Towards novel stem cell and protein based therapies for myocardium regeneration

Exploiting myocardial ischemia/reperfusion models and advanced proteomic tools

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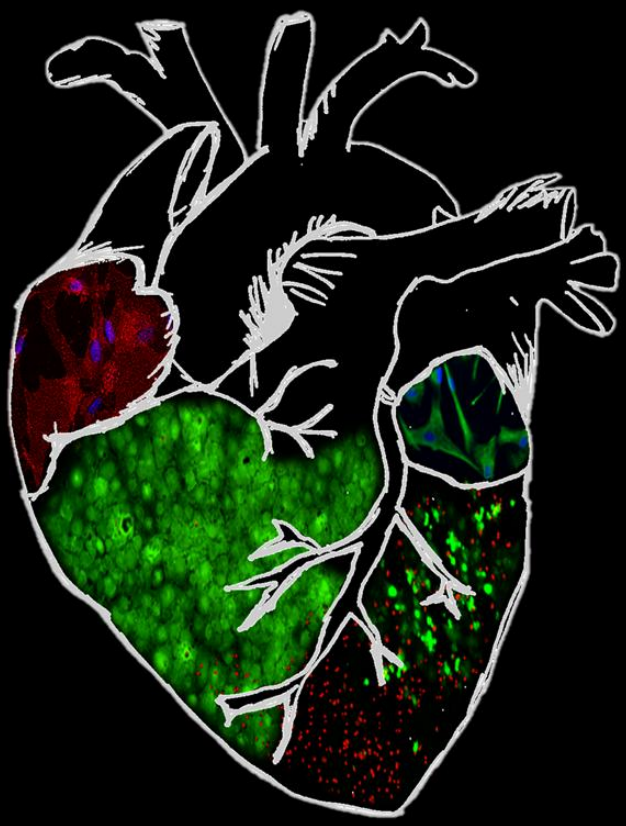

Dissertation presented to obtain the Ph.D degree in Sciences of Engineering and Technology, Biological Engineering

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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À minha família e ao Fred

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Abstract

Acute Myocardial Infarction (AMI) remains a leading cause of death worldwide. After AMI, clinical restauration of blood flow aggravates tissue damage (Ischemia/Reperfusion, I/R injury), critically decreasing the number of viable cardiomyocytes (CMs). Besides CMs, other myocardial cell populations such as cardiac stem/progenitor cells (CSCs) and cardiac fibroblasts (CFs) play key roles in tissue pathology and regeneration upon AMI. Several studies have demonstrated the relevant role of endogenous CSCs in myocardial repair after I/R injury, supported by the establishment of a paracrine cross talk between CSCs and the injured tissue. Due to their regenerative properties, human CSC (hCSC) transplantation has been arising as a promising therapy for AMI patients. Clinical trials using hCSC have demonstrated some physiological improvements, but the limited cell retention and engraftment in the heart still constitutes one of the main challenges that stand in the way of meeting hCSCs full clinical potential. The low cell engraftment efficiency further supports the hypothesis that the beneficial effects of hCSCs are mainly due to paracrine modulation. In fact, novel strategies for heart regeneration involve the direct protein and small-molecule based activation of endogenous CSCs populations. A better understanding of hCSC biology upon I/R in the context of allogeneic transplantation is therefore paramount, envisioning novel cell-based and cell-free therapies in order to fully avail and potentiate hCSC regenerative properties.

Moreover, human CFs (hCFs) also have a central role in myocardium pathophysiology, namely in fibrosis, a process of tissue reorganization upon I/R injury and other ischemic heart conditions. The study of this cell population has been limited by the lack of standardized and reliable

molecular markers, since none of the antigens currently used for characterization and isolation is specific for hCFs.

The main focus of this thesis is to characterize human cardiac cell populations in contexts of homeostasis and AMI, including hCSCs and hCFs, as well as to develop relevant I/R *in vitro* human cell models. In particular, advanced mass spectrometry (MS) tools were used to unveil hCSC mechanisms of action in a myocardial I/R context as well as to provide a comprehensive description of hCF membrane molecular landscape.

Envisioning at studying hCSC response to an AMI situation, the development of a two-dimensional (2D) *in vitro* cell model of myocardial I/R injury was explored in **Chapter II**. Here, a heterotypic co-culture was used, with hCSCs and human induced pluripotent stem cell derived CMs (hiPSC-CMs) , in order to better mimic the complexity of the *in vivo* paracrine milieu. This model was able to recapitulate important hallmarks of I/R pathophysiology, including hiPSC-CM death, the protective effect of hCSCs on hiPSC-CM viability and hCSC proliferation activation. This model also allowed us to probe hCSCs biology in response to I/R injury with a whole-proteome approach, enabling us to propose novel pathways involved in the regenerative process activated by hCSCs, including cell cycle regulation, proliferation through EGF signaling, and reactive oxygen species detoxification.

Following this work, another I/R injury *in vitro* model was established in **Chapter III**, taking advantage of three-dimensional (3D) hiPSC-CM spheroid cultures and stirred-tank bioreactor technology. 3D culture enables more extensive networks of cell-cell and cell-extracellular matrix (ECM) interactions, representing a step closer to the *in vivo* microenvironment architecture. On the other hand, bioreactor technology

ensures the possibility to control/ monitor environmental parameters such as pH and dissolved oxygen, critical in the context of I/R physiology. Using this setup we were able to once again recapitulate hallmarks of AMI, including hiPSC-CM death, secretion of pro-inflammatory and angiogenic factors and changes at a cell ultra-structural level, including disruption of sarcomeres and mitochondria organization.

In order to further investigate hCSC response to the factors secreted by injured hiPSC-CM, in **Chapter IV**, hCSCs were incubated with the conditioned medium from these bioreactor experiments, and their response was analyzed in terms of quantitative whole-proteome profiling. Besides investigating the paracrine cross-talk between hCSCs and hiPSC-CMs in an injury setup, in **Chapter V** we further explored the interaction of hCSCs with T-lymphocytes, aiming at better understanding the interactions of hCSCs with the host immune system in a allogeneic transplantation context. More specifically, the effect of hCSCs and hCSC conditioned media on T-lymphocyte proliferation were accessed, shedding new light on how hCSC immunomodulatory properties are not only depending on contact-dependent programmed death ligand 1 (PDL-1) mechanisms but also on tryptophan metabolism.

Chapter VI was focused on another myocardial cell population: hCFs. In this chapter, we examined hCF proteome profile with a focus on membrane proteins. In order to define a protein signature distinctive of this cell population, we further compared the membrane-enriched proteome of these cells with membrane-enriched fractions from another stem and cardiac cell populations, yielding a subset of 30 membrane proteins exclusively identified in hCFs, constituting a valuable source of information for further studies aiming at defining a membrane molecular signature of hCFs.

Overall, by developing and characterizing *in* vitro I/R cell models and applying advanced MS proteomic tools, this thesis contributed to generate novel and relevant knowledge on cell populations of the human heart with key roles in AMI pathology, regarding their molecular identity and mechanisms of action upon I/R injury. Moreover, we believe that the myocardial human I/R *in vitro* models established herein will constitute important tools for further studies, which will enable the development of novel therapies focused on activation, recruitment and improvement of the endogenous heart regeneration capacity.

Resumo

O infarte agudo do miocárdio (IAM) constitui uma das principais causas de morte a nível mundial. Após o IAM, a restauração do fluxo sanguíneo na área afectada agrava o dano causado no miocárdio (lesão de Isquémia/Reperfusão, I/R), diminuindo criticamente o número de cardiomiócitos (CMs) viáveis. Além dos CMs, outras populações celulares tais como células estaminais cardíacas (CSCs) e fibroblastos cardíacos (CFs) desempenham papéis fundamentais na patologia e regeneração após o IAM. Diversos estudos demonstraram que as CSCs endógenas têm um papel relevante na regeneração do miocárdio após a lesão de I/R, baseada no estabelecimento de comunicação parácrina entre estas células e o tecido afectado. Devido ás suas propriedades regenerativas, o transplante de CSCs humanas (hCSCs) tem vindo a surgir como uma nova e promisora terapia para pacientes de IAM. Vários ensaios clínicos demonstraram algumas melhorias a nível funcional, mas a limitadaretenção e inclusão das células no tecido cardíaco impedem o total aproveitamento das capacidades regenerativas destas células.

Além de hCSCs, os CFs humanos (hCFs) têm também um papel central na fisiopatologia do tecido cardíaco, nomeadamente na fibrose, um processo de reorganização do tecido que ocorre após a lesão de I/R e outras patologias de isquémia cardíaca. O estudo desta população celular tem sido restringido pela falta de marcadores moleculares uniformes e fidedignos, uma vez que nenhum dos antigénios utilizados actualmente para a caracterização e isolamento de hCFs é específico para os mesmos.

Esta tese teve como principal objectivo a caracterização de populações de células cardíacas humanas em contextos de homeostase e IAM, incluido hCSCs e hCFs, bem como o desenvolvimento de modelos

celulares humanos *in vitro* de lesão de I/R. Nomeadamente, foram utilizadas ferramentas avançadas de espectrometria de massa (MS) para estudar os mecanismos de acção de hCSCs em contextos de lesão de I/R, bem como para uma caracterização abrangente das proteínas de membrana dos hCFs.

Tendo como objectivo o estudo da resposta das hCSCs a uma situação de AMI, o **Capítulo II** explora o desenvolvimento de um modelo bi-dimensional (2D) *in vitro* de lesão de I/R. Neste estudo foi utilizada uma co-cultura heterotípica com hCSCs e CMs derivados de células estaminais pluripotentes humanas (hiPSC-CMs), com o fim de mimetizar a complexa rede de interacções parácrinas do tecido cardíaco. Este modelo demonstrou capacidade para recapitular aspectos importantes da fisiopatologia de lesão de I/R, incluindo a perda de viabilidade dos hiPSC-CMs, o efeito protector das hCSCs nos hiPSC-CMs e a activação da proliferação das hCSCs. Através deste modelo foi também possível investigar a resposta das hCSCs à lesão de I/R, através de um estudo de proteoma global, desvendando assim novas vias moleculares envolvidas nas capacidades regenerativas destas células, incluindo regulação de ciclo celular, proliferação através de sinalização por EGF e detoxificação de espécies reactivas de oxigénio.

