC TCA
Igfbp4	AGA AGC CCC TGC GTA CAT TG	TGT CCC CAC GAT CTT CAT CTT
Rbp4	AGT CAA GGA GAA CTT CGA CAA GG	CAG AAA ACT CAG CGA TGA TGT TG
Igf2	CGT GGC ATC GTG GAA GAG T	ACG TCC CTC TCG GAC TTG G
A2m	AGA TGG TGA GAT TTC GTG TTG TC	ACG GTC CTG CCT GAT TCT GTA
Apom	TAA CTC CAT GAA TCA GTG CCC T	CCC GCA ATA AAG TAC CAC AGG
Brachyury	CTC TAA TGT CCT CCC TTG TTG CC	TGC AGA TTG TCT TTG GCT ACT TTG
Gapdh	TCC CAC TCT TCC ACC TTC GAT GC	GGG TCT GGG ATG GAA ATT GTG AGG
Isl1	CTT AAG CAT GCC CTG TAG CTG G	CAG ACA GGA GTC AAA CAC AAT CCC
Nkx2.5	CCA CTC TCT GCT ACC CAC CT	CCA GGT TCA GGA TGT CTT TGA
Nodal	TGG CGT ACA TGT TGA GCC TCT	TGA AAG TCC AGT TCT GTC CGG
xMHC	GAT GGC ACA GAA GAT GCT GA	CTG CCC CTT GGT GAC ATA CT
Cited2	CGC ATC ATC ACC AGC AGC AG	CGC TCG TGG CAT TCA TGT TG
Lefty1	TGG ACA AGG CTG ATG TGG AA	TGG AAG GTT CTG GCT GAA CCT
Lefty2	TAC CCA CGT GAG GTC CCA GTA T	AGG TGA GTG GAG GTC TCT GAC A
Cdx2	CCT GCG ACA AGG GCT TGT TTA G	TCC CGA CTT CCC TTC ACC ATA C
FoxH1	AGA GTT CCC AAG GGG AGG TA	AGA GGT GGT CGG TAG TGT GG
FoxA2	GGC CCA GTC ACG AAC AAA GC	CCC AAA GTC TCC ACT CAG CCT C
Tbx3	TTA TTT CCA GGT CAG GAG ATG GC	GGT CGT TTG AAC CAA GTC CCT C
Pitx2c	CTT GGA GCA CCC AGC AGC	CTG GAA AGT GGC TTC CAG

2.2.5 Conditioned Medium preparation

To prepare the conditioned medium (CM), $3x10^6$ previously transfected E14T/CAGIP or E14T/FC2 ESC were plated on 0.1% gelatine-coated 10cm dish and supplemented with GMEM with LIF. This way cells were kept in stemness conditions and allowed a replication cycle of one day, enabling to have about double the number of cells plated. The following day, cells were washed twice with PBS (Sigma) and 10mL of GMEM without supplements (and without LIF) was added. As such, the cells initiated the differentiation and started releasing to

the extracellular medium the proteins that we aimed to study. Furthermore, the non-supplemented cultured medium allows us to identify only the secreted proteins upon the initiation of the differentiation. This way we were able to collect only the factors released by the cells under culture. After 16 hours, the differentiation was stopped, and the medium was collected and filtered through 0.45 µm in order to withdraw dead cells that could be on the medium. At the onset of differentiation, *Cited2*$^{fl/fl}$[Cre] ESC were treated with half CM and half differentiation medium with 10% total FBS. We thereafter followed the differentiation protocol as normal. Where at day 2, cells were treated with normal differentiation medium for the remaining of differentiation.

2.2.6 Zebrafish Microinjection and Developmental Study

The night before injection, male and female zebrafishes were set up in breeding tanks with dividers in order to share the same water though still remain physically separated. This way, we were able to increase total egg production. For these experiments we have chosen to microinject in the yolk of the zebrafish egg (Figure 13 A and B). As our candidates were secreted proteins we aimed to recreate as such by injecting them in the extracellular area of the egg, the yolk. This way, all cells that gave rise from following cell divisions had access to the microinjected content in the yolk in the same conditions.

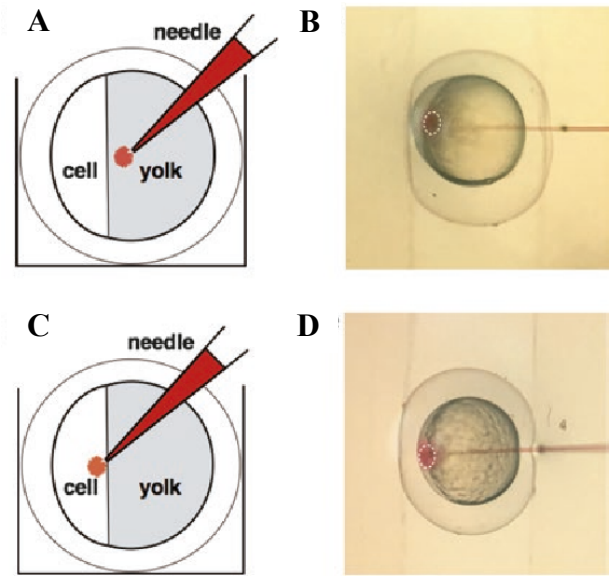


Figure 13 - Schematic of the microinjection. In A) and B) the microinjection has been done in the yolk of the egg whereas in C) and D) the microinjection was in the cell.

To achieve *Cited2* depletion, 1-cell stage zebrafish eggs were injected with 4.6nl of a solution with 5ng of custom anti-*Cited2* Morpholino (MO) designed to bind to the translation staring site (UAG-MO) (5'-CCATCATGCGGTCTACCATTCCC-3') with 3'-Carboxyfluorescein end modification combined with anti-*Cited2* MO designed to the splicing site of the 1st intron (Splicing-MO) (5´-AACTTTGTAACCTTTACCTCTCCGC-3') with 3'-Lissamine end modification (GeneTools) prepared in Danieau's solution. To perform *Cited2* depletion we used Danieau's solution with 5ng of the *Cited2* MO combined (1:1). Whereas to perform the rescue assays, the embryos were co-injected with 5ng of *Cited2* MO combined and 50ng of FGF10 recombinant protein.

Since the viability of the embryos was dependent on the progenitors age, all progenitors used were of similar ages. Moreover, when required to use progenitors of different tanks due to the need of furthermore embryos, controls for each progenitor's tank were taken into consideration. Further controls were not taken into consideration as this model has been previously validated in our lab. With this being said, our lab has previously analyzed if the micro-injection itself was accountable for any effect by micro-injecting with a random oligo solution. Moreover, due to previous experiments, we were confident of the MO's specificity to target *Cited2* sites since when co-injected with CITED2, our lab was able to rescue the MO's induced defects.

Upon micro injection the zebrafish embryos were kept in embryo medium at 28.5ºC with a photoperiod of 14 hours light and 10 hours dark. The plates with the embryos were scattered in piles of two plates to ensure that all plates received the same light intensity. Furthermore, at all-time points that required to assess the embryos outside the controlled environment (light and temperature), all plates were withdrawn and piled in the same workspace and at the same time. The developmental study was pursued as follow:

- **At 6hpf**
    - Assessed the fluorescent embryos. The MO microinjected had a fluorescent tag enabling to select the embryos that were properly microinjected
    - Non-fluorescent embryos were discarded

- **At 24hpf**
    - Assessed the mortality rate and discarded the dead embryos
    - Assessed the developmental delays

- **At 48hpf**
    - Assessed the mortality rate and discarded the dead embryos
    - Filmed 10sec videos of randomly chosen embryos
    - Counted the beating per minute of each embryo filmed
    - Assessed the average heart beating per minute

- **At 72hpf**
    - Assessed the mortality rate and discarded the dead embryos
    - Assessed both heart and other developmental defects

For heart rate determination, the beating per minute (bpm) were counted from 10-second videos of hearts acquired from randomly picked embryos at 48hpf. Live imaging and photography were captured on a Leica MZ 7.5 stereomicroscope (Leica Microsystems). All experiments were conducted in accordance with the regulation of the Directive 2010/63/EU (EU, 2010).

2.2.7 LC-MS

The CM was concentrated using centricons (MRCF0R030, Millipore). This 0.5 mL ultrafiltration centrifugal filter enables the concentration of biomolecules with a membrane NMWL of 30 kDa. These centricons that have a vertical membrane that reduces the concentration polarization for ultra-fast spin times and also has high concentration factors ranging 80 – 100x. We have spined the centricon at 14,000 x g for 20 min each time until all 5 ml of the CM were concentrated. Since the mass of the CM added from each condition (CM-control and CM-FC2) is different, the volumes added to the centricons were measured each time in order to make sure the centricons with the respective CM had the same weight when centrifugated.

After the CM samples were concentrated, they were stored and frozen in a -80ºC freezer. The LC-MS experiment was performed in I3S institute with a sample preparation according to kulak *et al.* 2014 (257). In this regard, it was applied the in-StageTip method since it is able to increase measurements and high reproducibility between replicates (R^2 = 0.97). Therefore, the samples were stored and sent in a recipient filled with dry ice. The LC-MS equipment used was a nanoUHPLC Ultimate Dionex 3000, MS Q-Exactive Orbitrap with nanoESI.

CHAPTER 3

Assessment of FGF10's ability to rescue cardiac defects caused by *Cited2* depletion *in vivo*

In our lab we have identified a wide range of secreted proteins important for the heart development which regulation may be under the influence of CITED2 (195). For example, in microarray experiments performed with extracts from *Cited2*-depleted cells, the expression of *Wnt5a*, *Wnt11* and *Fgf10* was reduced when compared to control cells (195). Moreover, these proteins have been previously described as secreted proteins with an important role in the cardiac development (225,230,255). In this regard, it has also been shown that the conditioned medium derived from *Cited2* overexpression cells (CM-CITED2) increases the number of beating colonies in cell cultures derived from *Cited2*-depleted mESC using EB differentiation protocol (255). Moreover, through a microarray analysis and qPCR, we validated the concomitant expression of *Cited2* with *Wnt5a*, *Wnt11,* and *Fgf10*. Additionally, the CM-CITED2 immunodepletion of these proteins alone impaired the ability of the CM-CITED2 to rescue *Cited2* depletion cardiac defects *in vitro* (255). Further experiments have showed that WNT5a and WNT11 supplementation can also rescue defects triggered by *Cited2* depletion *in vivo*, using a zebrafish model (255). As such, we have assessed the ability of FGF10 to rescue cardiac defects caused by *Cited2* ablation in zebrafish.

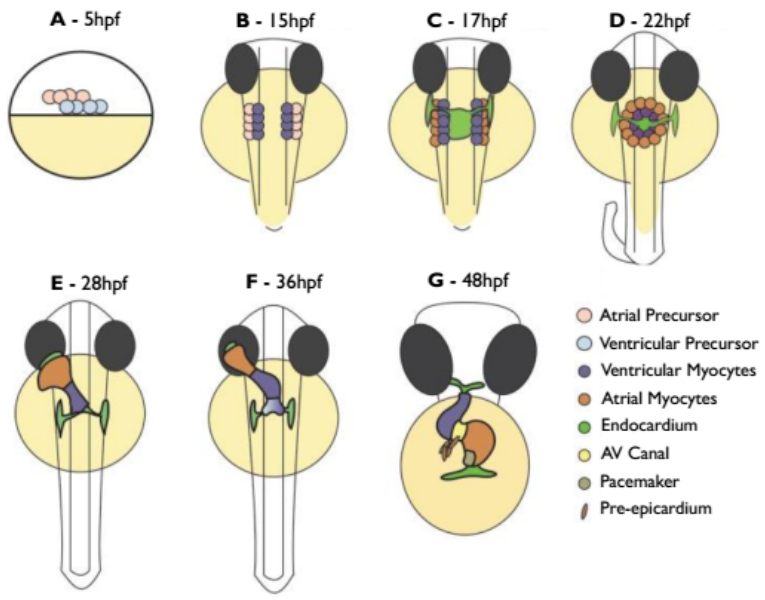


Figure 14 - Schematic representation of heart development in zebrafish. **A**) CPC are identified 5hpf, located in the lateral marginal zone. **B**) At 15hpf cardiac primordia are bilaterally aligned at the lateral plate mesoderm. **C**) and **D**) Endocardial progenitors are established at the midline before migrating towards the midline and fusing to form the primitive heart with ventricular cells at the apex and atrial cells at the base. **E)** The heart starts elongating and the primitive heart tube begins to contract. **F)** The heart tube undergoes looping and the distinct atria and ventricle compartments become evident. **G)** Cardiac maturation occurs, AV valve is formed and the proepicardium adheres to the heart surface (258).

The zebrafish, *Danio rerio*, is approximately 3.5 to 4.5cm in length, and its genes shares 70% homology with human genes (259). Like mammals, zebrafish's heart is the first organ to be fully developed (Figure 14). Interestingly, the zebrafish heart is not only developed within a short time frame (two days post-fertilization - 2dpf), but is also easy to visualize in embryos in culture, allowing easy access and detailed imaging (260). Similar to what happens in mice, Cited2 is expressed throughout zebrafish development. Indeed, Cited2 expression is detected as early as pre-gastrulation (5hpf). At 15hpf its expression is mostly found in the somites and eyes whereas further on (up to 17hpf) it is also detected in the branchial arches and even on the hindbrain by 30hpf. Actually, *Cited2* expression in the eyes, branchial arches and hindbrain, persists until the egg hatching (260).