Na sequência deste trabalho, no **Capítulo III** foi desenvolvido um modelo *in vitro* de lesão de I/R mais avançado, utilizando sistemas de cultura tri-dimensionais (3D) e bioreactores de tanque agitado. A utilização de sistemas de cultura 3D permite a criação de redes complexas de interacção célula-célula e célula-matriz extracelular (ECM), representanto assim um microambiente mais próximo ao tecido *in vivo*. O uso de bioreactores de tanque agitado possibilita a monitorização/controlo de vários parâmetros de cultura, tais como pH e oxigénio dissolvido, especialmente críticos no contexto de I/R. Utilizando

esta estratégia, foi possível recapitular uma vez mais aspectos da fisiopatologia de IAM, incluindo a morte de hiPSC-CMs, a secreção de factores angiogénicos e disrupção da organização de sarcómeros e mitocôndrias. Com o intuito de investigar em mais detalhe a resposta das hCSCs aos factores parácrinos secretados pelos hiPSC-CMs após a lesão de I/R, no **Capítulo IV**, as hCSCs foram incubadas com o meio condicionado das experiências de bioreactores (Capítulo III). A resposta das hCSCs foi analizada quantitativamente em termos de proteoma total. Além do estudo da comunicação parácrina entre hCSCs e hiPSC-CMs, a interacção de hCSCs com linfócitos T foi explorada no **Capítulo V**, num contexto de transplante alogénico de hCSCs. Nomeadamente, foi analizado o efeito das hCSCs e de meio condicionado das mesmas na proliferação de linfócitos T, permitindo elucidar que os mecanismos imunomodulatórios das hCSCs não dependem apenas dos previamente descritos mecanismos de contacto directo através do receptor PDL-1, mas também através do metabolismo de triptofano extracelular.

O **Capítulo VI** centra-se noutra população de células do miocárdio: CFs humanos (hCFs). Neste capítulo, o perfil proteómico destas células é analizado, com um foco em proteínas de membrana. Com o objectivo de definir uma assinatura molecular distintiva e específica de hCFs, foram comparados os perfis proteómicos destas células com outras populações de células cardíacas e estaminais, gerando um painel de 30 proteínas de membrana exclusivamente identificadas em hCFs, constituindo assim uma valiosa fonte de informação para novos estudos envolvendo esta população cardíaca.

Em suma, esta tese contribui para o estado da arte no campo de populações celulares cardíacas e na sua resposta em contextos de IAM, através do desenvolvimento de modelos *in vitro* de lesão de lesão de I/R e da aplicação de técnicas avançadas de proteómica. Os modelos

estabelecidos constituem ferramentas importantes para estudos posteriores que possibilitarão o desenvolvimento de novas terapias focadas em activação, recrutamento e melhoria das capacidades regenerativas endógenas do coração humano.

Thesis Publications

Sebastião, M.J., Pereira, R., Serra, M., Gomes-Alves, P., Alves, P.M. "Human Cardiac Fibroblast Membrane Proteome Characterization." Proteomics e1700446, 2018. doi:10.1002/pmic.201700446.

Sebastião, M.J., Pereira, R., Palacios, I., Serra, M., Gomes-Alves, P., Alves, P.M. "Myocardial ischemia/reperfusion injury human *in vitro* model to study human cardiac stem cells activation and regeneration mechanisms." (*submitted*).

Sebastião, M.J.*, Menta, R.*, Serra, M., Palacios, I., Alves, P.M., Lombardo E.[†], Gomes-Alves[†], P., "Study of immunomodulatory properties of human cardiac stem cells." * These authors contributed equally. [†]Co-corresponding authors. Stem Cell Research and Therapy (in press).

Sebastião, M.J., Reis I., Palacios, I., Serra, M., Gomes-Alves, P., Alves, P.M. "Merging bioreactor technology with 3D culture: a novel myocardial I/R in vitro model." (*manuscript in preparation*).

Book Chapters:

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Conference Proceedings:

Sebastião, M.J., Gomes-Alves, P., Pereira, R., Palacios, I., Serra, M., & Alves, P. (2017). LB41 – Unveiling human cardiac stem cells regenerative potential in ischemia/reperfusion injury. *Cytotherapy* (Vol. 19).

Sebastião, M. J., Paiva, M., Reis, I., Palacios, I., Serra, M., Gomes-Alves P., & Alves, P. (2018). P494 – Applying proteomic tools to disclose human cardiac stem cells regenerative potential in ischemia/ reperfusion injury. *Cardiovascular Research* (Vol. 114, Pages S120).

Sebastião, M. J., Pereira, R., Serra, M., Gomes-Alves P., & Alves, P. (2018). P466 – A step closer to disclose human cardiac fibroblasts molecular profile. *Cardiovascular Research* (Vol. 114, Pages S112-S113).

Additional Publications

Torán, J.L., López, J.A.,Gomes-Alves, P., Aguilar, S., Torroja, C., Trevisán-Herranz, M., Moscoso, I. **Sebastião, M.J.**, Serra, M., Brito, C., Sepúlveda, J.C. Martínez, F., Borlado, LR., Vázquez, J., Alves, P.M., Bernad, A. Definition of human cardiac progenitor cells core functions; genomic and proteomics comparative analysis with human mesenchymal stem cells. (*submitted*).

Correia, C., Koshkin, A., Duarte, P., Hu, D., Carido, M., **Sebastião, M.J.**, Gomes-Alves, P., Elliott, D.A., Domian, I., Teixeira, A.P., Alves, P.M., Serra, M. 3D aggregate culture improves metabolic maturation of human pluripotent stem cell derived cardiomyocytes. *Biotechnology and Bioengineering* 115 (3): 630–44. doi:10.1002/bit.26504.

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List of Abbreviations

2D two-dimensional

2DE two-dimensional gel electrophoresis

3D three dimensional

ACE angiotensin-converting-enzyme

AMI acute myocardial infarction

ASCs adipose-derived mesenchymal stem cells

bFGF basic fibroblast growth factor

CDCs cardiosphere-derived cells

CFs cardiac fibroblasts

CFSE carboxyfluorescein succinimidyl ester

CHF chronic heart failure

c-kit tyrosine kinase

CMs cardiomyocytes

CSCs cardiac stem/progenitor cells

CTGF connective tissue growth factor

CXCL6 granulocyte chemotactic protein 2

DDA dependent data acquisition

DDR2 tyrosine kinase discoidin domain receptor

DIA data independent acquisition

EB embryoid body

ECGM2 Endothelial Cell Growth medium 2

ECM extracellular matrix

ECs endothelial cells

EGF epidermal growth factor

ELISA enzyme-linked immunosorbent assay

ERS endoplasmic reticulum stress

ESCs embryonic stem cells

FACS fluorescence-activated cell sorting

FBS fetal bovine serum

FDA fluorescein diacetate

FDR false discovery rate

FSG Fish Skin Gelatin

G-CSF granulocyte colony-stimulating factor

hASCs human adipose-derived mesenchymal stem cells

hCFs human cardiac fibroblasts

hCSCs human cardiac stem/progenitor cells

hDFs human dermal fibroblasts

HGF hepatocyte growth factor

HIF-1α hypoxia-inducible factor 1 α

hiPSC-CMs human induced pluripotent stem cell derived cardiomyocytes

HLA human leukocyte antigen

hMSCs human mesenchymal stem cells

hPBMCs human peripheral blood mononuclear cells

HPLC high performance liquid chromatography

HUVECs human umbilical vein endothelial cells

I/R Ischemia/ Reperfusion

ICAT isotope-coded affinity tag

iCSCs induced cardiac stem/progenitor cells

IDA independent data acquisition

IDO indoleamine 2,3-dioxygenase

IFN-γ Interferon gamma

IGF-1 insulin-like growth factor 1

IHD ischemic heart disease

IMS ischemic mimetic solution

IPA ingenuity pathway analysis

iPSCs induced pluripotent stem cells

Isl-1 insulin gene enhancer protein

Kyn kyurenine

LAD left anterior descending

LC liquid chromatography

MACS magnetic –activated cell sorting

MHC major histocompatibility complex

MPTP mitochondrial permeability transition pore

MS mass spectrometry

PBMCs peripheral blood mononuclear cells

PCI percutaneous coronary intervention

PD-1 programmed cell death-1

PDL-1 programmed death ligand 1

Pen/Strep penicillin/streptomycin

PFA paraformaldehyde

PI propidium iodide

PSCs pluripotent stem cells

ROS reactive oxygen species

Sca-1 stem cells antigen-1

SCF stem cell factor

SDF-1 stromal cell derived factor 1

SILAC stable isotope labeling in cell culture

SMA smooth muscle actin

SMCs smooth muscle cells

SWATH sequential window acquisition of all theoretical mass spectra

T3 triiodothyronine

TCR T-cell receptor

TEM transmission electron microscopy

TGF-β transforming growth factor β

Trp tryptophan

TW traswells

UPR unfolded protein response

VEGF vascular endothelial growth factor

WHO World Health Organization

Chapter I

Introduction

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Contents

Introduction

1. Acute myocardial infarction: the unmet clinical need

Over the last decades, better medical care and living conditions have led to a gradual increase in average life expectancy, which reached a worldwide average of 71.4 years in 2015 (WHO). According to United Nations 2015 world population ageing report, the number of people aged 60 years or over increased from 607 million in 2000 to 901 million in 2015, with projections pointing to 1.2 billion in 2030 and 2.1 billion in 2050. Such increase in life expectancy has led and will lead in the coming decades to a higher prevalence of age-related diseases including cancer, neurodegenerative disorders and cardiovascular diseases.

Ischemic Heart Disease (IHD) is one of the most common types of cardiovascular disease, and a major cause of death worldwide (Benjamin *et al.*, 2017). IHD consists in atherosclerosis (lipid plaque deposits) in heart arteries inner walls, narrowing and reducing blood flow to the heart, ultimately leading to an Acute Myocardial Infarction (AMI), commonly known as heart attack. AMI consists on the cessation of blood flow to an isolated region of the heart, causing oxygen and nutrient supply depletion (ischemia), leading to myocardial tissue damage with loss of cardiomyocytes (CMs), the main cell type in the heart. Since the extent of tissue damage and cell death is influenced by both the magnitude and duration of ischemia, the revascularization and restauration of blood flow as soon as possible remains the clinical intervention of choice for AMI patients (Anderson and Morrow, 2017). However, this process, also known as reperfusion, although necessary to reestablish the delivery of oxygen and nutrients to the affected area, causes increased tissue damage (Ischemia Reperfusion I/R Injury). The elevation of molecular oxygen levels occurs at a toxic rate to cells, causing up to 50% of the

final damaged tissue size (Hausenloy and Yellon, 2013) and contributing to almost one fourth of AMI mortality (Yellon and Hausenloy, 2007).

Nevertheless, the current treatments are successful in reducing immediate mortality but the tissue damage is often too large (in average, about 1 billion CMs are lost during an AMI, Laflamme & Murry, 2011) to allow restauration of normal muscle function. Deposition of fibrous scar tissue leads to a decrease in myocardium tensile strength and progressive loss of cardiac output, often leading to Chronic Heart Failure (CHF), a highly fatal condition (survival rate of only 50% at 5 years, Cahill, Choudhury, & Riley, 2017) to which the only available clinical option is heart transplant. As it is known, this solution is not optimal in clinical practice due to the scarcity of available heart donors, high costs and need for immunosuppression (Lund *et al.*, 2014).