3.1 Experimental Strategy

We seek to test whether FGF10 recombinant protein rescues impaired cardiogenesis of *Cited2* depleted cells. Though, we were not able to test that hypothesis due to a contamination that altered the phenotype of the cells while they were pluripotent and impaired their ability to differentiate into cells with spontaneous contractibility. Nonetheless, we have tested the same hypothesis in an *in vivo* model, using the zebrafish.

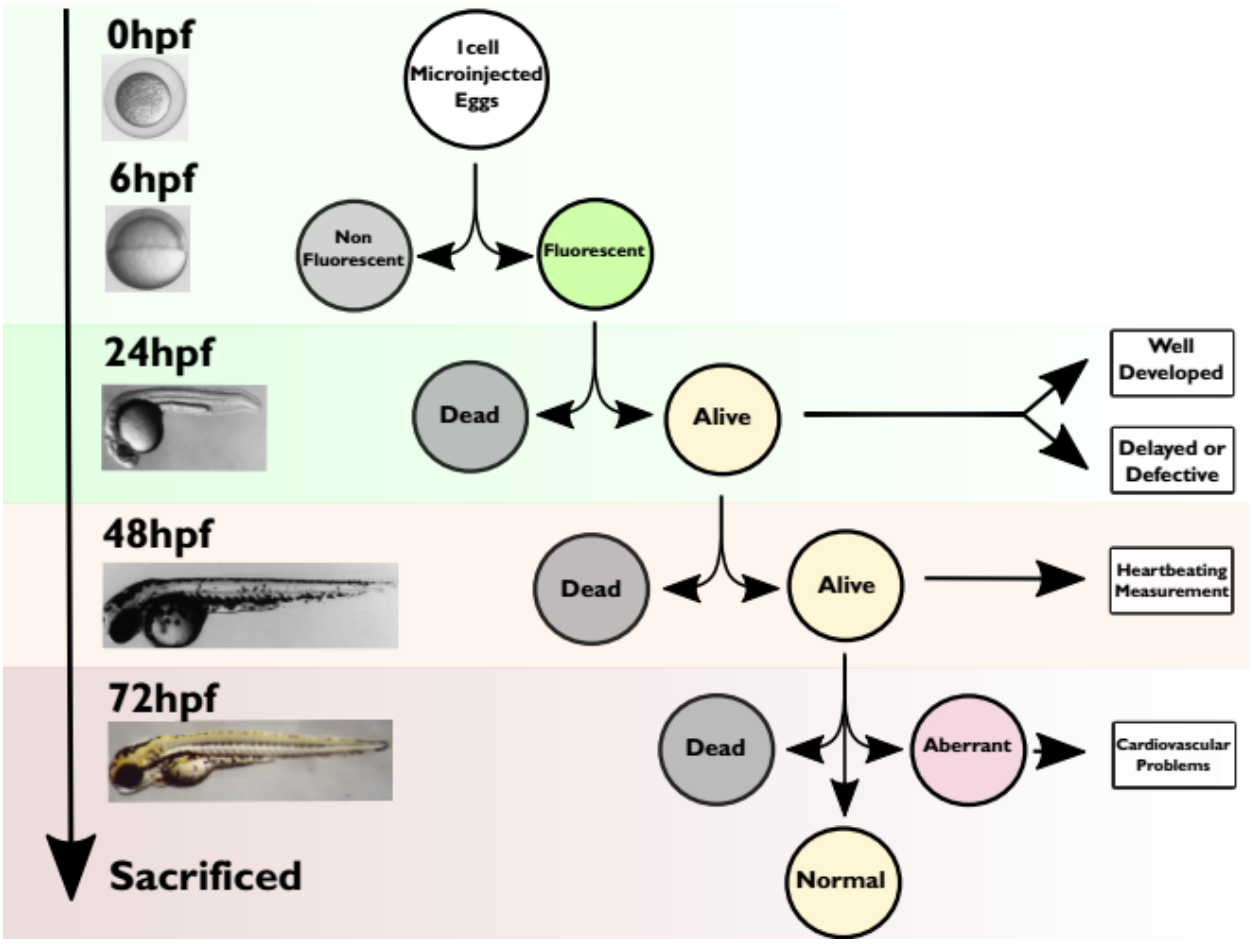


Figure 15 - Schematic representation of the experimental setting. 1-cell stage embryos were injected 15 to minutes after fertilization, and at 6hpf fluorescent embryos were selected. At 24hpf dead embryos were counted and removed and the remaining evaluated if they were developing correctly. At 48hpf dead embryos were counted and removed and heart beating rate was measured. At 72hpf larvae were separated in dead, normal or aberrant. Aberrant larvae were assessed for possible cardiac problems. Larvae were sacrificed at 72hpf. Embryos and larvae photos were obtained from (261).

In order to test this hypothesis, we have microinjected a combination of two morpholinos to target the mRNA of *Cited2*, more precisely one that blocks the translation initiation complex and other that blocks splicing sites. The set design of this experiment is the same as previously used on our lab with WNT5a and WNT11 (255). As such, the morpholinos and zebrafish model (Figure 15) have already been previously validated. Moreover, due to costs and time management, we have not repeated some of the expected controls. With this being said we have chosen only to have a control for the MO induced Cited2 knockdown, by comparing the non-microinjected embryos with the MO-microinjected embryos. This way, for the reasons stated above, several controls were not pursued for this thesis, i.e. we haven't controlled the microinjection itself, neither if the MO may be having some off targets.

With regards to the procedure for the zebrafish model (Figure 15), there were three different sets for the experiment, namely the control (non-microinjection embryos), the Cited2 knockdown embryos (MO microinjected embryos) and the rescue set (combined microinjection of the MO and the FGF10 recombinant protein). The Cited2 knockdown embryos were microinjected with approximately 5ng of the combined morpholinos at 1-cell stage embryo, whereas the rescue set of embryos were microinjected with the same morpholinos combination complementing with FGF10 recombinant protein (50ng/ml). Thereafter, at 6hpf, non-fluorescent embryos were discarded, and embryonic lethality was accessed every 24h where dead embryos were discarded. Moreover, upon 24hpf, embryo's morphology was observed, and developmental delays assessed. At 48pf, zebrafish's heart rate was filmed with 10sec videos and the average heart rate was determined. At 72hpf, the aberrant zebrafishes were analyzed to identify potential cardiovascular defects. All zebrafish larvae were sacrificed at 72hpf.

3.2 FGF10 rescues cardiac defects *in vivo*

To start the experiments, we first assessed if we were able to reproduce the same efficiency of injection as previously established on our lab (255). Both MO have fluorescent tags that can be detected 6hpf allowing us to select the embryos that have been properly microinjected. Indeed, at 6hps we were able to detect 47% and 60% of fluorescent embryos for the one microinjected with the combination of MO and the same combination with the FGF10, respectively (Figure 16A). Non-fluorescent embryos were thereafter discarded.

It has also been shown that at 24hpf, the microinjection of MO induces developmental delays or aberrant phenotype in approximately 60% of the zebrafish embryos (255). In the present experiments, we were not able to detect such drastic induced phenotype. In fact, only 3.6% of the embryos microinjected with both MO had some sort of developmental delay, whereas embryos microinjected with the combination of the MO with the FGF10 had a slight increase ending up with 5.4% of delayed embryos (Figure 16B).

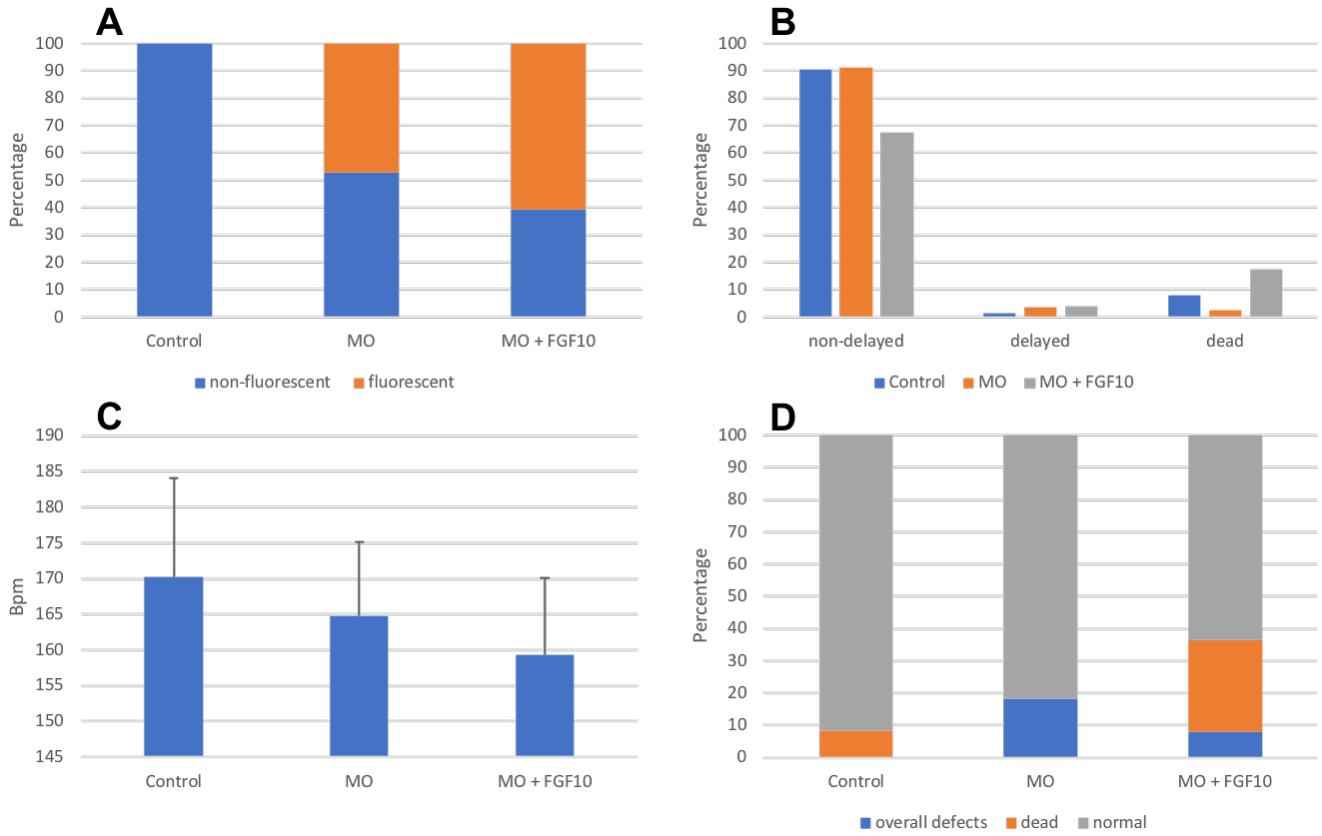


Figure 16 - Cited2 morphants display little disturbance in early developmental. **a** 1 cell-stage egg embryos were co-injected either with morpholinos targeting the transcriptional start site (UAG MO) + the splicing site in the exon 1 (SPL MO) 5ng or the same MO in combination with FGF10 100ng/ml. The fluorescent embryos were determined 6hpf. **b** Percentage of embryos that are non-delayed, delayed or dead at 24hpf after the same co-injection as described in **a**. **c** Average heartbeat at 48hpf of the same conditions as in **a**. 10-second videos of the zebrafish were recorded and average beating per minute (bpm) was determined. All recordings were performed at RT. **d** Percentage of embryos with normal morphology or overall defects as well as the death percentage (cardiovascular or morphology defects) at 72hpf.

At 48hpf the heart of the zebrafish is fully formed though the larvae (or at least most) should still be in the egg. Notwithstanding, in the present experiment, most of our zebrafish embryos that were microinjected (both conditions studied) had already hatched by this time point, in opposition to the control (not microinjected) as well as to the previous experiments ran on our lab (255). Still, embryos microinjected had a rather slow mobility, thereafter we were able to record 10secs videos to assess the average heart beating per minute (Bpm) (Figure 16C). We were able to measure an average of 170bpm for the control. Though, regarding the microinjected embryos with the MO we only had a minor decrease to 165bpm in opposition to the previous results of 120-130bpm (255). Regarding the combination of the MO with the FGF10 the average beating remained similar to the MO condition.

Additionally, we assessed the *Cited2* ablation induced defects at 72hpf (Figure 16D). In this regard, all control embryos showed a normal phenotype. On the other hand, microinjection of both MO, triggered cardiac defects in approximately 20% of the embryos. The co-injection of the MO with FGF10 protein, reduced the defects to 10% of the embryos. Though, we were still not able to detect as much death as on previous results (255). Essentially, we were not able to detect further death after the one assessed at 24hpf.

Since we were not able to replicate the MO induced phenotypes as shown previously (255) we hypothesized that the MO solution was out of validity and could have degraded and, therefore, be less efficient at targeting Cited2 transcripts and even have non-specific targets. To assess this hypothesis, we repeated the experiment but this time with a higher MO concentration (3x-fold more than the previous experiment) (Figure 17). Also, we aimed to determine whether 8R-CITED2 recombinant protein (C2) was able to rescue the defects caused by MO injection. If these delays, defects and death rates observed were not due to *Cited2* depletion, but due to off targets resulted of the degradation do the MO, we should not be able to see a rescue by the microinjection of C2 in combination with the MO (Figure 17).

Interestingly, when we increased the MO concentration by 3x-fold we were able to observe an increase in the delays and defects as well as a decrease of the bpm to closer values in accordance to the previous results on our lab (255).

In terms of efficiency of the microinjection, we were able to have between 60 and 80% of fluorescent embryos (Figure 17A), which was slightly better than what we had on the previous experiment (Figure 16A). Moreover, upon 24hpf (Figure 17B), we were able to observe around 38% of the MO embryos with developmental delays. Though, it is important to point out that the replicates did not have similar percentages. Actually, there was some variability since on one we were able to observe around 30% of delays whereas on the duplicate we observed around 47% of delays. Still, not as high the 60% of MO induced delays previously observed in our lab (255). Nonetheless, it's remarkable that the microinjection of FGF10 caused less delay in proportions to the rescue observed with C2 microinjection. The death rate at this time point, was higher than the control conditions, and we also observed a higher death rate with the FGF10 and C2 conditions.

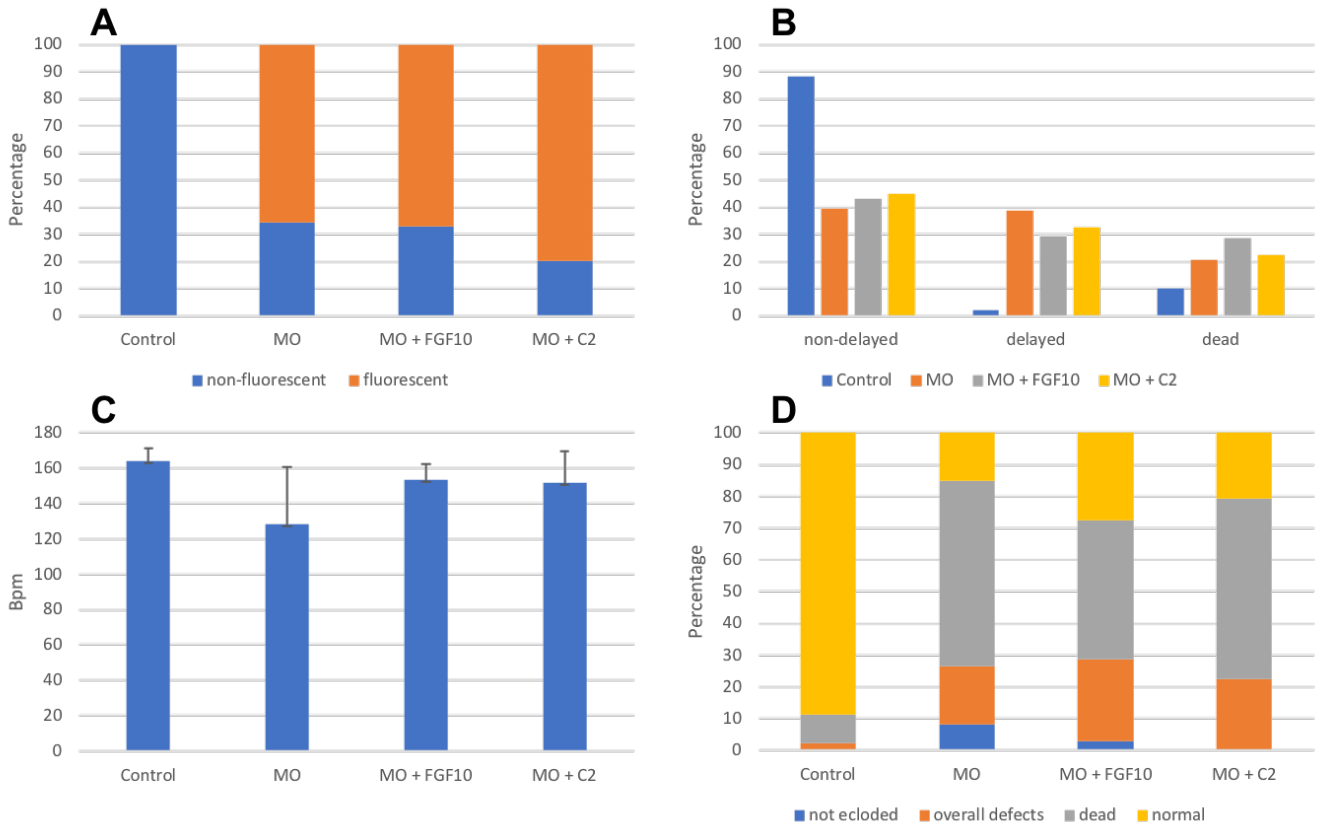


Figure 17 - Early developmental defects of Cited2 morphants are not reverted by FGF10. **a** 1 cell-stage egg embryos were co-injected either with UAG MO + SPL MO with 15ng of an unknown concentration or the MO in combination with FGF10 100ng/ml. The fluorescent embryos were determined 6hpf. **b** Percentage of embryos that are non-delayed, delayed or dead at 24hpf after the same co-injection as described in **a**. **c** Average heartbeat at 48hpf of the same conditions as in **a**. 10-second videos of the zebrafish were recorded and average beating per minute (bpm) was determined. All recordings were performed at RT. **d** Percentage of embryos with normal morphology or overall defects (cardiovascular or morphology defects) at 72hpf, as well as the percentage of the death rate between the selected embryos between 6hpf and 72hpf. All conditions were done in duplicate except for the condition MO + C2.

When assessing bpm's at 48hpf, we not only observed a decrease of the average heart rate of cited2 morphant embryos to 120bpm, but also an increase of this heart rate in FGF10 treated embryos to rates similar to those observed to morphants microinjected with C2 and the control embryos (Figure 1711C). In regard to the average heart beating, though there seems to be a rescue when adding the FGF10 to *Cited2* deficient embryos, the error bars of the MO alone embryos reached the average of the control.

Further on, by 72hpf (Figure 17D), we observed a different phenotype than the one observed on the first experiment (Figure 16D). Indeed, this time we were also able to observe some embryos that hadn't hatched yet. Moreover, it's interesting to point out that although the

condition with the FGF10 had a higher delays percentage at 24hpf, there were more normal embryos as well as less embryos that hadn't ecloded at 72hpf. Though, not reaching the level of control animals. Regarding the C2 condition, we were also not able to observe any significant rescue of either delays or survival.

3.3 Conclusion

It has been previously demonstrated that CM-CITED2 has the ability to rescue cardiac defects caused by *Cited2* depletion. Moreover, recombinant proteins such as WNT5A and WNT11 have been described to be able to exert the same rescue as the whole CM-CITED2. Furthermore, we showed now that FGF10, a candidate gene regulated by *Cited2* gene (255), is not able to fully rescue our MO induced defects. Furthermore, even when increased the MO concentration by 3x-fold, we had a higher impact on the zebrafish morphology and death, though we still weren't able to reach the same rate of defects, delays and death as previously demonstrated on our lab. Additionally, we were not able to rescue the MO induced phenotype co-microinjecting with CITED2 recombinant protein. Taking all this in consideration, there is a likelihood that the MO is indeed degraded and that most morphology defects, delays and death may be caused by off targets resultant of the free arbitrary combination of nucleotides. In agreement with this, if we assume that all defects, delays and death not rescued by CITED2 are accountable for off targets, the results of the FGF10 that resemble to the C2 condition, may be considered as a plausible rescue of Cited2 depletion induced defects. Though, in order to confirm this hypothesis further studies should be carried out and with a new MO oligos solution.

CHAPTER 4

Cited2-induced secretome/proteome analysis

CITED2 plays an important role in cardiac development of the zebrafish, mouse and humans (184, 195). CITED2 is also critical in embryonic stem cells (ESC) for their specification into cardiac cell lineages *in vitro*. Using ESC as a cellular model to study cardiogenesis, we have determined that the secretome of ESC overexpressing CITED2 (CM-CITED2) restores the cardiac differentiation capacity in *Cited2*-depleted cells. Indeed, as shown on Figure 9B, the CM-CITED2, when added to cells *Cited2*-depleted (4HT), upon differentiation, is able to increase the number of beating colonies emerging in culture.

Moreover, we have identified in the CM-CITED2 specific molecules with rescuing properties, such as WNT11 and WNT5a (255). Microarray data obtained in our group also revealed that several signalling molecules were potentially under the control of CITED2. Thus, other secreted molecules than WNT5a and WNT11 with rescuing properties may be also present in CM-CITED2.

4.1 Experimental Strategy

Conditioned medium from E14T CITED2-overexpressing cells (CM-CITED2) and its respective control CM-GFP from E14T cells transfected with a GFP expressing vector (CM-Control) were collected to be further analysed using the Mass Spectrometry (MS) in order to identify differentially expressed secreted proteins. Analysis of the MS results from CM-CITED2 indicated the potential presence of proteins that are not described as secreted proteins in the literature. However, studies of extracellular vesicles and exosomes have reported that these vesicles originating from the cells may contain intracellular proteins that are not normally secreted (198,199). Since we were mostly interested in known secreted proteins, we only pursued with those in the following analysis (Figure 18). From these secreted proteins we have chosen the ones differentially expressed and that are signalling proteins. Finally, we evaluated their expression patterns with the qPCR.

Figure 18 - Experimental strategy for the proteasome analysis of the conditioned medium of Cited2 overexpressing cells.

4.2 Secretomic differences between control and CITED2 overexpressing cells.

To identify the secretomic differences of mESC overexpressing CITED2 (CM-CITED2) and the respective control (CM-Control), conditioned media were collected from undifferentiated ESC and subjected to liquid chromatography coupled to tandem mass spectrometry (LC-MS).

Table 3 - MS data of the proteins with the highest PSM scores. The CM-control proteins PSM scores are on the left and CM-CITED2 PSM scores are on right in bold.

Gene Symbol	Coverage [%]	Peptides	PSMs
Hsp90ab1	64 / **63,5**	49,5 / **44,5**	232 /**187**
Fn1	49 / **46,5**	72,5 / **70**	182 / **161,5**
Ppia	71 / **67,5**	11 / **10**	98 / **73**
Hspg2	23,5 / **17,5**	57,5 / **43,5**	71,5 / **52**
Nid1	45,5 / **42**	36,5 / **34**	61 / **51,5**

In total, over 47 000 and 57 000 potential proteins were identified in CM-CITED2 and CM-control, respectively. Once we selected the proteins of interest to the purpose of this thesis, the ones that are secreted, we were able to refine the list of candidates to 174 proteins on the CM-CITED2 and 181 proteins on the CM-control. When we ordered these proteins according to their MS-PSM's we were able to elaborate a list of proteins that had a much higher PSM than all the others. Though, when filtering them to proteins that had at least 50 PSMs (Table 3) the proteins gathered were mainly the same and the PSMs didn't differ much, relatively, between each condition. As the objective of our work is to identify proteins differentially

expressed in CITED2 overexpressing cells, we didn't pursue with these top PSM's score proteins.

Table 4 - List of candidate genes representative of the proteins that are common to both MS data in regard to the secreted proteins. In green candidates' proteins more represented in CM-CITED2, in orange less represented and in black the ones that do not have differential expression.

List of common candidates to both MasSpec data			
Adam23	Cpb2	Itih4	Pxdn
Afm	Cpxm1	Kng1	Pzp
Aimp1	Cst3	Lama5	Qsox1
Alb	Ctsb	Lamb2	Rgn
Angptl3	Ctsd	Lamc1	Rnase4
Anxa2	Dag1	Lefty1	S100a11
Apoa1bp; Naxe	Dnpep	Lefty2	Scpep1
Apoe	Fbln1	Lgals3	Serpinb1a
Bcam	Fetub	Lifr	Serpinb6a
C1qbp	Fgf4	Loxl2	Serpinc1
C3	Fstl1	Lpl	Serpine1
Calr	Gars	Lum	Serpine2
Cap1	Ggh	Manf	Serpini1
Capg	Gpc3	Mcam	Sfrp1
Cd9	Gpi1	Mdk	Smoc1
Cfl1	Hmgb2	Mfge8	Sod1
Chga	Hnrnpa2b1	Nodal	Sorl1
Clstn1	Hspa5	Pcolce	Stc2
Clu	Htra1	Pdcd6ip	Tcn2
Coch	Hyou1	Pdgfc	Thbs1
Col18a1	Igfals	Pdia3	Thbs4
Col1a1	Igfbp2	Pla2g7	Timp1
Col1a2	Ilf2	Pltp	Trh
Col3a1	Ilf3	Pnp	Txn1
Col5a1	Inhbb	Postn	Vegfc
Copa	Itih1	Ppt1	Ybx1
Cpa1	Itih2	Pros1	

To assist on the analyzes of these candidates list it was used the EnrichR platform (262). Indeed, when we ran all the list on the EnrichR, the cell type that popped up with the highest adjusted p-value was the cardiomyocytes (Table 5).

Table 5 - Cellular association of genes presented in table 4 using EnrichR platform. The genes association was performed using the Human Gene Atlas dataset.