1.1. Pathophysiology of myocardial ischemia/reperfusion

The two phases of I/R injury have distinctive physicochemical properties and therefore distinct pathophysiology mechanisms affecting myocardium tissue and CM death (figure 1.1).

During ischemia, the deprivation of oxygen and nutrients results in a series of biochemical and metabolic cellular changes including decrease of ATP and pH levels, increase in intracellular lactate and accumulation of intracellular Ca^{2+}. Without oxygen, CMs stop oxidative phosphorylation, leading to a decrease in ATP synthesis and an increase in anaerobic respiration by glycolysis and lactic acid fermentation with accumulation of intracellular lactate. While increased glycolysis helps to compensate for the lack of aerobic ATP, this pathway binds less ATP hydrolysis-generated H^+, leading to lower pH. The intracellular accumulation of H^+ activates the Na^+/H^+ exchanger (figure 1.1). The lack

of ATP during ischemia also causes CM contraction arrest and inactivation of $3Na^+/2K^+$ exchanger ATPases, leading to intracellular Na^+ accumulation. In response, the activation of the $2Na^+/Ca^{2+}$ pump results in intracellular Ca^{2+} overload (Hausenloy and Yellon, 2013; Kalogeris *et al.*, 2017) (figure 1.1)

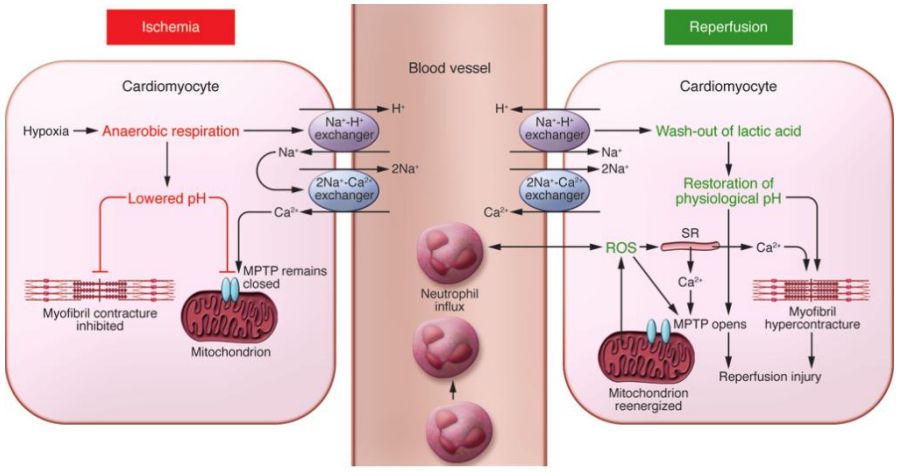

Figure 1.1. **Schematic illustration of the main biochemical mechanisms underlying myocardial ischemia/reperfusion induced cardiomyocyte cell injury and death.** During ischemia, metabolism is switched to anaerobic respiration, resulting in production of lactate, low ATP and low pH. Ionic channels activation leads to intracellular accumulation of Na^{2+} and Ca^{2+}. During reperfusion, the electron transport chain is reactivated, generating ROS that, together with higher Ca^{2+} accumulation, lead to MPTP opening and CM contracture. Neutrophils are recruited to the site of injury in response to the release of ROS and cytokines, contributing to tissue inflammation (Hausenloy and Yellon, 2013).

Restauration of blood flow during reperfusion provides oxygen and metabolic substrates required for aerobic ATP generation. However, the reactivation of the mitochondrial electron transport chain leads to production of cytotoxic reactive oxygen species (ROS). Acidosis is also corrected by the Na^+/H^+ exchanger which results in additional intracellular Na^+ accumulation, resulting in the activation of $2Na^+/Ca^{2+}$ exchanger. The release of extra Ca^{2+} from sarcoplasmic reticulum, adding to the ischemic Ca^{2+} accumulation culminates into a large overload of intracellular Ca^{2+}, which in turn promotes hypercontracture

(sarcomeres myofibrillar contraction) (Hausenloy and Yellon, 2013; Kalogeris *et al.*, 2017). ROS synthesis, the increase in pH and the Ca^{2+} overload result in the opening of the mitochondrial permeability transition pore (MPTP), a non selective channel of the inner mitochondrial membrane. MPTP opening results in mitochondrial membrane depolarization and uncoupling of the electron transport chain, leading to ATP depletion, mitochondria swelling with mitochondria membrane rupture and release of apoptotic factors culminating in activation of apoptosis and cell death (figure 1.1) (Hausenloy and Yellon, 2013; Kalogeris *et al.*, 2017).

In response to the stress caused by reperfusion, CMs secrete several growth factors (including insulin-like growth factor 1 IGF-1, epidermal growth factor EGF, hepatocyte growth factor HGF, vascular endothelial growth factor VEGF), cytokines, chemokines and other pro-inflammatory molecules, which together with the released ROS recruit immune cells to the site of injury (figure 1.1). Neutrophils are key players in the post AMI inflammatory process: by secreting ROS, proteases, chemokines and other cytotoxic molecules, these cells further enhance inflammation and tissue damage (Frangogiannis, 2014). Phagocytic leukocytes also clear dead cells and matrix debris, setting the stage for fibrous collagen-based scar tissue deposition by activated cardiac fibroblasts (see section 5).

2. Current treatments for myocardial I/R injury

Duration of the ischemia phase is a major contributor to the extent of myocardial tissue damage. As so, the intervention of choice for a patient with AMI symptoms is the rapid reperfusion of the affected artery. The reperfusion is done mechanically by a balloon-inflated metal mesh stent

(percutaneous coronary intervention, PCI), by pharmacological anti-platelet and fibrinolytic (anti-thrombotic) agents, or by a combination of both (e.g., drug eluting PCI stents). Another alternative to PCI is coronary bypass surgery, in which a healthy vessel is used to divert the blood flow around the blocked artery (Anderson and Morrow, 2017). In addition, angiotensin-converting-enzyme (ACE) inhibitors and β-blockers are commonly used in the clinic to downsize myocardial scar formation (Anderson and Morrow, 2017). A substantial decrease of in-hospital AMI-related mortality rate of 7-18% was registered over the last two decades (Cahill, Choudhury and Riley, 2017). Such improvement in AMI patients prognosis is a testament to advances in PCI and pharmacological therapies as well as implementation of preventative measures. While immediate unclogging of the affected artery remains the keystone for the treatment of AMI patients, several approaches focusing in reducing the damage caused by the reperfusion are being developed and tested in clinical trials.

In contrast to classic unimpeded reperfusion, ischemic post-conditioning, also designated as slow reperfusion, consists in intermittent reperfusion, with brief repetitive interruptions of blood flow. This on/off reperfusion is thought to trigger a cascade of cell protection mechanisms that translate into a reduced myocardial injury size. Although promising results were registered in animal models, human clinical trials remain inconclusive (Giustino and Dangas, 2017). Another strategy showing more promising results in humans is remote ischemic per-conditioning, in which tissues other than the heart (usually limbs) are exposed to ischemia, resulting in the activation of systemic mechanisms of defense and myocardial protection (McLeod, Iansavichene and Cheskes, 2017).

A multitude of pharmacological approaches have also been developed to reduce the adverse impact of reperfusion injury, and several are currently

being evaluated in clinical trials. One such example is cyclosporine A, an inhibitor of MPTP opening. However, a recent meta-analysis has shown that the use of this drug before PCI does not translate into a better clinical outcome (Rahman *et al.*, 2018).

Besides mechanical and pharmacological-based treatments, cell-based approaches have been emerging in the last years as a novel strategy to treat infarcted myocardium.

3. Novel therapeutic strategies for myocardial I/R injury

For many years, the adult mammalian heart has been considered an organ without regenerative potential. In 2003, Beltrami and colleagues identified cardiac stem/progenitor cells (CSCs) in the mouse heart for the first time (Beltrami *et al.*, 2003), and in the following years CSCs were identified in other animals including humans (Bearzi *et al.*, 2007). As most adult stem cells, CSCs have the ability to self-renew and to differentiate into tissue-specific lineages [CMs, vascular smooth muscle cells (SMCs) and endothelial cells (ECs)] (Beltrami *et al.*, 2003).

The discovery of CSCs is an hallmark in AMI-related research, as it challenges the previous paradigm in cardiovascular research, in which the heart was seen as a post-mitotic organ without endogenous regenerative capacity. This finding opened novel avenues in regenerative medicine strategies for recovery of infarcted myocardium, including cell therapy and protein-based stimulation of endogenous heart repair.

3.1. Cell therapy

In cell therapy-based approaches several cell types were already applied in clinical trials, including “first generation” stem cell sources such as

bone-marrow mononuclear cells (e.g., REPAIR-ACS- NCT00711542, BOOST-NCT00224536), bone marrow-derived mesenchymal stem/stromal cells (e.g. BOOST trial, NCT00224536), and adipose tissue-derived mesenchymal stem/stromal cells (e.g., APOLLO trial, NCT00442806).

After “first generation” stem cell sources, more focus is now being devoted to therapies with purified and homogeneous “second generation” stem cell sources, such as CSCs.

3.1.1. Cardiac stem cells in cell therapy

CSCs are considered by several authors as the preferable candidate cell therapy for cardiac diseases, mainly due to their physiologic location and function in the heart, their potential to differentiate into myocardial lineages, and the promising regenerative effects of CSCs transplantation in myocardial infarction preclinical models. In a recent preclinical meta-analysis, an estimated improvement of 10.7% of left ejection fraction was registered, with superior effects of CSCs compared with other cell types in mice (Zwetsloot *et al.*, 2016). However, such trend seems to be lost when moving to large animal models (Zwetsloot *et al.*, 2016).

The first clinical trials using CSCs were based on autologous therapies (Chugh *et al.*, 2012; Makkar *et al.*, 2012; Ishigami *et al.*, 2015) (table 1.1), which have the great advantage of not presenting immunogenicity risks to the patients. However, autologous cell therapy is associated with serious limitations that compromise widespread “off-the-shelf” clinical applications such as the difficult logistic, economic and time-constraints in patient specific tissue harvesting and expansion. Moreover, cell’s phenotype, regenerative potential, and quality will be highly variable and dependent on patient’s age, co-morbidities, and genetic background

(Dimmeler and Leri, 2008; Wu *et al.*, 2016; Sharma *et al.*, 2017). To overcome such limitations, the field has been moving towards allogeneic clinical approaches, such as the clinical study ALLSTAR and more recently, the CAREMI trial (Sanz-Ruiz *et al.*, 2017; Fernández-Avilés *et al.*, 2018), which had success in demonstrating safety and lack of rejection of transplanted cells, as well as improvements in infarct size (table 1.1). Moreover, a cross-talk of transplanted allogeneic c-kit$^+$ hCSCs with innate natural killer cells has been shown to result in attenuation of myocardium inflammation and prevention of adverse scar tissue formation (Boukouaci *et al.*, 2014). The same group has also identified programmed death ligand 1 (PDL-1) interaction with T regulatory cells as one of the mechanisms involved in this immunomodulatory capacity and suggested PDL-1 as a marker to identify and select low immunogenic risk allogeneic c-kit$^+$ CSCs (Lauden *et al.*, 2013). Another cell therapy approach being explored is the use of CSCs derived from pluripotent cell populations such as the ESCORT trial, in which CSCs derived from embryonic stem cells (ESC-CSCs) have been applied (table 1.1).