Term	Adjusted P-value	Genes
CardiacMyocytes	1.5986973061722097E-17	COL1A1; POSTN; SERPINE2; COL5A1; STC2; SERPINE1; LAMC1; THBS1; FSTL1; LOXL2
SmoothMuscle	8.00377439469341E-14	COL1A1; POSTN; COL5A1; STC2; SERPINE1; LAMC1; THBS1; FSTL1; LOXL2

Since the purpose of this work was to identify secreted proteins whose regulation may be influenced by CITED2 expression and that may rescue cardiogenic defects caused by Cited2-depletion, we first selected the genes determined to be associated with CardiacMyocytes, using the EnrichR platform. (Table 5). Interestingly, the biological activity of cardiomyocyte-related genes is mostly associated to extracellular matrix (ECM) organization and shaping activity (Table 6).

Table 6 - Biological functions of the 10 genes associated with Cardiomyocytes presented in table determined using EnrichR platform. The gene association was performed by analysis of GO Biological Process 2018 dataset.

Term	Adjusted P-value	Genes
extracellular matrix organization	1.4434190074112908E-8	COL1A1; POSTN; COL5A1; SERPINE1; LAMC1; THBS1; LOXL2
regulation of plasminogen activation	2.750530324884236E-5	SERPINE2; SERPINE1; THBS1
negative regulation of protein processing	6.943761080481358E-5	SERPINE2; SERPINE1; THBS1
collagen fibril organization	4.628857145059027E-4	COL1A1; COL5A1; LOXL2
regulation of blood coagulation	4.0987737121797845E-4	SERPINE2; SERPINE1; THBS1

When further pursuing these candidate's activity regarding the cardiomyocytes list gathered from the first Enrichr data run, we were also able to confirm that they mostly come from the endoplasmic reticulum lumen. Indeed, when choosing the gap Ontologies from EnrichR platform, more precisely the GO Cellular Component 2018, only the endoplasmic reticulum lumen had an adjusted p-value higher than 0.001 (Table 5).

Table 7 - Cellular component of all 10 genes associated to CardiacMyocytes on table 5 assessed with the EnrichR platform. The genes association was done taken into account the GO Cellular Component 2018 dataset.

Term	Adjusted P-value	Genes
endoplasmic reticulum lumen	5.240619083383741E-7	COL1A1; COL5A1; STC2; LAMC1; THBS1; FSTL1

As previously stated, the purpose of this work was not only to find candidates that are secreted proteins associated with of CITED2 expression but also to identify proteins which are secreted modulators of signaling pathways. This comes with high importance since in future experiments we aim to attempt to rescue developmental defects caused by CITED2 dysfunction in animal models. Therefore, we have chosen to put aside the complement and collagen related proteins. Moreover, we have chosen to run just the candidates that were differentially represented in terms of PSM score between CM-CITED2 and CM-Control (Table 4 – green and orange candidates). Using the EnrichR platform to determine to what GO Biological process these genes have been associated, we identified that the genes with the highest adjusted p-value were classified in the extracellular matrix organization category (Table 8). Nonetheless, we were now able to distinguish also genes associated with the regulation of the exocytosis and cellular protein metabolic process.

Table 8 -* GO Biological Process *of all genes, except genes related to complement and collagen family, which are differentially detected in CM-CITED2 and presented in table 4.

Term	Adjusted P-value	Genes
extracelular matrix organization	3.189449405503528E-5	LAMA5; POSTN; LUM; LAMB2; PXDN; DAG1; HTRA1; LAMB1; LAMC1; LOXL2
platelet degranulation	2.879281329670533E-5	ITIH4; ALB; VEGFC; QSOX1; CLU; LEFTY2; SOD1; MANF
regulated exocytosis	7.609222224858054E-5	ITIH4; ALB; VEGFC; QSOX1; CLU; LEFTY2; SOD1; MANF
cellular protein metabolic process	1.1589366153268175E-4	ITIH2; LAMB2; IGFBP2; ALB; QSOX1; LAMB1; LAMC1; IGFALS; APOE; MFGE8; SORL1; LOXL2

Most striking was the fact that when choosing the gap GO Molecular Function 2018, there was no hit with an adjusted p-value higher than 0.001 and therefore not represented here on this thesis. Though, of note that the TGF-β regulation was one of the top hits on this gap and the genes associated to this function, according to the EnrichR platform, were *Lefty1* and *2*, *Inhbb* and *Nodal*.

When evaluating the outcome results from the gap GO Cellular Component 2018, we still had the endoplasmic reticulum lumen as the main hit, nonetheless, using this list of genes we also had a hit on the secretory granule lumen and platelet alpha granule lumen (Table 7).

Table 9 - Cellular component of all genes coloured on table 4, with the exception of the complement and collagen family, assessed with the EnrichR platform. The genes association was done taken into account the GO Cellular Component 2018.

Term	Adjusted P-value	Genes
endoplasmic reticulum lumen	1.7817657014818436E-10	PDIA3; ITIH2; HSPA5; LAMB2; LAMB1; LAMC1; PDGFC; ALB; DAG1; QSOX1; HYOU1; APOE; CALR; MFGE8
secretory granule lumen	1.2602393161214441E-8	CAP1; ITIH4; ANXA2; GGH; VEGFC; CLU; ILF2; PNP; ALB; QSOX1; CTSD; LEFTY2; S100A11
platelet alpha granule lumen	5.523048130494675E-4	ALB; VEGFC; QSOX1; CLU; LEFTY2

Due to their important role in embryonic development (61-65), and the fact that they were detected in the CM-CITED2 secretome more abundantly (Table 4), we assessed by qPCR *Lefty1*, *Lefty2* and *Nodal* transcriptional expression from day 1 (D1) to D5 of differentiation in *Cited2*-depleted cells and respective control (Figure 19). As previously reported (195), we verified that the supplementation of 4HT at the onset of differentiation reduced *Cited2* expression (Figure 19). However, the impact of Cited2-depletion on *Lefty1/2* and *Nodal* expression is erratic during the time course of differentiation (Figure 19). Indeed, all 3 genes were downregulated at D1 and D3. Lefty1/lefty2 and Nodal expression was increased at D2 and D5.

Since the ACTIVIN/NODAL pathway is important for the specification of endoderm and mesoderm embryonic development, we assessed the expression of important for endoderm and mesoderm specification such as FoxA2 and FoxH1 (Figure 19). In this regard, there is an indication that FoxH1 is downregulated in Cited2 depleted cells except at D3, whereas FoxA2 was upregulated at D1 and D5 but downregulated at D2, D3 and D4. We have also analyzed the expression of *Cdx2*, as an important gene in mesoderm and cardiac specification (58,263). Interestingly, *Cdx2* expression was down throughout all time frame. Lastly, we analyzed Pitx2c as one of the NODAL downstream effectors and a Cited2 direct regulate gene (163). Though its expression was mostly downregulated, it had a peak of expression at D3 and not at the same time point as Nodal (D5).

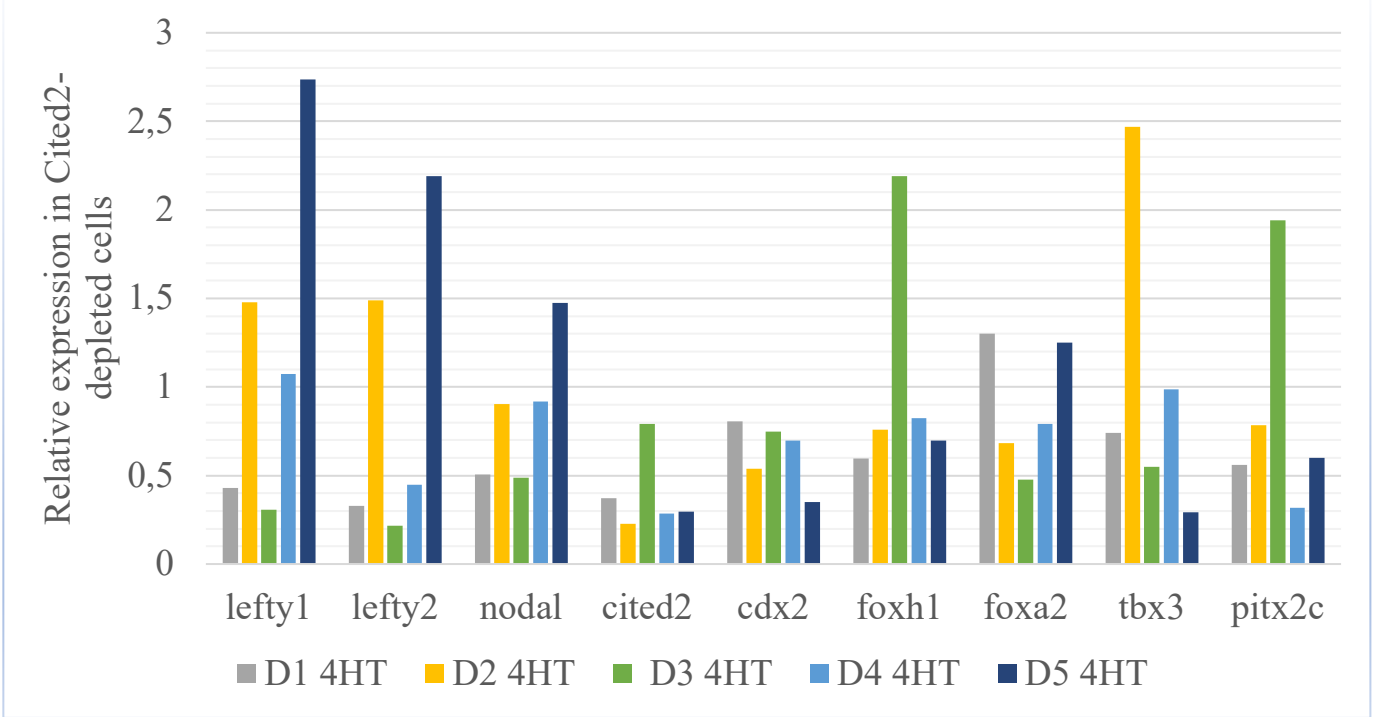


Figure 19 - Relative expression of the indicated genes encoding secreted proteins as well as Cited2 and markers representative of different time points important to the embryonic differentiation (Cdx2, FoxH1, FoxA2, Tbx3 and Pitx2c). The expression was determined by qPCR from day 1 to day 5 of differentiation in cultures derived from $C2^{fl/fl}$[Cre] ESC with ethanol or 4HT at the onset differentiation.

In order to compare the results presented in Figure 19 with data sets reported by other research groups, we assessed the expression patterns using the HeartExpress platform (

Figure **20**). The HeartExpress platform is an interface that allows the query and visualization of the expression of genes during *in vitro* and *in vivo* cardiogenesis, based on the comparison of previously published datasets. Interestingly, both *Lefty1, Lefty 2* and *Nodal* expressions are high between D0 and D2 and dramatically reduced in the following days. Regarding *Cdx2*, in opposition to our results, it was expected to increase its expression from D3 onwards. According to HeartExpress data, FoxH1 was expected to have a peak of expression by D3 (

Figure **20**). Our *Cited2*-depleted cells had a higher relative expression pattern of *FoxA2* (Figure 19) and in opposition, this gene predictable expression pattern was decreased (

Figure **20**). Moreover, although we did not observe a peak of expression on D3 as on Figure 19, *FoxA2* relative expression went up from D3 to D5, reaching a point of overexpression at D5. Finally, the days of the peaks of expression observed on our *Cited2*-depleted cells for the *Tbx3* and *Pitx2* do not match to the predictable peaks of expression

according to the HeartExpress. Though, these results are only indicative as they do not have replicates.

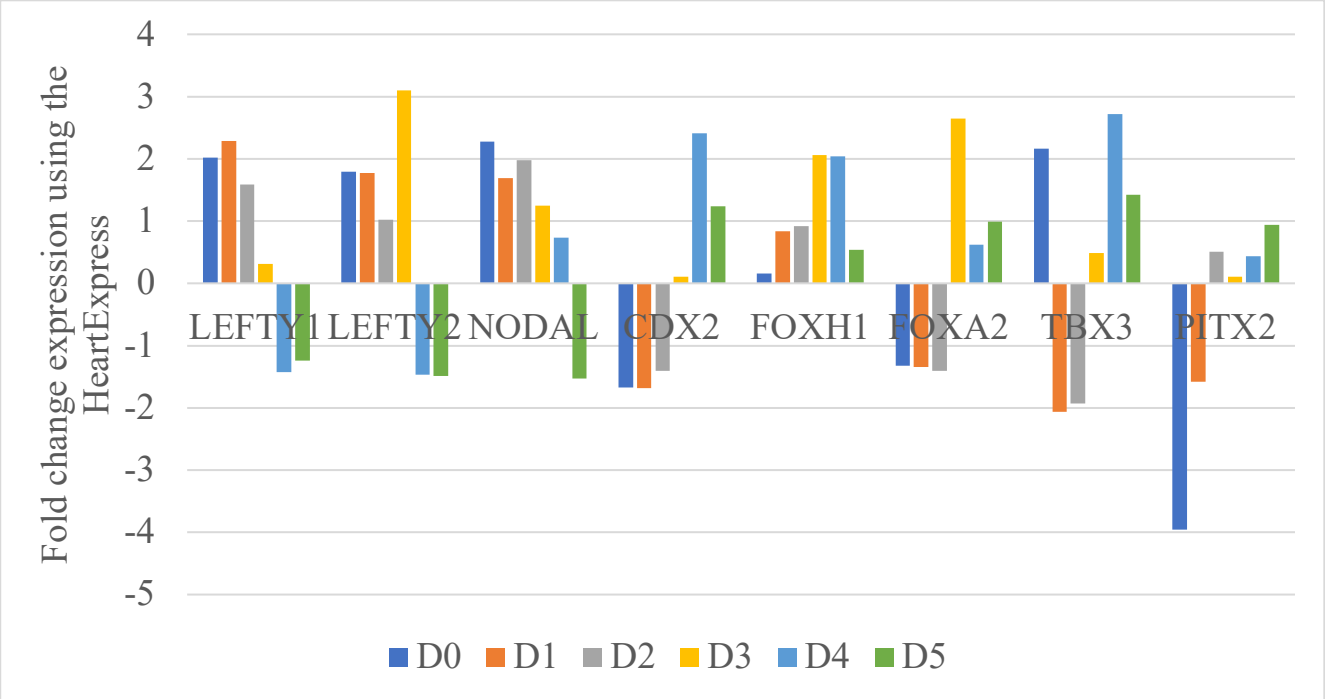


Figure 20 - Fold change expression of the indicated genes encoding secreted proteins as on Figure 19. The expression data was obtained through a meta-analysis using the HeartExpress platform.