For all different CSC subpopulations tested, clinical trials have demonstrated some physiological improvements, namely increase in viable tissue and in heart functional outcome (table 1.1), but very limited cell retention and engraftment in the heart was observed, regardless of the route of administration and cell dosage. Within 24 hours of delivery, less than 10% of injected cells remain at the targeted location, and most of the successfully retained cells die, probably due to the inflammatory environment in the infarct and infarct border zones of the myocardium (Hong and Bolli, 2014; Mathur *et al.*, 2017). In order to further improve the physiologic benefit of cell transplantation, several strategies have been pursued to increase cell retention, including preconditioning of cells

to be transplanted, for example with an hypoxia cultivation priming phase (Hosoyama *et al.*, 2015; Hernandez *et al.*, 2018), preconditioning the target tissue (Assmus *et al.*, 2013), repeated cell dosage (Tokita *et al.*, 2016), and biomaterial-based approaches (Hosseinkhani *et al.*, 2010; Kryukov, Ruvinov and Cohen, 2014; Rajabi-Zeleti *et al.*, 2014; Gaetani *et al.*, 2015; Menasché, Vanneaux, Hagège, *et al.*, 2015).

3.2. Cell-free therapeutic approaches

The low cell engraftment efficiency in preclinical and clinical studies strongly supports the hypothesis that the beneficial physiological effect of transplanted cells is due to paracrine modulation rather than to cells engraftment and differentiation (Madonna *et al.*, 2016).

In fact, novel strategies for heart regeneration involve the activation of endogenous CSCs populations directly with growth factors. Examples of growth factors investigated include IGF-1 and HGF (Urbanek *et al.*, 2005; Ellison *et al.*, 2011; Koudstaal *et al.*, 2014; O'Neill *et al.*, 2016; Blanco Blazquez *et al.*, 2017), granulocyte colony-stimulating factor (G-CSF) (STEMMI-NCT00135928), erythropoietin (REVIVAL-3-NCT0039083), as well as VEGF (NORTHERN-NCT00143585). Such single growth factor therapies showed only little benefit in AMI patients, possibly due to the rapid diffusion and short half-lives of the injected molecules (Awada, Hwang and Wang, 2017). Other approaches try to overcome this by combining growth factor therapy with cell therapy, such as ALCADIA trial, in which a sustained release of basic fibroblast growth factor (bFGF) from a gelatin hydrogel sheet was used in order to augment the effect exerted by the transplanted CSCs (Takehara *et al.*, 2012) (table 1.1).

Table 1.1. Clinical Trials with Transplantation of hCSCs.

	Phase	Identifier	CSC type	Patients	Route of administration	Dosage	Status	Functional outcomes	ref.
SCIPIO [N1] - Cardiac Stem Cell Infusion in Patients With Ischemic Cardiomyopathy	I	NCT00474461	Autologous c-kit$^+$ CSCs	MI (pre-CABG) (LVEF<40%)	Intracoronary injection	Single dose (0.5-1x10^6)	Completed	↑LVEF; ↓infarct size; ↑contractile function	Bolli *et al* 2011; Chugh *et al* 2012.
CADUCEUS- Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction	I	NCT00893360	Autologous CDCs	Recent MI (LVEF<45%)	Intracoronary injection	Single dose (12-25x10^6)	Completed	↓infarct size, ↓viable mass; ↑contractile function	Makkar *et al* 2012
ALLSTAR- Allogeneic Heart Sem Cells to Achieve Myocardial Regeneration	I/II	NCT01458405	Allogenous CDCs	Recent and chronic MI (LVEF≤45%)	Intracoronary injection	Single dose (12.5 and 25x10^6)	Phase I completed; Phase II ongoing	Phase I: ↓infarct size; ↑ infarcted segments wall thickening	Chakravarty *et al* 2016
HOPE - Halt Cardiomyopathy Progression in Duchenne	I/II	NCT02485938	Allogenous CDCs	Duchenne muscular dystrophy cardiomyopathy	Intracoronary injection in each of the three left ventricle territories (anterior, lateral, inferior)	Single dose (75x10^6)	Ongoing	NA	[N2]
DYNAMIC - Dilated Cardiomyopathy Intervention with Allogeneic Myocardially-Regenerative Cells	I	NCT02293603	Allogenous CDCs	Severe heart failure (LVEF≤35%)	Intracoronary injection	Single dose	Ongoing	NA	[N2]
ALCADIA - Autologous Human Cardiac-Derived Stem Cell to Treat Ischemic Cardiomyopathy	I	NCT00981006	Autologous CSCs + bFGF	Ischemic ardiomyopathy (LVEF ≤35%)	Intramyocardial injection; gelatin hydrogel patch with bFGF implanted on epicardium injection sites	Single dose (0.5x10^6/kg); 200mg bFGF	Completed	↑LVEF; ↓infarct size; ↑Maximal aerobic exercise capacity	[N2]
CAREMI - Safety and Efficacy Evaluation of Intracoronary Infusion of Allogeneic Human Cardiac Stem Cells in Patients With AMI	I/II	NCT02439398	Allogenous c-kit$^+$ CSCs	STEMI	Intracoronary injection	Single dose (35x10^6)	Ongoing	↓infarct size	Fernández-Avilés *et al* 2018

	Phase	Identifier	CSC type	Patients	Route of administration	Dosage	Status	Functional outcomes	ref.
ESCORT - Transplantation of Human Embryonic Stem Cell-derived Progenitors in Severe Heart Failure	I	NCT02057900	ESC derived CD15+ Isl-1+ CSCs	Severe heart failure (LVEF≤35%)	Epicardial delivery of cells embedded in fibrin patch	Single dose ($4x10^6$)	Recruiting	NA	Menasche *et al* 2005
TICAP - Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single Ventricle Physiology	I	NCT01273857	Autologous CDCs	Pediatric patients with hypoplastic left heart syndrome	Intracoronary injection	Single dose ($0.3x10^6$/kg)	Completed	↑RVEF; ↑somatic growth	Ishigami *et al* 2015
PERSEUS - Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease	II	NCT01829750	Autologous CDCs	Univentricular heart disease	Intracoronary injection	Single dose ($0.3 x10^6$ cells/kg)	Completed	↓infarct size; ↑somatic growth; ↑ factors production	Ishigami et al 2017
CONCERT-HF - Combination of Mesenchymal and C-kit+ Cardiac Stem Cells as Regenerative Therapy for Heart Failure	II	NCT02501811	Autologous BM_MSCs, c-kit+ cCSCs alone and in combination	Ischemic cardiomyopathy	Transendocardial injection	Single dose ($150x10^6$ BM-MSCs; $5x10^6$ CSCs)	Ongoing	NA	[N2]
Transplantation of Autologous Cardiac Stem Cells in Ischemic Heart Failure	II	NCT01758406	Autologous CSCs	Ischemic heart failure	Intracoronary injection	Single dose ($5\text{-}100x10^6$)	Recruiting	NA	[N2]
APOLLON - Cardiac Stem/Progenitor Cell Infusion in Univentricular Physiology	III	NCT02781922	Autologous CSCs	Pediatric patients with heart failure	Intracoronary injection	Single dose ($0.3x10^6$ cells/kg)	Recruiting	NA	[N2]
TAC-HFT-II - The Transendocardial Autologous Cells (hMSC) or (hMSC) and (hCSC) in Ischemic Heart Failure Trial	I/II	NCT02503280	Combination of autologous MSCs and c-kit$^+$ CSCs or MSCs alone	ischemic left ventricular dysfunction and/or heart failure secondary to MI	Transendocardial injection	Single dose ($200x10^6$ BM-MSCs or $190x10^6$ BM-MSC + $1x10^6$ CSCs)	Recruiting	NA	[N2]

Abbreviations: Cardiac stem/ progenitor cells (CSCs); Cardiosphere-derived cells (CDCs); Embryonic stem cells (ESC); Bone marrow-derived mesenchymal stem cells (BM-MSCs); mesenchymal stem cells (MSCs); Myocardial infarction (MI); Coronary artery bypass grafting (CABG); Left ventricular ejection fraction (LVEF); Segment elevation myocardial infarction (STEMI); Right ventricular ejection fraction (RVEF). NA – not available [N1]: subject to expression of concern; [N2]: Information were obtained from www.clinicaltrials.gov, accessed March 2018.

Despite promising, several challenges still need to be addressed in order to reach the full clinical potential of CSCs. Further understanding of CSC biology is needed in order to discover and modulate molecular pathways involved in the regenerative potential of these cells. Novel findings will be pivotal for the development of improved clinical strategies, including enhanced activation of endogenous hCSCs, preconditioning of cells to be transplanted and protein-based therapies. New relevant human cell-based models of cardiac tissue damage should also be pursued to characterize CSC response to myocardial injury *in vitro*.

International collaborative consortiums and multicenter studies such as Translational Alliance for Regenerative Therapies in Cardiovascular Syndromes (TACTIC) (Fernández-Avilés *et al.*, 2017), Consortium for Preclinical Assessment of Cardioprotective Therapies (CAESAR) (Jones *et al.*, 2015), and Cardio Repair European Multidisciplinary Initiative (CARE-MI) (www.cordis.europa.eu) have been emerging as important platforms to bring basic researchers and clinicians together to discuss and define common goals and strategies, as well as to standardize protocols and analytical techniques in preclinical and clinical studies.

4. The role of endogenous cardiac stem cells in cardiac repair

Endogenous CSCs play important roles in cardiac homeostasis and in response to physiological stress and cardiac injury. The mammalian adult heart harbors a small percentage of endogenous CSCs (about one stem cell per 8000-20000 CMs (Anversa *et al.*, 2006)), which are located in organized hypoxic niches within the myocardium, more abundant in lower hemodynamic stress areas such as the atrium and apex. Within these

niches, CSCs are typically clustered together with early committed cells and adult CMs (Leri *et al.*, 2014).

Since their discovery in 2003 (Beltrami *et al.*, 2003), efforts have been made to isolate, cultivate, characterize and study the regenerative mechanisms of these cells.

4.1. Identification and isolation of endogenous CSCs

Several CSCs subpopulations have been described and isolated from the adult heart according to their phenotypic profile and differential expression of several surface molecular markers. These subpopulations include c-kit+, Sca1+, and Isl-1+ CSCs (reviewed in detail in Santini et al. 2016).

4.1.1. C-kit+ CSCs

C-kit$^+$ cells were the first population of CSCs to be identified in the adult mouse heart in 2003 (Beltrami *et al.*, 2003). These cells are characterized by expression of the stemmness marker c-kit, expression of cardiac lineage transcription factors such as GATA4, Nkx2.5, and Mef2C, and absence of hematopoietic lineage markers such as CD34 and CD45 (Santini *et al.*, 2016). c-kit$^+$ CSCs are one of the most extensively studied CSCs subpopulations, already employed in several clinical trials such as SCIPIO, CAREMI, CONCERT and TAC-HFT-II (table 1.1)

4.1.2. Sca-1+ CSCs

Sca-1$^+$ CSCs are characterized by the expression of the endothelial marker stem cells antigen-1 (Sca-1). These cells have been identified in mice and human adult heart and express the cardiac transcription factors GATA4, Mef2C and Tef1, but lack other cardiac lineage markers such as Nkx2.5, the

hematopoietic markers CD34 and CD45, and mature endothelial markers such as CD31 (Santini *et al.*, 2016).

4.1.3. Isl-1+ CSCs

Another population of CSCs, characterized by the expression of the LIN-homeodomain transcription factor insulin gene enhancer protein (Isl-1) has also been described in murine and human hearts. These cells do not express c-kit, Sca-1, or CD31 while expressing the cardiac transcription factors Nkx2.5 and GATA4. The expression of Isl-1 is closely associated with age: Isl-1^{+} cells can be found predominantly in fetal and neonatal myocardium, yet very low levels of this population could be found in adult hearts, which limits their clinical application (Weinberger *et al.*, 2012).

4.1.4. CSC isolation methods

CSC subpopulations are usually isolated from the adult human heart tissue either from cadavers or from biopsies of patients undergoing heart surgery. Experimental approaches for the isolation of hCSCs from cardiac tissue can be separated into two categories: cardiosphere-based and immunoselection-based, taking advantage of the expression of specific molecular markers (figure 1.2).

The first method to isolate CSCs from human heart biopsies was reported in 2004 (Messina *et al.*, 2004) (Patent number WO2005012510). Cardiac tissue explants were enzymatically digested and cultured as adherent explants. These explants gave rise to cellular outgrowths that spontaneously aggregate, generating three-dimensional (3D) structures, named cardiospheres, which are composed of mixed cell populations, with undifferentiated cells expressing stem cell markers in the core and differentiating cells expressing markers characteristic of cardiac, vascular

endothelial and stromal commitment in the periphery (Davis *et al.*, 2010). Cardiospheres are then collected, replated in fibronectin adherent culture dishes and further expanded as monolayers to yield cardiosphere-derived cells (CDCs) (Cheng *et al.*, 2014; Kapelios, Nanas and Malliaras, 2016).

Several preclinical studies using myocardial injury models (Kanazawa *et al.*, 2016) have demonstrated the beneficial effects of transplantation of CDCs in improving cardiac function and decreasing scar tissue size (for detailed reviews see Marbán 2014; Kapelios, Nanas, and Malliaras 2016). These studies have already been translated into several human clinical trials such as CADUCEUS and ALLSTAR (table 1.1, page 12). However, the cell population heterogeneity inherent to the cardiosphere isolation method has been pointed as a disadvantage in terms of obtaining robust and predictable clinical outcomes.

Another method for isolating hCSCs from heart tissue is by using the enzymatic digestion of tissue explants followed by immune-selection for stem cell markers such as the surface receptors c-kit, Sca-1, Isl-1 and immunodepletion for hematopoietic and mesenchymal markers such as CD45 and Tryp, using magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS) (Barile *et al.*, 2007; Lauden *et al.*, 2013; Goichberg *et al.*, 2014). In particular, CSCs immunoselected for c-kit$^+$ represent the most extensively characterized CSC population. Although controversy exists regarding the differentiation capability of c-kit$^+$ CSCs (Ellison *et al.*, 2013; Nadal-Ginard, Ellison and Torella, 2014; van Berlo *et al.*, 2014) and the stability of c-kit marker expression during *in vitro* culture (Forte *et al.*, 2011), numerous preclinical studies (for a more detailed review consult Nigro et al. 2015) and clinical trials (table 1.1, page 12) demonstrate that c-kit$^+$ CSCs have relevant regenerative properties, since transplantation of this cell population results in improved cardiac tissue function and reduction of scar tissue size.

4.1.5. Derivation of cardiac stem cells from other cell sources

Besides donated human hearts, other sources for CSC have emerged (figure 1.2). Pluripotent stem cells (PSCs), including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), have unlimited self-renewal capacity and the potential to differentiate *in vitro,* holding great promise for the clinical translation of cell therapies. In particular, hiPSCs hold the advantage of allowing for autologous therapies without invasive isolation procedures. In fact, as development of less invasive surgical interventions techniques is further explored, the availability of tissue for isolation of autologous and allogeneic hCSCs will be scarcer in a near future. Efforts have been made throughout the years to obtain CSCs from pluripotent stem cells (PSC-CSCs).

The first study reporting PSC-CSCs (Isl-1^+) was performed using murine ESCs, demonstrating the proliferative capacity of these derived CSCs on a feeder layer of mesenchymal cells (Moretti *et al.*, 2006). Similarly, in following studies, human PSC (hPSC) were differentiated into CSCs either through an embryoid body (EB)-based spontaneous differentiation step originating Isl-1^+ hCSCs (Bu *et al.*, 2009) or a bone morphogenic protein 2 (BMP-2) directed differentiation originating stage specific embryonic antigen-1 (SSEA1^+) hCSCs (Tomescot *et al.*, 2007; Blin *et al.*, 2010). Both types of hPSC-CSC have shown similar regenerative potential to the CSCs isolated from human fetal hearts in both rat and non-human primate models. More recently, these advances in the differentiation of hPCS-CSCs led to the translation from bench scale to a cell-based medicinal product (Menasché, Vanneaux, Fabreguettes, *et al.*, 2015) culminating in the ongoing clinical trial with hESC-CSCs for patients with severe heart failure (ESCORT Trial, table 1.1 page 12). More recently, efforts have been made to achieve more efficient protocols for differentiation and expansion of CSCs

from hPSCs in a chemically defined medium under feeder- and serum-free culture conditions resulting in high purities of SSEA1^{+} mesoderm posterior protein 1 positive (MESP1^{+}) cells (Cao *et al.*, 2013).

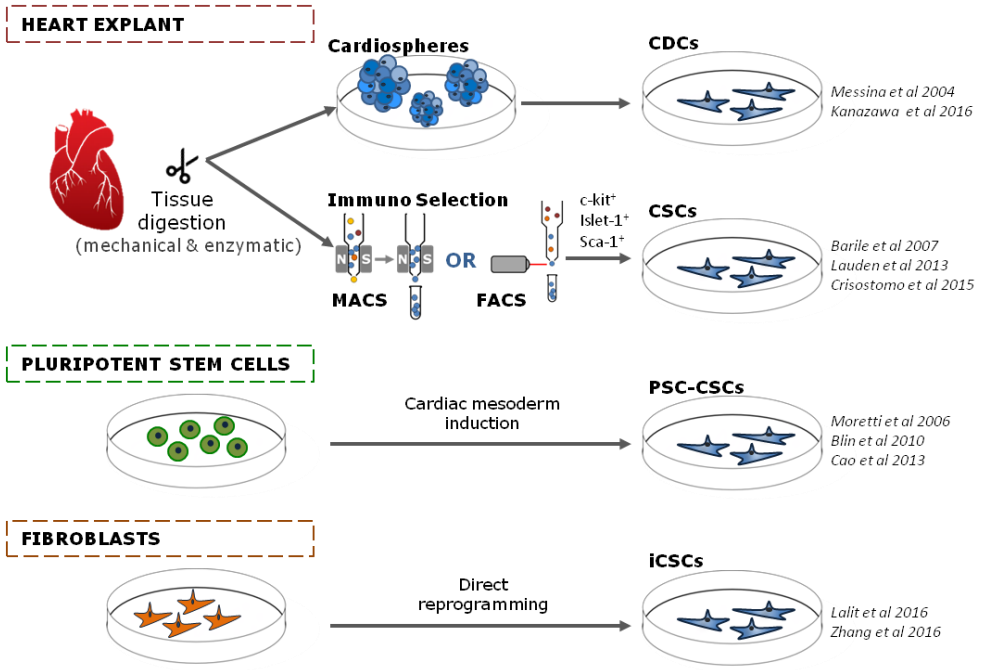

Figure 1.2. Sources currently available for obtaining cardiac stem/progenitor cells (CSCs). Abbreviations: Magnetic activated cell sorting (MACS); Fluorescence activated cell sorting (FACS); Cardiosphere derived cells (CDCs); Pluripotent stem cell derived cardiac stem cells (PSC-CSCs); induced cardiac stem cells (iCSCs). Examples from literature are included. Adapted from Sebastião *et al* 2018.

Several questions still arise today on how these hPSC-CSCs compare to the CSCs isolated from the human heart. Within this context, several studies have been trying to bring light on the different cardiac progenitors generated during heart development, and which signaling pathways may be manipulated to obtain those (Birket *et al.*, 2015). In a more recent study, global transcriptomic analysis of patient epicardium-derived CSCs and hPSC-CSCs was carried out showing that more than three thousand genes were differentially expressed between the two cell types, with hierarchical clustering analysis denoting a pronounced separation between the two

types of CSCs (Synnergren *et al.*, 2016), confirming the phenotypic differences between these two populations.

Other innovative strategies for the generation of CSCs have emerged through direct reprogramming of fibroblasts (induced CSCs, iCSCs) (figure 1.2). These iCSCs have been described to i) express cardiac signature genes, ii) have extensive proliferative capacity, and iii) be able to differentiate into the three main cardiac lineages *in vitro* and *in vivo* after transplantation into infarcted mouse hearts (Lalit *et al.*, 2016; Zhang *et al.*, 2016). Although these iCSCs have shown promising *in vivo* regeneration capacity, their direct comparison to isolated endogenous CSCs or hPSC-CSCs is still lacking. In addition, these studies were performed using murine cells and further studies with human cells are still required (Chen and Wu, 2016).

4.2. Cardiac stem cells regenerative mechanisms

Endogenous CSCs play important roles in cardiac tissue cell turnover, as well as in response to physiological stress, including I/R injury. Numerous studies suggest that these cells become activated after injury, having an immuno-supressive role, proliferating, differentiating into cardiomyogenic lineages, migrating to the site of injury, and secreting important paracrine factors involved in the modulation of cell proliferation, angiogenesis, vasculogenesis and pro-survival of CMs (figure 1.3).