In our MS analysis, we have also identified less represented peptides in terms of PSM score. However, some of these proteins were either specifically identified in CM-Control or CM-CITED2 media. These condition-specific proteins were mostly identified in the first MS experiment.

The expression profile of these proteins was also assessed using the HeartExpress platform. Interestingly, the expression of genes encoding for the set of proteins increased in CM-CITED2 medium displayed an expression which was expected to be low at the early steps of differentiation (Day 0 to Days 3-4) and increased by Days 4-5 (

Figure **20**). On the other hand, the genes encoding for proteins specific for CM-Control are abundantly expressed in undifferentiated ESC and the early events of cardiac differentiation (Figure 21).

Therefore, the overexpression of CITED2 in ESC may promote an early expression and secretion of proteins which are important for normal cardiogenesis at later stages. A similar effect was previously described on the expression of pro-cardiogenic genes which are transcription factors such as *Isl1, Gata4, Tbx5* (230), and secreted proteins such as Wnt5a and

Wnt11 (255) in mouse ESC overexpressing *Cited2*. Thus, the overexpression of *Cited2* in mouse ESC triggers the early expression of pro-cardiogenic genes which are only normally

expressed in differentiated cells and may prime cardiac cells differentiation from ESC.

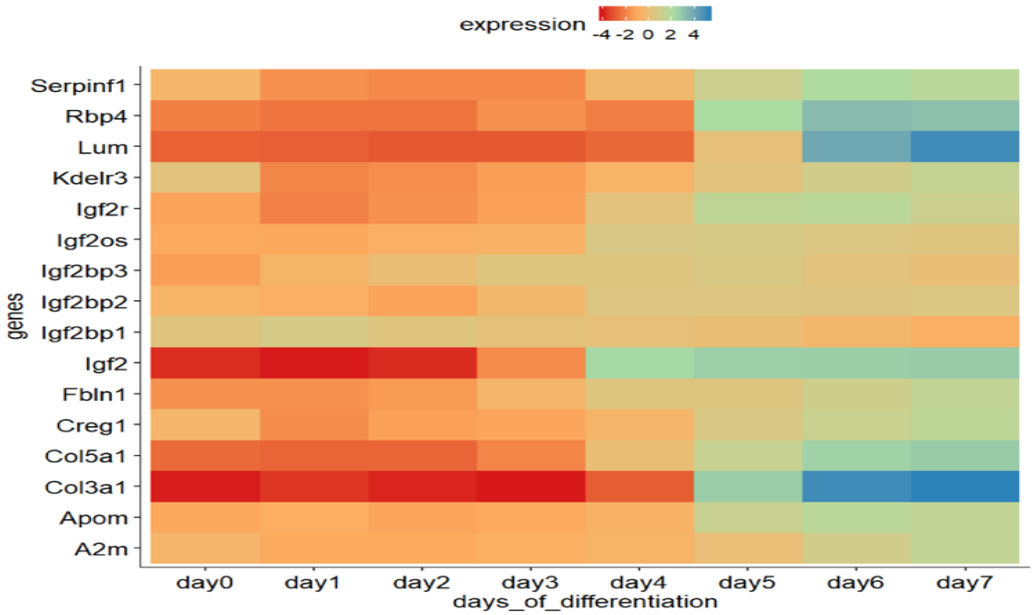


Figure 21 - Heatmap of the time course transcripts of selected proteins identified by MS Analysis (CM-control proteins on the top panel and CM-CITED2 proteins on the bottom panel). The expression data was obtained through a meta-analysis using the HeartExpress.

In addition, we analysed the expression of genes uniquely expressed in each condition. When focusing the attention to these specific genes, we were able to see that on the CM-Control, the *Mmrn2* and *Mmp11* were expected to be expressed at the onset of differentiation but not the *Igfbp4*, which was only expected afterward. The reason that we were able to detect IGFBP4 may be due to the fact that we used different cells than the ones used on HeartExpress platform. Moreover, this platform is based only on a limited number of article experiments. Though, it's interesting to observe on Figure 22 (on the top) that most of the gene's expression goes down by D1 to later on increase their expression in a similar way to *Cited2*. Even more interesting is when addressing the genes specific to the CM-CITED2 (Figure 22, on the bottom), where none of the genes were to be expected to be expressed before day 3 of the differentiation.

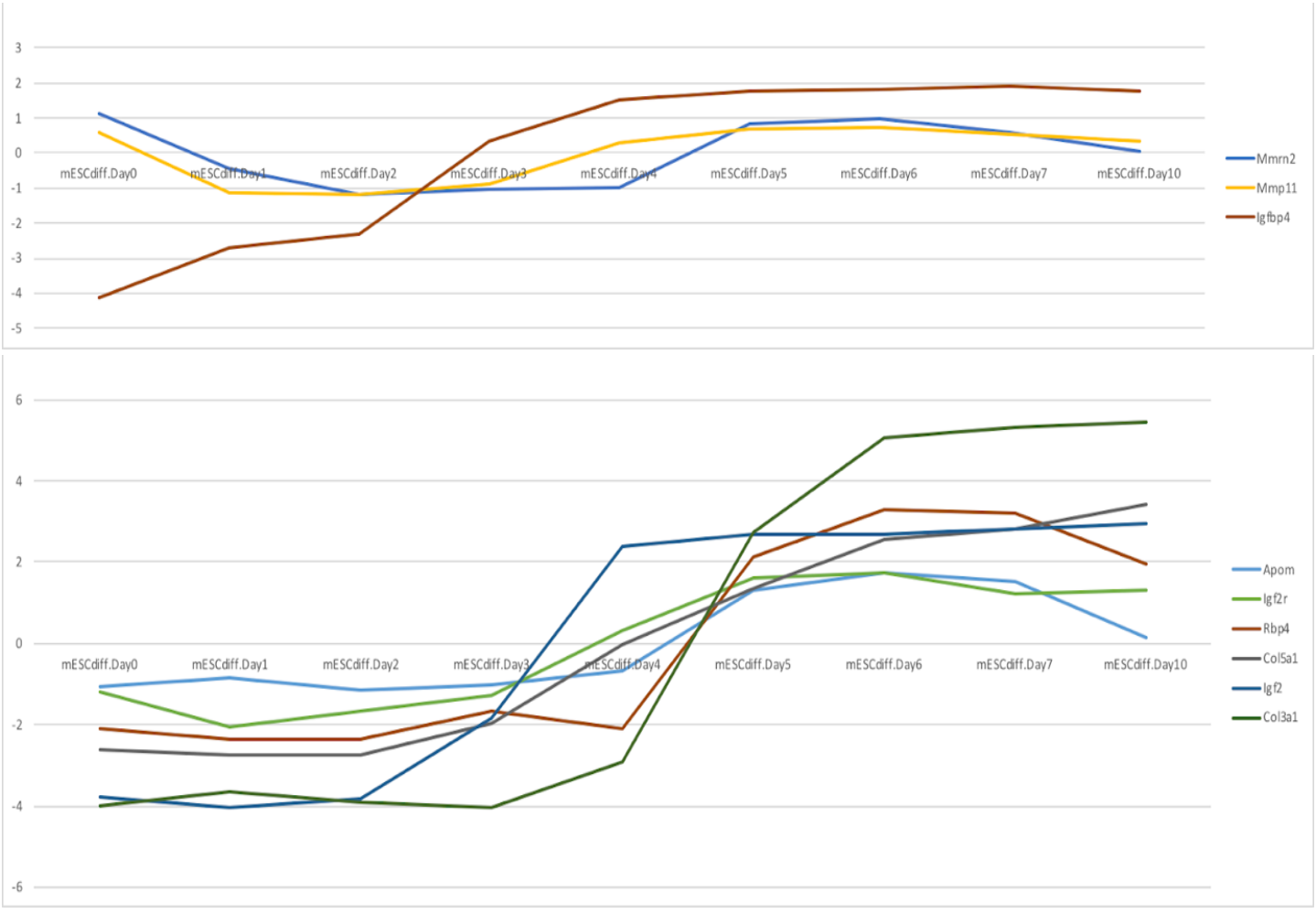


Figure 22 - Time course of transcripts expression of genes identified by MS Analysis unique to CM-Control (top) or to the CM-Cited2 (bottom). The expression data was obtained through a meta-analysis using the HeartExpress platform.

To confirm the expression of these genes we evaluated the gene expression patterns through qPCR in undifferentiated ESC (at day 0) and Day2 (Figure 23), we prioritized once more signaling molecules and discarded genes such as *Col5a1* and *Col3a1* which do not belong to this category. Moreover, we have chosen to add another candidate that was not on the list, the A2M. Although A2M is not one of the proteins with the highest PSM, it was on the top proteins that were detected in the CM-CITED2 in the first MS set of results.

First, we wanted to confirm whether the expression of transcripts encoding for these genes which are enriched in conditioned media, were affected by *Cited2* expression using the qPCR. For this purpose, we analyzed the gene expression of *Cited2* overexpressing cells 16h after the onset of differentiation (Figure 23 – H16). In this regard, as observed on Figure 23, we were able to perceive that the CM-CITED2 candidates (*Rbp4*, *Igf2*, *A2M* and *Apom*) were upregulated on *Cited2* overexpressing cells. Unexpectedly, one of the CM-control candidates, the *Mmrn2*, was also upregulated on these *Cited2* overexpressing cells whereas the other candidate, the *Igfbp4*, was under expressed as anticipated. Another interesting feature observed was that the *Brachyury* was downregulated whereas other cardiac mesoderm and cardiac markers such as *Isl1*, α*MHC* and *NKX2.5* were upregulated.

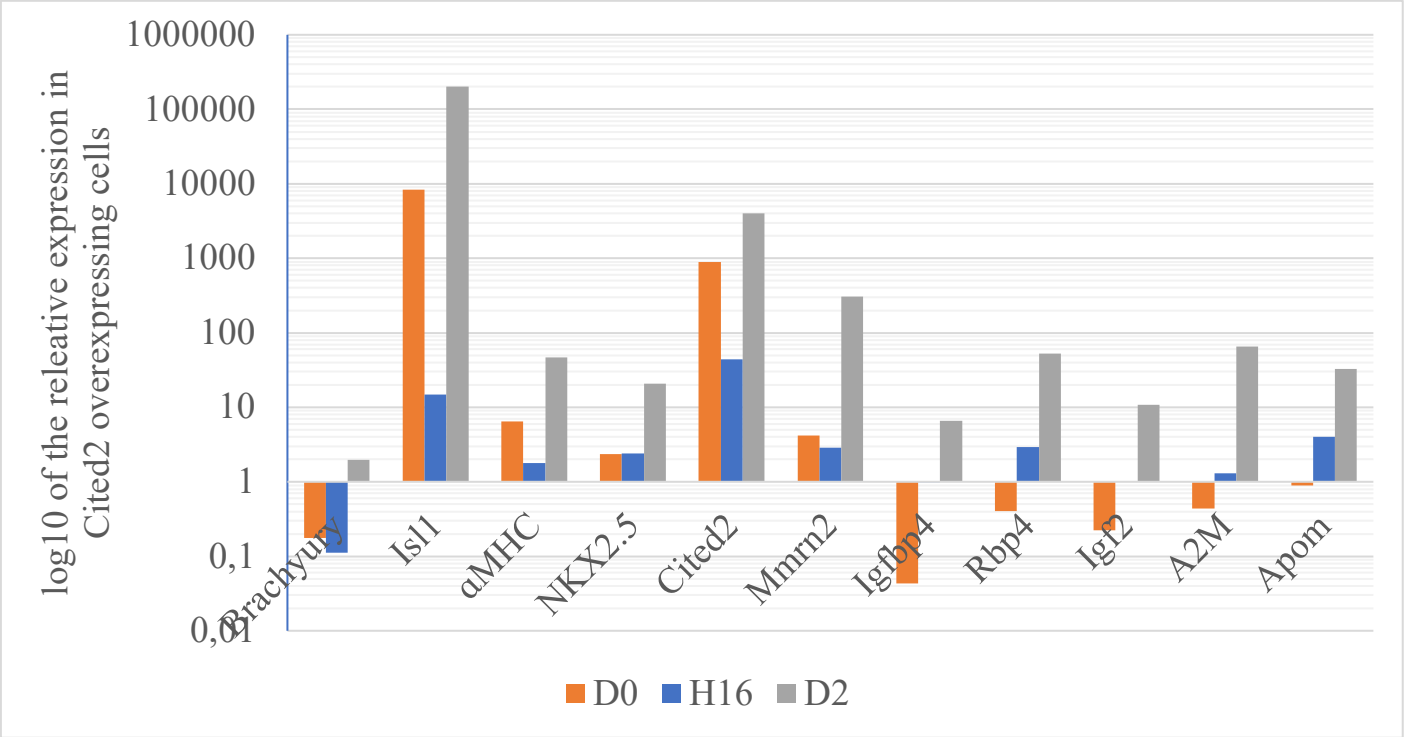


Figure 23 - Relative expression of the indicated genes encoding secreted proteins selected from the CM-control (Mmrn2 and Igfbp4), from the CM-CITED2 (Rbp4, Igf2, A2M and Apom) as well as Cited2 and markers representative of different time points important to the cardiac differentiation (Brachyury, Isl1, αMHC, NKX2.5). The expression was determined by qPCR in E14/T ESC transfected with a plasmid expressing flag-tagged CITED2 or the control empty plasmid. Experimental timeline was at day 0 (ESC), H16 (16h after initiation of the differentiation protocol, equivalent to the time point used for the LC-MS CM analysis) and at day 2.