Although one of the hallmarks used to define CSCs is their ability to differentiate *in vitro* into the three major cells in the myocardium (CMs, SMCs and ECs), doubts and controversy still exist regarding the ability of endogenous and transplanted CSCs to differentiate *in vivo* upon injury. While some authors defend that endogenous CSCs can differentiate *in vivo* into functional cardiomyocytes, contributing to tissue regeneration (Ellison *et*

al., 2011, 2013; Vicinanza *et al.*, 2017, 2018), others defend that CSCs differentiation to cardiac lineages occurs at a very low rate, making it unlikely to be the main mechanism for CSCs-induced tissue recovery(van Berlo *et al.*, 2014, 2018). Additionally, the origin of new CMs during tissue homeostasis and upon injury is still a matter of debate, with different authors defending different origins, ranging from CSCs to division of pre-existing CMs (Senyo *et al.*, 2013; Sereti *et al.*, 2018), reviewed in more detail in (Cahill, Choudhury and Riley, 2017).

Despite such doubts regarding the differentiation capacity of CSCs, there is a general consensus in the scientific community regarding CSCs-derived paracrine mechanisms. CSCs have been shown to exert potent paracrine regenerative effects both *in vivo* and *in vitro* (Li et al. 2012; Sharma et al. 2016; Miyamoto et al. 2010; Torella et al. 2007). Upon injury, CMs activate the expression and secretion of a large number of growth factors and cytokines (e.g. IGF-1, HGF, stem cell factor SCF and stromal cell derived factor 1 SDF-1) (X. Li *et al.*, 2014a) that bind to receptors expressed by CSCs (Gomes-Alves et al. 2015; Ellison et al. 2012; Li et al. 2014; Urbanek, Rota, et al. 2005). Consequently, an auto/paracrine cross-talk is established between CMs and CSCs, leading to a continued production of growth factors by CSCs, which have been shown to contribute to the modulation of angiogenesis, vasculogenesis, pro-survival of CMs and activation of CSCs proliferation and migration as well.

Cardiac stem cell secretome studies have revealed that these cells secrete a large array of signaling molecules, including the growth factors IGF-1, EGF, VEGF, transforming growth factor beta (TGF-β), HGF and several interleukins and chemokines (Stastna *et al.*, 2010; Albulescu *et al.*, 2015; Sharma *et al.*, 2015; Park *et al.*, 2016; Torán *et al.*, 2017). Several of these cytokines and growth factors have shown to induce CSCs proliferation *in vitro* and *in vivo*, namely IGF-1 (Ellison *et al.*, 2011, 2012; Koudstaal *et al.*,

2014; Waring *et al.*, 2014), EGF (Aghila Rani and Kartha, 2010), and connective tissue growth factor (CTGF) (Stastna and Van Eyk, 2012). CSCs are also proposed to exert potent cytoprotective effects in cardiomyocytes through factors such as IGF-1 (Linke *et al.*, 2005; Kawaguchi *et al.*, 2010; Miyamoto *et al.*, 2010), HGF (Linke *et al.*, 2005) and VEGF (Miyamoto *et al.*, 2010). Other factors secreted by CSCs have also been shown to promote neovascularization in infarcted hearts, including the pro-angiogenic factors VEGF (Miyamoto *et al.*, 2010; Wang *et al.*, 2014), TGF-β (Park et al. 2016; Ellison et al. 2012) as well as several interleukins (Valiente-Alandi *et al.*, 2016). Exosomes have also emerged recently as a novel player in CSC paracrine regenerative properties, with observed cardiac functional improvement after administration of CSC-derived exosomes in mouse (Ibrahim, Cheng and Marbán, 2014), rat (Barile *et al.*, 2014) and pig (Gallet *et al.*, 2016; Nguyen *et al.*, 2018) infarcted myocardium. Several micro-RNAs have been described as having a role in this exosome-mediated protection, including miR-210, miR-132 and miR-146, miR-181 and miR-451 (Chen *et al.*, 2013; Barile *et al.*, 2014, 2017; Mol, Goumans and Sluijter, 2017).

Upon AMI, CSCs also home and migrate to the site of injury, a process activated by ischemia through hypoxia-inducible factor 1α (HIF-1α) transcription factor and SDF-1 (Ceradini *et al.*, 2004; Rota *et al.*, 2008). During myocardial infarction, there is a sudden elevation of SDF-1 levels in the myocardium, which improves CSC homing to the site of injury (Penn *et al.*, 2012). However, the repair potential of endogenous CSCs is thought to be restricted by the short half-life of SDF-1 in the myocardium due to its degradation by proteases such as DPP4. In mice, treatment with DPP4 inhibitors has shown to improve cardiac function after myocardial injury (Zaruba and Franz, 2010). In addition to SDF-1, other molecules have showed to have an effect on CSC migration, including granulocyte

chemotactic protein 2 (GCP-22/ CXCL6) (Torán *et al.*, 2017), EGF and HGF (Urbanek *et al.*, 2005; Boucek *et al.*, 2015).

Cardiac stem cell immunologic studies have also revealed that these cells have immunosuppressive effects, including the inhibition of T-lymphocyte proliferation (Lauden *et al.*, 2013; van den Akker *et al.*, 2018), by directing natural killer cells to secrete anti-inflammatory cytokines (Boukouaci *et al.*, 2014), as well as by modulation of monocytes, macrophages and dendritic cells towards an anti-inflammatory phenotype (Dam *et al.*, 2017).

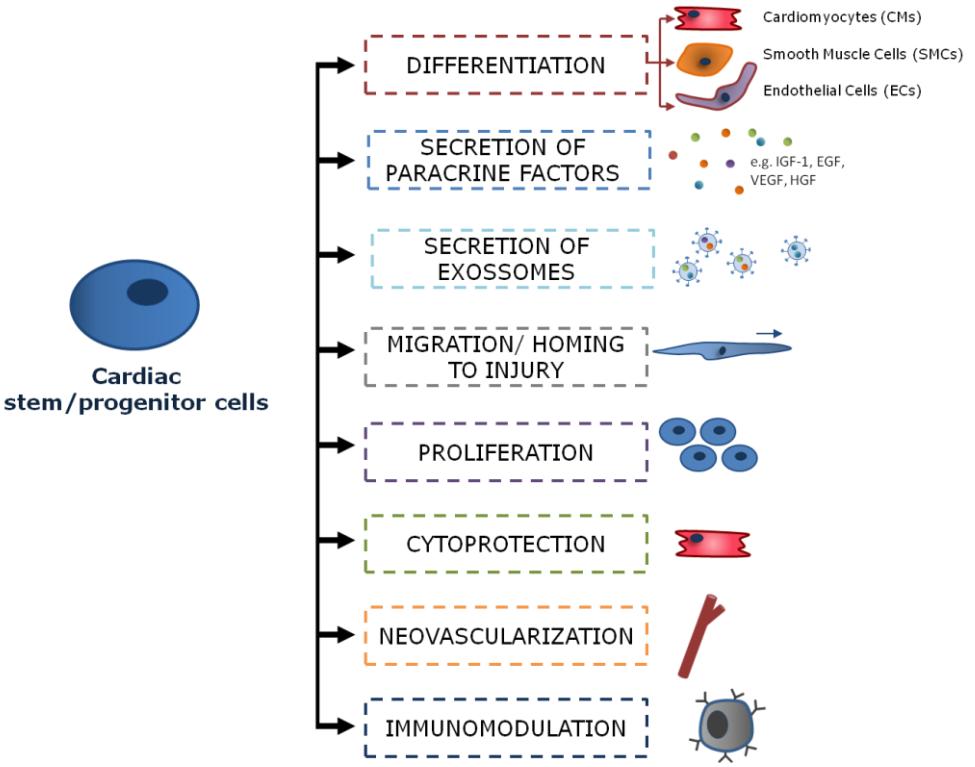

Figure 1.3. Overview of the main regenerative properties described for cardiac stem/progenitor cells (CSCs) in cardiac regeneration and repair. CSCs are described to be able to differentiate into the three main cardiac lineages, secrete an array of paracrine factors and exosomes, proliferate, migrate to the site of injury, protect the endogenous myocardial tissue, promote neovascularization and exert immune-suppressive roles. Adapted from Sebastião *et al* 2018.

5. Cardiac Fibroblasts: key players in heart homeostasis and disease

Besides CSCs and CMs, other myocardial cell populations including endothelial cells (ECs), SMCs and cardiac fibroblasts (CFs) play key roles in I/R pathology.

Namely, CFs are one of the most numerous non CM cell types in the human heart. While most studies account them for representing about 30-60% of the total cell number in myocardial tissue (Camelliti, Borg and Kohl, 2005; Krenning, Zeisberg and Kalluri, 2010), a more recent study, using a combination of genetic tools and cellular markers, points to percentages from 5-10 % (Pinto *et al.*, 2016). Regardless of their percentage in the heart, CFs play key roles both in homeostasis and in response to injury. CFs are the main cell type involved in synthesis and turnover of extracellular matrix (ECM), function as local immune modulators, and are a constituent part of the cardiac electrophysiology network (Furtado, Costa and Rosenthal, 2016). Upon myocardial disease and injury, activated fibroblasts (myofibroblasts) become one of the predominant cell types in the healing of the infarcted area. Their origin is still debatable, with different authors defending different origins of myofibroblasts, including activation of resident cardiac fibroblast populations, cardiac pericytes modulation, endothelial to mesenchymal transdifferentiation, and recruitment of circulating fibroblasts (Saxena, Russo and Frangogiannis, 2016). Myofibroblasts proliferate and secrete an excess of ECM proteins (mainly collagen), preserving the integrity of the tissue, avoiding major tissue distension and rupture, and ensuring that coordinated beating function of the heart is maintained. This process is referred as fibrosis and can occur in response to CM death (reparative fibrosis) our in response to changes in myocardial load or inflammation (responsive fibrosis). Fibrotic tissue is stiffer and less conductive, which leads to impaired normal heart function, with increased

tissue workload and arrhythmias (Furtado, Costa and Rosenthal, 2016). Fibrosis is a pathological feature present in numerous forms of heart disease, including AMI and heart failure. It is estimated that about 45% of all deaths in the developed world involve cardiac fibrosis (Wynn, 2007).

5.1. Cardiac Fibroblasts molecular identity

Attending to their central role in cardiac fibrosis, a lot of attention has been draw to the study of CFs. Although a lot of progress has been made in the last years on unveiling CFs mechanisms in homeostasis and pathological settings (reviewed in Dostal, Glaser and Baudino, 2015), the lack of a proper molecular marker definition has been one of the major impediments to the characterization, isolation and study of this cell type both *in vivo* and *in vitro*. Some of the most commonly used markers for CFs are collagens, vimentin, tyrosine kinase discoidin domain receptor (DDR2) and Thy1 cell surface antigen (CD90). None of these molecules is neither expressed by all CFs neither unique for this cell type (Furtado, Costa and Rosenthal, 2016). Recently, a whole human heart proteome analysis revealed a panel of 25 cell membrane receptors shown to be preferably enriched in CFs, when compared to adipose fibroblasts, ECs and SMCs (Doll *et al.*, 2017).

6. Modeling myocardial I/R injury

Investigation of the molecular mechanisms underlying function and response of different heart cell populations requires suitable experimental models, both in homeostasis and in disease settings. These models can be divided in *in vivo* (animal models), *ex-vivo* (isolated whole heart preparations) and *in vitro* (cell cultures) (figure 1.4).

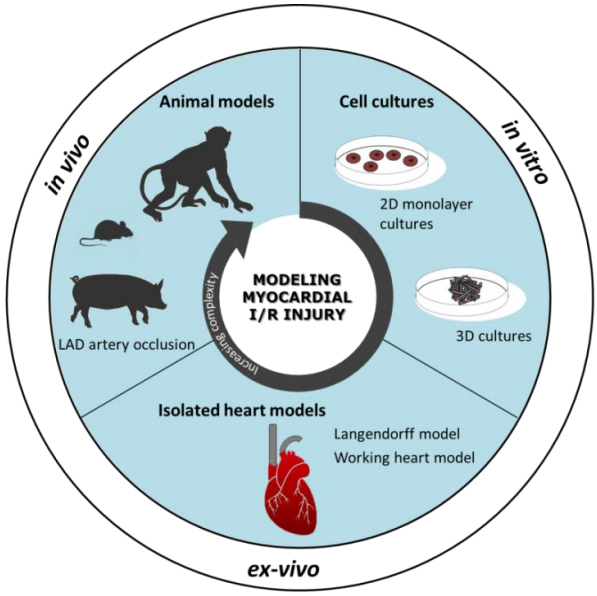

Figure 1.4. Main methodologies applied to model ischemia reperfusion (I/R) myocardial injury. The most commonly used *in vivo* models for IR injury include mouse, rat, pigs and non-human primates. Ex-vivo heart preparations from mouse and rats are also used to study acute myocardial biology. *In vitro* models rely on the use of primary cultures or cell lines from murine origin or on human pluripotent derivatives. Cells can be cultured as monolayers (two dimensional, 2D) or in three-dimensional (3D) setups.

Cardiovascular research has traditionally relied on animals to study AMI and I/R injury. In both small and big animal models, the most common methodology to recapitulate AMI is the occlusion of the left anterior descending coronary (LAD) artery, either by suture ligation (open-chest models) or balloon stent inflation (closed-chest models) (Fliegner *et al.*, 2015). Small animals such as mice and rats are often used since they are easy to house, have a short gestation time and many genetically modified variants are available. However, despite their widespread use, the small size of murine hearts makes them physiologically distant to humans and mice hearts have been consistently reported to be more tolerant to drugs. In fact, cardiotoxicity is still one of the main causes for therapy withdrawal during clinical trials (Han, Zhou and Liu, 2017). Larger animals, such as pigs and non-human primates resemble better human cardiac physiology and

have a higher translational predictive power in terms of drug safety. *Ex-vivo* approaches are also employed, namely the Langendorff and the working heart model, that consists on the perfusion of isolated whole hearts (usually from mouse or rat) with plasma or blood like medium. Such models have the advantage of allowing to impose different degrees of ischemia and flow rates, also allowing for studies on drug dose responses, as their concentration can be more accurately controlled than in a whole animal model (Vidavalur *et al.*, 2008).

Although preclinical animal models represent a biological complexity level closer to humans, and have unquestionable value in AMI cardiovascular research, their predictive power is still limited: about 90% of candidate therapies that showed efficacy in preclinical studies fail in human trials (Olson *et al.*, 2000), generating massive costs in the drug development chain. Moreover, the logistical, and financial restraints of *ex*-vivo and animal models, associated with the growing ethical implications represented by the concepts of the 3Rs (replacement, reduction and refinement), make them not amenable for high-throughput studies in earlier phases of drug development. The inherent cell complexity of tissues also makes it difficult to interpret single cell or single cell-population mechanisms.

6.1. *In vitro* models

The inherent limitations of animal studies and lack of translational success from preclinical to clinical trials illustrates the need for the development of additional platforms for filtering therapeutic targets and better understanding pathology mechanisms of action. *In vitro* models provide a complementary approach to *in vivo* and *ex vivo* models and support earlier stages of research, being cheaper, faster and with higher-throughput.

6.1.1. Cell sources

When modeling cardiovascular systems *in vitro*, murine cell populations have been extensively used throughout the years. This includes primary fetal, neonatal and adult CMs, mouse atrial HL-1 cells and rat ventricular H9c2 cell lines (Parameswaran *et al.*, 2013). Fetal and neonatal CMs are easier to maintain in culture and are very useful to study adaptive mechanisms and cardiac development. However, fetal and neonatal cells have a highly glycolytic metabolism, while adult CMs metabolism depends more heavily in fatty acids, rendering neonatal cells more resistant to hypoxia and I/R injury (Robertson, Tran and George, 2013). In fact, after removal of the cardiac apex or coronary artery ligation-induced AMI, the neonatal mouse heart has been reported to be almost entirely regenerated, a capacity that seems to be limited to the first 7 days after birth, coinciding with postnatal maturation of the mouse heart (Soonpaa *et al.*, 1996; Porrello *et al.*, 2011). HL-1 is a cell line that can continuously divide while maintaining a cardiac phenotype and spontaneous contractility *in vitro*. However, the metabolism and phenotype of these cells is significantly different from primary adult CMs, including key aspects of mitochondrial bioenergetics (Kuznetsov *et al.*, 2014). H9c2 are rat myoblasts, with a phenotype very distinct from adult differentiated CMs (Hescheler *et al.*, 1991).

Murine and human cell physiology greatly differs at the cellular and molecular level. For example, repolarization of the cardiac action potential is governed by different ionic channels in mice and human. Also, although highly conserved at the exons, human and mice only share 50% of similarity at the intron level (Yue *et al.*, 2014), which translates into major differences in miRNAs, already shown to have an important role at cardiovascular homeostasis and pathological processes (Roux, Gonzàlez-Porta and Robinson-Rechavi, 2012). As previously mentioned (section 4.1.5), the

advances in hPSCs, including hiPSCs and hESCs has accelerated human cell biology research *in vitro*. Similarly with hPSC-derived CSCs, many strategies have been explored in the differentiation of CMs from hPSCs, including cultivation as embryoid bodies (EBs, 3D PSCs aggregates formed in suspension) (Kehat *et al.*, 2001) and small-molecule based differentiation strategies that focus on the modulation of key signaling pathways (e.g., BMP, TGF/Activin/Nodal and Wnt) (Talkhabi, Aghdami and Baharvand, 2016). However, most protocols for cardiac differentiation yield fetal-like CMs, presenting immature electrophysiological properties, a highly glycolytic metabolism, disorganized sarcomeres and expression of typical cardiac fetal genes (Tan and Ye, 2018). Similar to what is described for fetal and neonatal murine cells, immature hPSC-CMs are more resistant to hypoxia and I/R injury. In fact, by adding a fatty acid-based metabolic maturation phase, Hidalgo *et al* were able to significantly decrease the resistance of hESC-CMs and hiPSC-CMs to an I/R injury (*in vitro* setup) (from 5% cell death at day 16 of differentiation to 30% of cell death after an extra 8-day maturation phase) (Hidalgo *et al.*, 2018) (table 1.2).Besides modulation of metabolism (Correia *et al.*, 2017; Hidalgo *et al.*, 2018), other strategies have been applied to improve the maturation state of hPSC-CMs, including long term culture (Lundy *et al.*, 2013; Wei *et al.*, 2017), electrical and mechanical stimulation (Xia *et al.*, 1997; Ronaldson-Bouchard *et al.*, 2018), co-culture with other cell types (Kim *et al.*, 2010), culture as 3D aggregates (Correia *et al.*, 2018) and hormone supplementation (Yang *et al.*, 2014; Ribeiro *et al.*, 2015). Although many advances have been made in generating more physiological relevant hPSC-CMs, there are still few studies that utilize human cell populations to study I/R injury *in vitro* (table 1.2).

Table 1.2. Summary of studies using *in vitro* I/R injury setups to study human cardiac cell populations

Cell population	Maturation Strategy	Culture format	I/R Injury Setup		I/R Injury effect on cells	Major outcomes	Ref.
			Ischemia	Reperfusion			
hCSC-CMs	not performed	2D planar culture	2 or 14 h incubation in hypoxic chamber (95% N_2, 5% CO_2); glucose-free and serum-free culture medium.	2 h incubation in normoxic conditions (95% air, 5% CO_2); complete culture medium.	Increase in percentage of apoptotic cells; activation of endoplasmic reticulum stress response.	Levosimendan drug protects CMs from I/R injury and ER-stress.	Li *et al* 2014
hESC-CMs	not performed	Embryoid bodies	2.5 h incubation in hypoxic atmosphere (95% N_2, 5% CO_2); nutrient-free and serum-free HEPES-based salt solution with 20mM lactate and pH=6.4.	2 h incubation in normoxic conditions (95% air, 5% CO_2); complete culture medium.	Increase in percentage of apoptotic cells.	SNAP (nitric oxide chemical donor) protects CMs from I/R injury cell death.	Pálóczi *et al* 2016
hiPSC-CMs	not performed	2D planar culture	2 h incubation with 100 µM H_2O_2 and 10 µM 2-deoxy-glucose (glycolysis inhibitor).	4 h incubation in complete culture medium.	Increase in LDH release (measurement of cell death).	ROS scavenging inhibition restore isoflurane cardioprotective effects in elevated glucose conditions.	Canfield *et al* 2016
hiPSC-CMs	Long culture time: 40-50 days of differentiation	2D planar culture	45 min incubation in hypoxic chamber (95% N_2, 5% CO_2); nutrient-free and serum-free salt solution.	3 h incubation in high oxygen conditions (95% O_2, 5% CO_2) in complete culture medium	Increase in apoptosis; decrease in ATP production; ROS generation; abnormal Ca2+ transients.	Danshen (herbal medicine) protects CMs from I/R injury cell death, improving Ca^{2+} handling.	Wei *et al* 2017
hESC-CMs	not performed	2D planar culture	4 h incubation in serum-free culture medium, followed by 16 h of incubation in hypoxic atmosphere (95% N_2, 5% CO_2, 0.5% O_2).	10 h incubation in high oxygen conditions (95% O_2, 5% CO_2) in complete culture medium	Increase in apoptosis.	THF drug protects CMs from I/R injury cell death.	Li *et al* 2017
hESC-CMs and hiPSC-CMs	8 day metabolic maturation with fatty acids	2D planar culture	2 h incubation in hypoxic incubator (0% O_2) HEPES-based salt solution pH=6.2 with or without glucose.	4 h incubation in normoxic conditons (20% O_2) in solution with glucose.	Increase in LDH release (measurement of cell death); Increase in apoptotic cells.	Novel I/R model combining mature hPSC-CMs, low pH and glucose availability during ischemic phase.	Hidalgo *et al* 2018

Abbreviations: human cardiac stem cell-derived cardiomyocytes (hCSC-CMs); human embryonic stem cell derived cardiomyocytes (hESC-CMs); human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs); two dimensional (2D); Ischemia/ Reperfusion (I/R); Reactive Oxygen Species (ROS).