As one of the key moments of the *Cited2* expression patterns is its downregulation from D0 to D2, we were also interested in finding the expression patterns of our candidates at the onset of differentiation (D0) and at the second day of differentiation (D2) using *Cited2* overexpressing cells. Interestingly, the transcripts encoding for proteins potentially enriched in CM-CITED2 were all downregulated at D0 in cells overexpressing *Cited2* compared to control cells. Interestingly, these genes showed an increased expression in the subsequent days of differentiation. Remarkably, *Igfbp4*, as one of the CM-control candidates, had a rather lower expression at D0 but as the differentiation kept going on, its expression started to go up. In addition, the other CM-control related candidate, Mmrn2, was upregulated at all time points evaluated. Though, these results were not reproducible in a subsequent experiment, *Cited2* expression was increased in both sets of cells transfected with the *Cited2*-overexpressing vector (Figure 23, Figure 24). However, *Igf2* remained downregulated at both time points evaluated (D0 and D2) and the remaining candidates did not have an expression differential as striking

as on the first qPCR (Figure 24). The CM-control candidates had differed on their expression arrangements as well. *Mmrn2* expression went down to a point of being downregulated by D2, opposing the result obtained on the first qPCR (Figure 23), whereas Igfbp4, although not at the same level of expression, still went from downregulated at D0 to upregulated at D2. Also, noticeable, the fact that both *Isl1* and *NKX2.5* were downregulated by D0 though they had later on increased their expression to a point of being upregulated by D2.

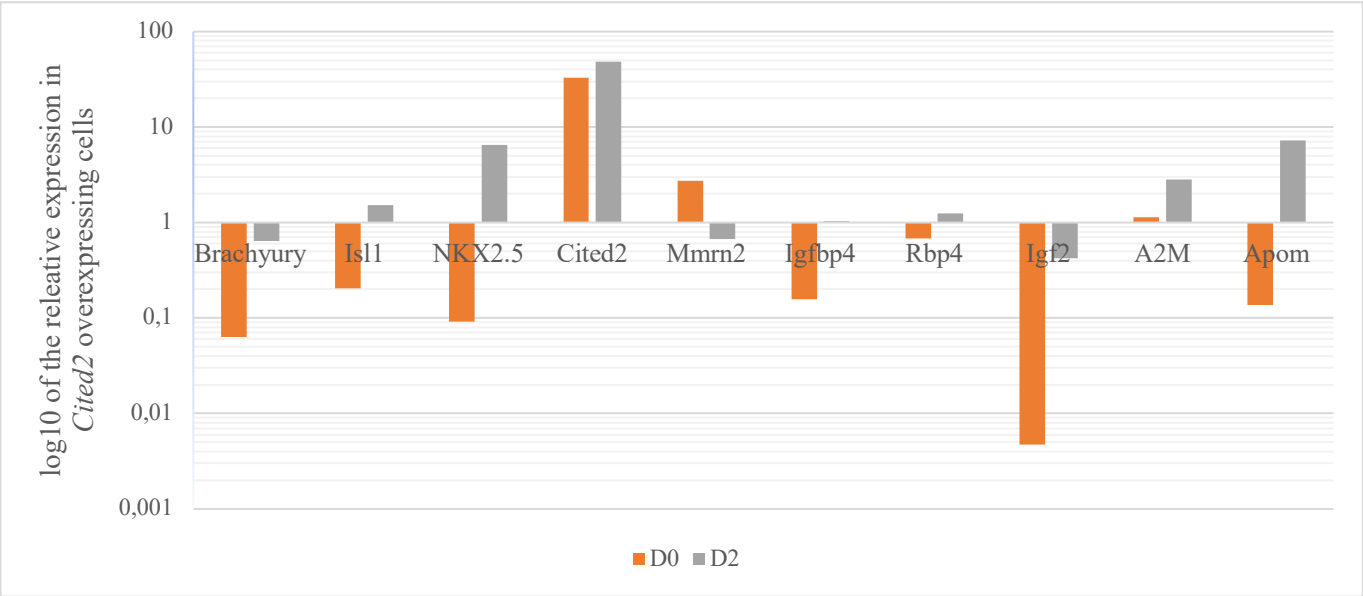


Figure 24 - Relative expression of the indicated genes encoding secreted proteins selected from the CM-control (Mmrn2 and Igfbp4), from the CM-CITED2 (Rbp4, Igf2, A2M and Apom) as well as Cited2 and markers representative of different time points important to the cardiac differentiation (Brachyury, Isl1, □MHC, NKX2.5). The expression was determined by qPCR in E14/T ESC transfected with a plasmid expressing flag-tagged CITED2 or the control empty plasmid. Experimental timeline was at day 0 (ESC analysis) and at day 2.

Thus, the results are inconclusive and need to be repeated. Nevertheless, we assessed how the expression of the candidate's genes was affected upon *Cited2*-depletion, using a line of cells which allow a conditional knockout of *Cited2* (C2KO). As expected, a decrease of *Cited2* expression was observed in cells treated with 4HT to excise *Cited2* gene (Figure 25). If these genes were to be dependent on CITED2 function, either directly or indirectly, we would expect them to be downregulated in *Cited2*-depleted cells. Indisputably, we were able to observe that candidate genes tested, except *Rbp4*, were downregulated in *Cited2*-depleted cells (Figure 25). Interestingly, when looking to the CM-control results (Figure 25), *Igfb4* was slightly downregulated by D2 which is in accordance to the fact that this gene is overexpressed at D2 when using *Cited2* overexpressing cells (Figure 23, **Figure** 24) even though it was estimated it should be upregulated according to the MS analysis. On the other hand, *Mmrn2* is

upregulated in *Cited2*-depleted conditions as expected by the results obtained by MS since we were only able to detect the protein in CM-control. Also, important to point out that *Brachyury* is upregulated and, though *Isl1* is also upregulated, *NKX2.5* is downregulated (Figure 25).

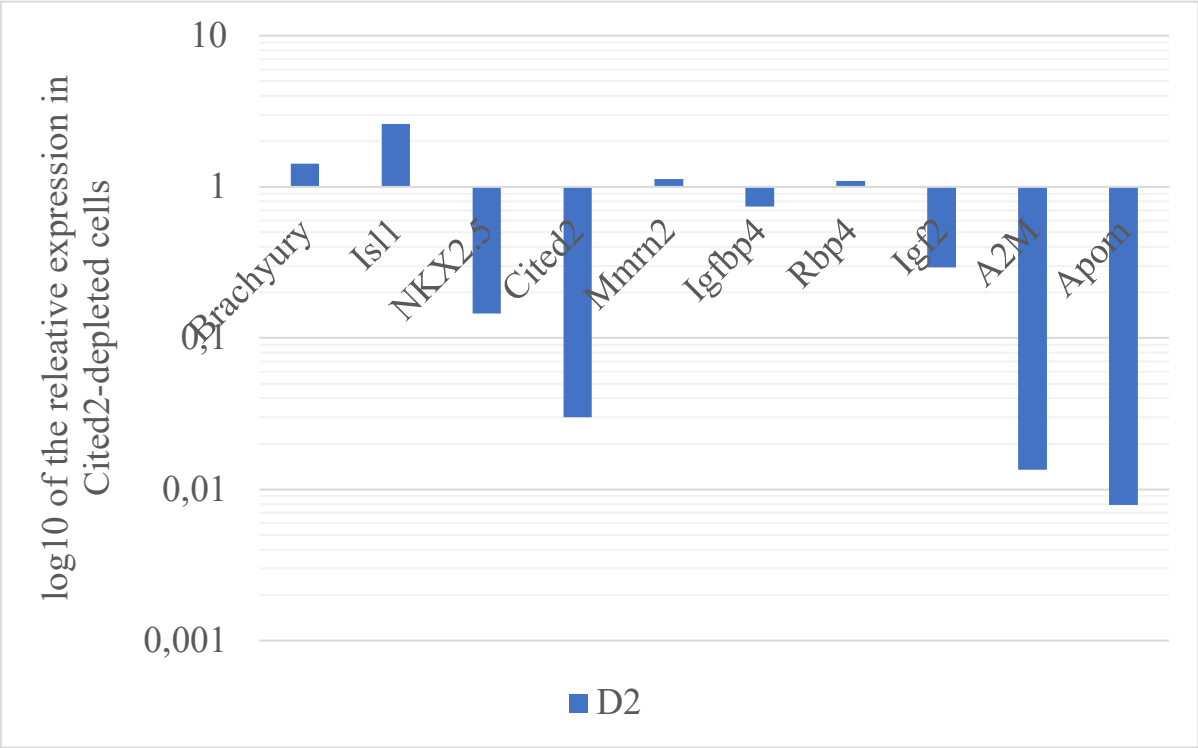


Figure 25 - Relative expression of the indicated genes encoding secreted proteins selected from the CM-control (Mmrn2 and Igfbp4), from the CM-CITED2 (Rbp4, Igf2, A2M and Apom) as well as Cited2 and markers representative of different time points important to the cardiac differentiation (Brachyury, Isl1, □MHC, NKX2.5). The expression was determined by qPCR at day 2 of differentiation in cultures derived from $C2^{fl/fl}$[Cre] ESC with ethanol or 4HT at the onset differentiation.

4.4 Conclusion

We have used Mass Spectrometry analysis to identify secreted proteins present in CM-CITED2 and CM-Control. Some of the secreted proteins that were differentially expressed have been associated with an expression in cardiomyocytes. Though most of these proteins were mostly responsible for extracellular matrix organization. Furthermore, there is an indication that CITED2 overexpression may be able to alter the gene expression patterns of the *Lefty1* and *-2*, nodal and of markers for the endoderm (FoxA2) and mesoderm (FoxH1).

Interestingly, we identified proteins that were either specifically expressed in CM-Control or CM-CITED2. Moreover, and the analysis if the transcripts encoding for these

proteins either by qPCR or using the HeartExpress platform, suggested that these proteins are expressed during ESC differentiation at critical cardiogenic steps. Overall, this work demonstrates that overexpression of CITED2 may promote the early onset expression of genes normally expressed in cardiac differentiated cells.

CHAPTER 6

Conclusion, discussion and future perspectives

The importance of CITED2 in heart development and CHD has been previously established but its role and mechanisms are still largely unknown. In our laboratory, we used mESC as a model system to study cardiogenesis, as ESC can differentiate into all cells that comprise the heart with molecular mechanisms comparable to developmental process.

Previously, we have demonstrated that the overexpression of *Cited2* in undifferentiated mESC stimulates the expression and secretion of cardiopoietic proteins. Moreover, this induced secretome seems to be able to rescue cardiogenic defects caused by the depletion of *Cited2* in mESC. Our lab showed that the rescue of the cardiogenic defects caused by *Cited2*-depletion was in part due to the increased secretion of WNT5a and WNT11 proteins (255). Preliminary results obtained during this study revealed that FGF10 may also be involved in the rescuing process *in vitro*.

Interestingly, it was previously showed in our lab, that if FGF10 was immunodepleted from the CM-CITED2 not only the rescue was not happening anymore, but the residual cardiogenic capacity of *Cited2*-deppleted cells was drastically decreased (JB unpublished data). Furthermore, FGF10 has been showed to promote cardiomyocytes differentiation (264,265). Here, we attempted to rescue the developmental defects caused by *Cited2* depletion in the zebrafish embryos with the supplementation of FGF10.

Even though, we were not able to have a rescue with the FGF10 in our very preliminary experiments, there are some indications of a potential positive effect. For instances, the MO alone condition wasn't able to induce as much delays, defective fishes and death as it should. The MOs used in this experiment were ordered more than a year ago, and although they were kept at -80ºC free from freezing-thawing cycles, it is known that MOs easily degrade over time. Thus, observing the poor effects on zebrafish embryos development after MO injection, we thought that the MOs could have been degraded. Since we had not enough time to order new MOs and perform more experiments, we decided to increase the concentration of the MOs micro injected, even though we were aware that degraded MOs may have some non-specific off targets and side effects. Indeed, the increase of the concentration of MOs micro injected resulted in the increase of developmental defects as expected.

To assess whether the developmental defects observed upon micro injection of the MOs were accountable of Cited2 depletion, a set of experiments was made by micro injecting MOs in combination with a bacterially produced recombinant 8R-CITED2 protein fused to 8 arginines at its N-terminal domain (195). If the defects observed by Cited2-MOs micro injection were specific to Cited2-depletion, we expected to have a reversion of the defects by providing 8R-CITED2 to the embryos. However, 8R-CITED2 showed a poor capacity to

rescue the defects suggesting there may be off-target effects in our conditions (or 8R-CITED2 is itself degraded). For this reason, if we assume that the defects, delays and death percentage that were residually rescued by CITED2 supplementation, is the percentage accountable for Cited2 depletion, we are able to observe a partial rescue effect when adding the FGF10. Though, all or most of the MOs induced phenotypes are most likely due to the off targets of the short oligos randomly formed out of the degradation. For instances, some of the off-targets may me potentiating the FGF10 effect and, thus, the effect observed being just by chance.

As previously stated, one of the goals of this thesis was to find candidate proteins with the potential to rescue cardiac defects induced by *Cited2* depletion. Although, the microarrays are an outstanding technology, they reflect the transcriptional expression levels of the genes and, not the protein levels. For this reason, we thought that the secretomics analysis would provide a good insight on the impact of CITED2 in the composition of proteins that are secreted. These proteins are of most interest as they not only modulate the ECM but also enable the cross-talk between other cells (132,133). As such, these secreted proteins play a scope of important roles in the biological stimuli response and homeostasis of the body (142).

Of notice, during this work, we did not use protocols to perform a directed cardiomyocyte differentiation. Indeed, we tried to mimic as best as we could the embryonic development *in vitro*, though with a focus on the cardiogenesis which is best achieved by EB formation. This detail is rather important as during the development there are many cells types that will secrete and induce changes on other cells and this may, thereafter, be important to the cardiomyocyte cell fate. For instances, the CNCCs, though they are not a cardiac cell type, they are very important to the cardiac development. Indeed, CNCC ablated chick embryos keep the SHF progenitors in a proliferative state, delaying their proper migration and myocardial differentiation (3,28,81). Furthermore, though the mechanism is still not well understood, the CNCCs contribute to the development of the conduction system and cardiac valves (266). Indeed, some of the novel human trials with cell-based therapies are being carried out not with a single type of cells but with combined CPCs, ECs, SMC and cardiac fibroblasts (129).

MS-based proteomics is nowadays a current and indispensable method to analyze a broad number of proteins of complex samples such as the secretome. As such, for the purpose of finding new candidates that are secreted in a CITED2-dependent manner we have used this technology with the following specifications: low-resolution linear ion trap coupled with an Orbitrap mass analyzer (197,198). For instances, this method allows to analyze the whole proteomics present on the secretome. Nonetheless, for the MS analysis we ended up using a protein concentration kit which may have withheld some proteins on the column. Moreover,

on the MS there were many proteins other than secreted proteins, with much higher detection hits (PSMs), that may have obscured other proteins at lower concentration rates. Although this effect could be diminished if we have pursued single protein expression, the purpose of this analyzes was the knowledge of the whole proteomics expression patterns on the secretome.

Interestingly, in our CM-CITED2 the LC-MS detected over 47 000 proteins whereas on the CM-control over 57 000 proteins were spotted. This largely differs from the 4 832 proteins identified when Wolling *et al.*, 2018 (142) differentiated human pluripotent stem cells (hPSCs) into cardiomyocytes *in vitro*. However, Wolling *et al.*, 2018 used a protocol specific to cardiomyogenic differentiation and hPSCs whereas we used mESC. Out of all proteins identified, we selected the ones that were either more or less represented in terms of PSM's.

To assist on the analyzes of these candidates list it was used the EnrichR platform. Indeed, when we ran all the list on the EnrichR, the cell type that popped up with the highest adjusted p-value was the cardiomyocytes. Though, when we ran the list of proteins that were allegedly associated to the cardiomyocytes, most were related to the extracellular matrix organization and original from the endoplasmic reticulum lumen. These candidates would not attend this thesis purpose since we wanted to identify candidates that were not only dependent on CITED2 but that were also signaling proteins. For this reason, we pursued this analysis taking out the complement, collagen and serpine family candidates. Even after we withdrawn such candidates, we were still only able to associate them with the extracellular matrix organization, exocytosis regulation and the endoplasmic reticulum lumen as well as the secretory granule lumen on the cellular components. This information may lead to think that CITED2 may also play a role in the regulation of the secretory pathway in terms of preparing the ECM plausibly to a cardiac specification, more precisely to the cardiomyocyte's specification. For this reason, the endoplasmic reticulum (ER) response may be, through an unknown process, partially regulated by CITED2.

There is a family of proteins that bind Ca^{2+} and have the ability to bind many extracellular proteins serving as anchors of the (ECM) (267,268). Indeed, there was a protein, with such ability, detected on the MS, the Thrombospondins (Thbs), more precisely TBSH1. Though, the THBS family comprises a group of five proteins. Thbs1 and -2 form homo- and hetero-trimers and contain a procollagen binding domain, a type 1 repeat domain that binds TGFβ as well as other domains that bind β1 integrins, calreticulin and CD36 and CD47 receptors (268). On the other hand, Thbs3, -4, and -5 form homo- and hetero-pentamers and lack both procollagen and type 1 receptors (267,268). All five Thbs genes are expressed, though in low amounts, in many tissues, but their expression is highly promoted when injury, stress,

or acute remodeling events occur (268). For instance, any of the Thbs1, -2, -3 or -4 are induced upon heart hypertrophy or infarction (139,267). Moreover, Thbs1 and -4 reside within the ER before being secreted from the cell, depending on the Ca^{2+} levels (269). As THBS1 was detected on the MS and the release of this proteins being dependent on the Ca^{2+} levels, CITED2 involvement on the ER may this way be on the regulation of the Ca^{2+} clearance. Moreover, the Ca^{2+} is up most importance on the cardiac development and maturation, thus nurturing the possibility of CITED2 involvement on the Ca^{2+} regulation.

The release of Ca^{2+} from the ER is involved in the regulation of several biological events, such as cell contraction, excitability, migration, secretion, growth and differentiation (270–273). This release is made through the inositol-1,4,5-triphosphate ($InsP_3$) at the ER, induced by the activation of phospholipase C in answer to extracellular growth factors, hormones or neurotransmitters (157). In terms of cardiac cell, the role of Ca^{2+} is easily linked to the contraction and relaxation, notwithstanding, the intracellular Ca^{2+} has been associated to cardiac cell differentiation and heart development as well. Indeed, Ca^{2+} handling proteins expression changes throughout the development, either for early stages or later on. Some of which are the ryanodine receptor 2 (RyR2), the sarcoplasmic reticulum (SR) pump (SERCA2) (274). ES-cell derived cardiomyocytes with impaired $InsP_3$ signaling or $Calr^{-/-}$ lead to different phenotypes than the ones deficient ryanodine 2 receptor (RyR2). Indeed, $RyR2^{-/-}$ cardiomyocytes shows no inhibition on the contractile ability neither on the flux of Ca^{2+}, but only impairs the beating frequency (275). Moreover, although Calsequestrin, the major SR Ca^{2+} binding protein, is only expressed from E8 on, Calreticulin (CALR), the major ER Ca^{2+} binding protein is highly expressed rather earlier in the embryonic heart (176).

Intriguingly, CALR had more PSMs hits in the CM-control than in the CM-CITED2 (Table 4). Though it was only slightly different between conditions, this may imply that CITED2 may be reducing the storage of Ca^{2+} on the ER. This way Ca^{2+} is more available on the cytoplasm and therefore able to play its role as a co-factor on several biological processes.

As previously mentioned, the TGFβ is a superfamily that includes not only the TGFβ itself, but also nodal, activin and BMPs that are as well dependent on Ca^{2+}. Indeed, TGFβ family and the FGF family both contribute to the cardiogenic morphogenesis. Stimulation of the TGFβ receptors will promote a response and in turn, the activation of Mitogen activated protein kinase kinase kinase (MAPKKK) – TAK. TAK will thereafter act, through the Ca^{2+} sensitive MAP Kinase pathway, on the transcription factors CREB and ATF2. ATF2 binds to SMAD's which in turn will promote the expression of cardiac transcription factors. Interestingly, Dishevelled (Dsh), a mediator of the canonical Wnt pathway, has been recently

shown to activate a Ca^{2+} dependent pathway including CamK through the Planal Cell Polarity (PCP) pathway. Additionally, Dsh deletion leads to vertebrate embryos with an immature heart (160). Moreover, Wnt11, a non-canonical cardiogenic member of the Wnt family, activates a Ca^{2+} dependent pathway, including Calmodulin dependent kinase II (CamKII), the protein kinase C (PKC) and the c-Jun Kinase (JNK) (160). There are also many transcription factors that play a role in cardiac specification and they are integrated in a transcriptional network that is to some extent, dependent on Ca^{2+}. Some of these are *Nkx2.5, Mef2c, GATA4, -5, -6, Tbx5* and *-20* (37). Indeed, it has been shown on our lab that *Wnt11* is not only upregulated on *Cited2* overexpressing cells but is also able to rescue CITED2 ablated cardiac defects both *in vitro* as well as *in vivo* (255). Additionally, if the secretion of certain proteins, accountable of the ECM modulation for cardiomyocytes, are stimulated by CITED2 (Table 5 and 6), it may be reasonable to idealize this protein over all biological role as preparing the cells to differentiate with a cardiomyocyte specification.

The pivotal role of intracellular Ca^{2+} in mouse cardiogenesis was inferred out of the phenotype of mice deficient in CALR. These knockout mice die in utero due to heart failure (174). Moreover, *in vitro* differentiation using the same EBs method we used for this work, when lacking Calr, ES cells-derived cardiomyocytes were not able to set their contractile apparatus. This may be due to the MEF2c misallocated in the cytosol, failing to translocate to the nucleus and, thereafter, not promoting the expression of many cardiac key transcription factors such as *Mlc2v* (276). On the other hand, the low amount of MLC2v that may exist requires to be phosphorylated to be added to the sarcomeric units, though such feature fail to happen in the absence of CALR (276). $Calr^{-/-}$ cardiomyocytes consequently have a disorganized contractile apparatus and, therefore fail to contract properly. This phenotype can be mimicked depleting Ca^{2+} using BAPTA or by inhibiting CamK. Also, the same phenotype seems to be rescued by adding an agonist that allows the intracellular Ca^{2+} flux in an $InsP_3$ dependent manner, through angiotensina II or ATP (176). This may lead to think of Ca^{2+} as a checkpoint at early stages of differentiation into further develop of mature cardiomyocytes with the full ability to contract.

All these findings show that Ca^{2+} has an impact regulating cardiac transcriptional program. Ca^{2+} not only regulates intracellular localization of the transcription factors but also their activity. For instances, the phosphorylation of histone deacetylase (HDAC) by Ca^{2+}-dependent CamK creates a docking site for the 14-3-3 protein. This will in turn break the MEF2c/HDAC complex, relieving MEF2c to be translocated to the nucleus (277). In a similar way, CamK-IV phosphorylates the serum response factor (SRF)/HDAC4 complex, allowing

the SRF to the activated and promote the expression of many other factors important to the cardiac cell differentiation (278).

Another level of Ca^{2+}-dependent regulation of the cardiogenesis includes the Nuclear Factor of Activated T cells (NFAT). For instances, Ca^{2+}-sensitive phosphatase calcineurin dephosphorylates NFAT allowing it to be translocated to the nucleus. Though, it only binds to DNA when associated to other cardiac specific transcription factors such as GATA4 (177). The compromised Ca^{2+} homeostasis in $Calr^{-/-}$ mice leads to a deficient calcineurin activity (279).

Though all the possible ways presented that may impact the endoplasmic reticulum response in terms of a possible CITED2 dependent manner, we decided to pursue with other candidates. In fact, it is of special note that on one of the duplicated CM analyzed we were able to detect proteins that were unique on each condition (CM-CITED2 and CM-control). As this may be an indication that these proteins expression may be dependent of CITED2 we were intrigued and pursued it further. Interestingly, when running the proteins unique to the CM-CITED2 on the platform HeartExpress we were able to infer that the CM had proteins that should be expected only at later stages of the differentiation and not at the onset of differentiation. Indeed, these proteins were only to be expressed by day 3 and 4 onwards. This is rather a good indication that CITED2 is promoting, directly or indirectly, the premature expression of these proteins.

For instances, apolipoprotein M (apoM) was one of the unique candidates present on the CM-CITED2. Remarkably, this protein has many biological functions important to the heart and may have a plausible role in the cardiogenesis. Ghaderian *et al* 2012 has shown that pediatric CHD patients have significantly higher levels of serum lipids (280). Furthermore, epidemiological studies suggest the protective role of HDL might be due to sphingosine-1-phosphate (S1P) (281–283). Additionally, S1P is carried by apoM inside the HDL mediating many of the beneficial effects of the HDL (282,284,285). ApoM is also important for the formation of nascent HDL and HDL-mediated cholesterol efflux (286,287). Both HDL, S1P and APOM polymorphisms have been correlated with the risk of coronary heart diseases (287,288).

Another interesting candidate that was present in CM-CITED2 as unique to this condition, was retinol-binding protein 4 (RBP4). RBP4 is a secreted protein that transports vitamin A (retinol) in circulation (289). This protein is elevated in insulin resistant states such as obesity and type 2 diabetes in both rodents and humans and contributes to systemic insulin resistance (290). Interestingly, it has been shown that RBP4 is a novel proinflammatory factor that activates Toll-like receptor 4 (TLR4)-dependent signaling and causes insulin resistance

(291,292). For instances, TLR4 is a modulator of the innate immunity and therefore triggers cytokine activation (293). Interestingly, TLR4 is also involved in mal-adaptive ventricular remodeling through the activation of cardiac inflammation (294). Furthermore, inhibition of TLR4 has a protective effect against cardiac hypertrophy in mice (295,296). Likewise, there have been emerging evidences that associated the high levels of RBP4 in the serum with cardiovascular diseases such as atherosclerosis and coronary artery disease (297). Lastly, RBP4 has been shown to be able to induce cardiomyocyte hypertrophy through the activation of TLR4/MyD88 pathway (298).

It has recently been shown that the secretome of regulatory T cells promotes cardiomyocytes proliferation during pregnancy and after myocardial infarction (299). Moreover, on the same study it was identified IGF2 as one of the key proteins of such effect. Indeed, either alone or in combination with other proteins detected on the regulatory T cells (CST7, TNFSf11, IL33, FGL2, MATN2), IGF2 was able to promote proliferation of rat neonatal cardiomyocytes (299). Nonetheless, it has also been shown that hyperglycemic conditions cause cardiomyocyte apoptosis, both *in vivo* and *in vitro*, through the over activation of IGF2R pathway (300). Thus, IGF2/IGF2R, one of the candidates that was unique to the CM-CITED2, has a role in the cardiac homeostasis, and thereafter may as well have role within cardiogenesis, though it requires a tight regulation of its expression/activation.

As indicated with the meta-analysis, all the candidates analyzed from the CM-CITED2 may be, at least to some extent, dependent on CITED2 and have a role within the heart homeostasis. For this reason, we pursued to explore their gene expression on both overexpressing and conditional ablation of *Cited2* cells. Though *Cited2* expression was concomitant with overexpression or *Cited2* knock down conditions, the overexpressing cells on one of the duplicates had much higher levels (Figure 23, Figure 24). This may be a causative reason for why, for example, *Isl1* expression on the duplicate (Figure 24) isn't as high as on the first qPCR data (Figure 23). Therefore, we decided to analyze these qPCRs in separate. Moreover, we also tried to replicate the cells conditions we had when gathered the CM for the MS. In this regard, we initiated the differentiation protocol but only extended it for 16h (same time point of the CM collection for the MS) and collected the cells at this time point (Figure 23 – H16). Interestingly, not all candidates (either CM-CITED2 or CM-control) matched the expression patterns expected according to the MS intel. Indeed, when looking to both CM-control candidates analyzed, only *Igfb4* was downregulated and just marginally, whereas *Mmrn2* was overexpressed. About the CM-CITED2 candidates, *Igf2* and *A2M* were just marginally overexpressed whereas *Rbp4* and *apoM* had a rather more striking peak of

expression. It's intriguing the fact although IGFBP4 was not detected on the first MS analyzes of the CM-CITED2 and that according to the HeartExpress platform, its expression is only expected to pop up by day 3, on both qPCRs made of CITED2 overexpressing cells, we not only observed an increase on *Igfbp4* expression between D0 and D2, but also reaching the overexpression by D2 (Figure 23, Figure 24). Though, when *Cited2* was ablated (Figure 25) *Igfbp4* was only marginally downregulated by D2. This may very well be a possible consequence of *Cited2* overexpression in regard to preparing the cell to the cardiac specification. For instances, IGFBP4 has been shown to promote cardiogenesis through the inhibition of canonical Wnt signaling (301) or even to induce cardiomyocytes proliferation (302). Though the reason for not detecting this protein on the MS is not clear. This may happen due to the fact that CITED2 may be promoting *Igfbp4* expression but at the cellular level still not be able to secrete IGFBP4 within the first 16h of differentiation. In the case of *Mmrn2* it seems to be rather independent of CITED2. Indeed, when looking to the *Cited2* overexpressing qPCRs (Figure 23, Figure 24), this gene doesn't even replicate the expression patterns. On one of the qPCRs it increases the expression between D0 and D2 whereas on the other qPCR it has an opposite expression behavior with a decreasing expression. Moreover, the *Cited2* ablation (Figure 25) didn't affect much *Mmrn2* expression. The expression of this protein may be very much just a consequence of the differentiation process and/or cellular density, as it has an anti-angiogenic activity (303).

When giving a closer look the CM-CITED2 candidates, *A2M* and *apoM* expression responded directly to *Cited2* expression patterns (Figure 23-19). Though, *Igf2* was only overexpressed when *Cited2* peaked its expression by D2 and only on the cells with the highest *Cited2* expression (Figure 23). On the duplicated qPCR for the *Cited2* overexpression, *Igf2* remained under expressed, but it's interesting to point out that its expression augmented dramatically and could possibly reach the overexpression rate if we had analyzed further on until later stages of the differentiation. If this was to be true, it could indicate that *Igf2* expression is not only dependent of CITED2 but also that higher CITED2 expression promotes earlier increase in the expression of *Igf2*. Since, IGF2 has been shown to be involved in the cardiac homeostasis (300) and cardiomyocyte proliferation (299), these data may very much be indicative of its role promoting earlier cardiac commitment in *Cited2* overexpressing cells. Regarding *Rbp4*, its expression didn't seem to be directly dependent of CITED2. Indeed, *Cited2* ablation (Figure 25) didn't decrease *Rbp4* expression, neither did *Cited2* overexpression increased much its expression, at least when looking to the qPCR that had a lower increase in *Cited2* expression (Figure 24). Though, when *Cited2* was dramatically overexpressed (Figure

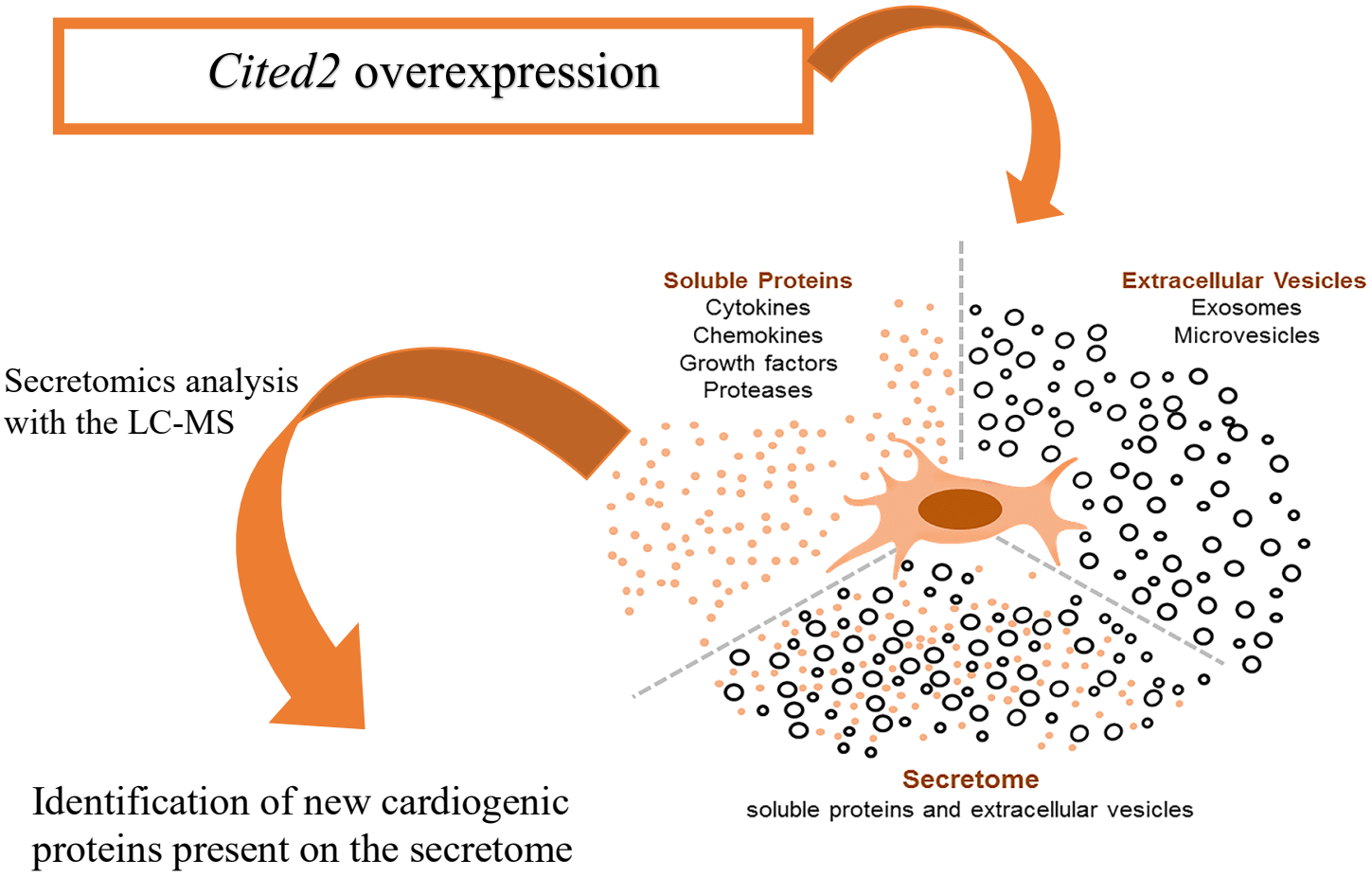
23), *Rbp4* reached a peak of expression by D2. This may indicate that Rbp4 is not dependent of CITED2 on its own, but its expression may be induced as a consequence of the cell specification induced by CITED2. Nonetheless, it may be of interest to study as RBP4 as it has been shown to induce cardiomyocyte hypertrophy (298).

All things considered, this work demonstrated that there are innumerous proteins that are secreted in response to *Cited2* overexpression. Moreover, there are some genes encoding such secreted proteins identified whose expression is somehow dependent on the CITED2 levels. Remarkably, different levels of overexpression of *Cited2* induce different patterns of expression on the candidates that were studied. For this reason, a CITED2 dose-effect study would be of most interest to pursue. Nonetheless, this dose-dependent effect may be a reason for such differences between replicates either with the qPCR or the CM LC-MS results. For this reason, it would also be interesting to use a line with integrated *Cited2* plasmid to induce the overexpression in order to ensure similar expression levels between replicates.

Though, we were still able to identify candidates that had a cardiogenic potential and that would be interesting to pursue. In this regard, it would be of interest to replicate the gene expression levels of each candidate in a more controlled *Cited2* expression condition and also studies at their protein level. These studies should be carried out with western blots for the extracellular medium as well as the intracellular medium since these proteins' abundance may be different in both sides of the cell. For instances, as explained on this thesis, THBS1 and -4 are stored in the ER and their secretion is dependent on the Ca^{2+} concentration. Another important study to be carried out is the CM immunodepleting for each candidate. This way we would asses if the candidates are needed for the CM rescue of the *Cited2*-depleted induced defects. Additionally, it is of interest to run experiments to assess if any of the candidates is sufficient to replicate the rescue effect of the CM. This can the tested by adding the recombinant protein for the respective candidates in the differentiation medium and asses the beating colonies of the *Cited2*-depleted cells, in a similar way as done on this thesis. Moreover, it would also be relevant to run expression analyzes of genes important at each step of differentiation. To sum up, future experiments should consider the schematic showed on Figure 26.

Overall, we have demonstrated that CITED2 expression may promote the early onset expression of genes normally expressed in cardiac differentiated cells. Also, CITED2 seems to induce the secretion of important cardiac proteins. Nonetheless, we failed to induce Cited2-ablation defects in our *in vivo* model (zebrafish) and this way were not able to assess one of previous experiments candidates ability to rescue such defects, nor were we able to replicate experiments for other newly identified candidates. Notwithstanding, CITED2 induced secreted

proteins may as well hold a potential for human therapies. This comes with upmost interest since the health care advances, early diagnosis, medical treatment, new and better surgical methods as well as regarding the postoperative care, trigger the prevalence of CHDs in adults and consequently the transmission to their offspring.



1. Does the gene expression match to the selected secreted proteins expression?
2. Is the "new identified protein" immunoprecipitated conditioned medium able to rescue *Cited2* ablation *in vitro*?
3. Is this "new cardiogenic protein" able to rescue on its own the beating foci upon *Cited2* ablation *in vitro*?
4. Is the new candidate able to recue Cited2 ablation in an in *vivo model* (zebrafish)?

Figure 26 - Schematic of the workflow to be considered in further experiments carried out to identify novel secreted cardiogenic proteins dependent on CITED2.

CHAPTER 9

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