6.1.2. Mimicking AMI physiology

Both with murine and human cell populations, different strategies have been applied throughout the years to mimic and study AMI physiology in the lab. One of the most conventional and employed strategies to reproduce ischemia *in vitro* is by placing the cells at low oxygen (usually ranging from 1-4% of oxygen), in oxygen depleted hypoxic chambers (P. C. Li *et al.*, 2014; Wei *et al.*, 2017), analyzing them directly (without reperfusion phase) or after re-establishing oxygen levels at atmospheric oxygen (21%) (Pálóczi *et al.*, 2016; Chen *et al.*, 2018). In order to mimic the lack of nutrients in the ischemia phase, cells can also be incubated in medium with glycolysis inhibitors (Canfield *et al.*, 2016), or in serum-free and nutrient depleted media (Li, Wang and Tang, 2017; Wang *et al.*, 2017). Other parameters, such as osmolarity, pH and lactate concentration have also shown to be important to efficiently mimic I/R. For instance, Lu *et al* identified lactate concentration as a critical contributor to cardiac ischemia injury, when using rat primary CMs (Lu *et al.*, 2005).

6.1.3. 3D culture strategies

Most strategies for *in vitro* I/R modeling are typically performed using 2D planar systems, usually known as monolayers. 2D cell cultures are well accepted and have significantly contributed to the advance of our understanding on different cardiac cell populations response to I/R injury, and on elucidating many associated pathways. However, these strategies fail to recapitulate the *in vivo* cellular organization and microenvironment, where cells are embedded in a 3D tissue structure, presenting extensive networks of cell-cell and cell-extracellular matrix (ECM) interactions (reviewed in Serra *et al.*, 2012). Although being more difficult to analyze, 3D *in vitro* cardiac models allow for enhanced cell-cell and cell-ECM contacts, establishment of molecular concentration gradients and have

shown to better recapitulate physiologic electrical and contractile function when comparing to 2D cultures (Zhang *et al.*, 2013; Daily *et al.*, 2015; Correia *et al.*, 2018). Currently used *in vitro* 3D models for cardiovascular research include scaffold-free aggregates and culture with biomaterial-based approaches such as scaffolds, hydrogels and ECM-derived matrices (reviewed in Ryan et al. 2016). Culturing cells as aggregates is one of the most commonly employed method, since they are relatively easy to maintain and handle, allowing cells to self-assemble, the secretion and accumulation of endogenous ECM and the generation of molecular gradients along the construct. Recently, Correia et al demonstrated that culturing iPSC-CMs in 3D aggregates resulted in improved cell metabolic maturation and cardiac functionality, when compared to the same differentiation and maturation methodology used in 2D monolayers (Correia *et al.*, 2018).

There are few studies that utilize 3D cardiac models to study I/R injury *in vitro*. In 2010, Katare *et al* embedded neonatal rat CMs in a ring-shapped collagen-I scaffold with induced mechanical stretching, and studied the construct response to ischemia (Katare *et al.*, 2010). More recently, a 3D culture system formed by multiple stacked layers of paper containing hydrogel-suspended primary rat neonatal CMs and CFs was developed by Mosadegh *et al* to study CMs and CFs paracrine interaction upon ischemic stress (Mosadegh *et al.*, 2014).

6.1.4. Bioreactors

Several methodologies have been used to culture 3D models, including the hanging-drop method, micro-fluidic supports, non-adhesive substrates and stirred culture systems (Fennema *et al.*, 2013). Stirred culture systems such as spinner flasks and stirred-tank bioreactors allow cell cultivation in a homogenous environment, with improved gas and nutrient transfer

properties over static culture systems. Stirred tank bioreactors can be operated in different culture feeding modes (batch, fed-batch, perfusion) and also allow for non destructive sampling and precise control and monitoring of the culture environment parameters such as oxygen level and pH (reviewed in Serra *et al.*, 2012), especially relevant in the context of I/R. Bioreactor technology as already been used to modulate ischemia in brain (Santos *et al.*, 2005; Amaral *et al.*, 2010), and liver models (Allen and Bhatia, 2003), but still no study has been performed to modulate cardiac I/R injury.

There are still some disadvantages associated with cell culture in stirred culture bioreactors, namely the “hydrodynamic stress” undertaken by cells caused by the agitation to the medium causes the formation of small areas of intense turbulence, causing localized shear stress on cells. Animal cells lack a cell wall, being therefore sensitive to these forces. Such hydrodynamic effects can range from sublytic cell responses, such as slower growth, slower protein synthesis, or denaturation of membrane proteins, to cell apoptosis and disruption. In the case of stem cells, changes in differentiation potential may also occur (Zhiwei, Boyan and Yang, 2012).

7. Proteomics as powerful tool for characterization of cardiac populations

Proteins integrate and control essentially all cellular processes by exerting enzymatic catalysis, molecular signaling and structural functions. Protein-based knowledge is essential for the understanding of cellular pathways that occur under physiological and pathological conditions. In this context, the study of protein expression, localization, post-translational modifications, and interactions, collectively referred as proteomics, has become a powerful analytical tool for unveiling cellular mechanisms in

several cardiovascular contexts, including *in vivo* tissue and *in vitro* cardiac cell models.

Proteomic studies can be divided into targeted and non-targeted global approaches. While target methods involve the examination of hypothesized or known targets, global non-targeted methods are discovery-based and hypothesis-free, providing an unbiased insight into physiology or pathology, often leading to the discovery of novel molecular mechanisms and biomarkers. One of the most challenging aspects of non-targeted proteomics is the reliable and consistent identification of low abundant proteins. Advances in separation techniques (including liquid chromatography, LC), mass spectrometry (MS), bioinformatics tools and the existence of more robust and complete sequence and annotation databases, allow the identification of thousands of proteins per MS run, with increased sensitivity and consequently high proteome coverage. For example, large proteomic datasets of mouse (8,064 identified proteins, Lau *et al.*, 2016) and human (10,700 identified proteins, Doll *et al.*, 2017) heart tissue have been recently reported.

A typical workflow for non-targeted, label-free proteomics is shown in figure 1.5. Briefly, after extraction from biological samples, proteins are enzymatically digested (usually with trypsin), and the peptide mixture is desalted and loaded into a LC column for further separation. Usually, LC columns are connected on-line with MS equipments, were peptides are ionized, guided by electric fields, isolated and further fragmented (in MS/MS) and separated according to their mass over charge (m/z) ratio. MS_1 (intact ion peptides) or MS/MS (peptide ion fragments) m/z spectra are generated and compared with aminoacid sequences and theoretical spectra by bioinformatics tools (Lam *et al.*, 2016).

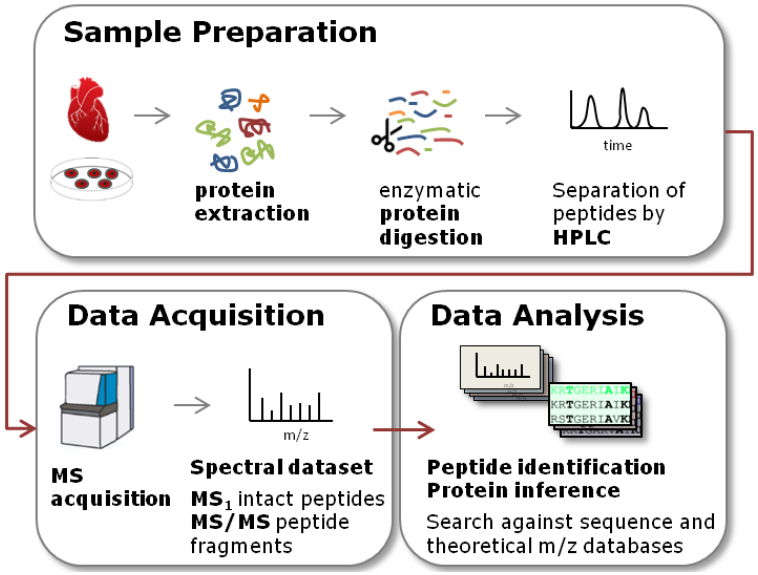

Figure 1.5. Typical workflow of non-targeted proteomic approaches. Biological samples derived from tissues or cell cultures are processed to extract the proteins which are then digested to peptides. The generated peptide mixture is desalted and separated using a high performance liquid chromatography (HPLC) column, which is usually directly coupled to a mass spectrometer (MS) instrument. In MS, peptides are ionized and mass to charge ratio (m/z) spectra are generated. Finally, the peptide sequencing data obtained from the m/z spectra are searched against sequence and theoretical spectra databases for protein identification.

Although the intensity of a peptide ion in a spectrum does not directly correspond to the quantity of the parent protein in the sample (due to peptide-inherent variability in accessibility to the protease, solubility and ionization efficiency), relative quantifications can be inferred by the peak height of the same peptides in different samples (Lam *et al.*, 2016). Also, labels (such as stable isotopes) can be added to one of the experimental conditions in order to get a more accurate relative quantification (for example SILAC, stable isotope labeling in cell culture; ICAT, isotope-coded affinity tag techniques; ICPL, non-selective isotope-coded protein labeling; iTRAQ, isobaric tags for relative and absolute quantitation and TMT, tandem mass tag, reviewed in DeSouza and Siu, 2013; Hsu and Chen, 2016). Label-free approaches are cheaper than labeling approaches, making high-

throughput analyses more feasible. One example of a recent label-free and high-throughput approach is SWATH-MS (sequential window acquisition of all theoretical mass spectra). This method combines data independent acquisition (DIA), sequential m/z windows and spectral libraries. Peptide ions are fragmented and identified (MS/MS) independently (DIA) of precursor peptide ions (prior to fragmentation, MS_1 scan) information, therefore generating spectra for all generated peptides in defined sequential m/z windows. A pre-selected and pre-analyzed peptide library is used to compare with the spectral datasets and for quantification.

Several authors have taken advantage of non-targeted, label-free MS-based proteomics approaches to characterize different cardiac populations in different settings, including *in vitro* studies on murine CSCs (Stastna *et al.*, 2010), hCSC receptome(Gomes-Alves *et al.*, 2016) and secretome (Sharma *et al.*, 2017; Torán *et al.*, 2017), *in vitro* characterization of rat H9C2 response to I/R injury (X. Li *et al.*, 2014a) and quantitative studies of mouse models in response to AMI (Datta *et al.*, 2017) and human heart tissue without injury (Doll *et al.*, 2017).

8. Aims and scope of the thesis

The main goal of this PhD thesis was to develop novel relevant myocardial I/R injury *in* vitro models and to use advanced proteomic tools to advance the knowledge on human cardiac cell populations, including endogenous hCFs, endogenous hCSCs and hiPSC-CMs. LC-MS proteomic methodologies were used to provide a comprehensive description of hCF membrane molecular landscape and also to unveil hCSC and hiPSC-CM mechanisms of action in a AMI context. The major goals of this thesis and specific aims of each chapter are represented in figure 1.6.

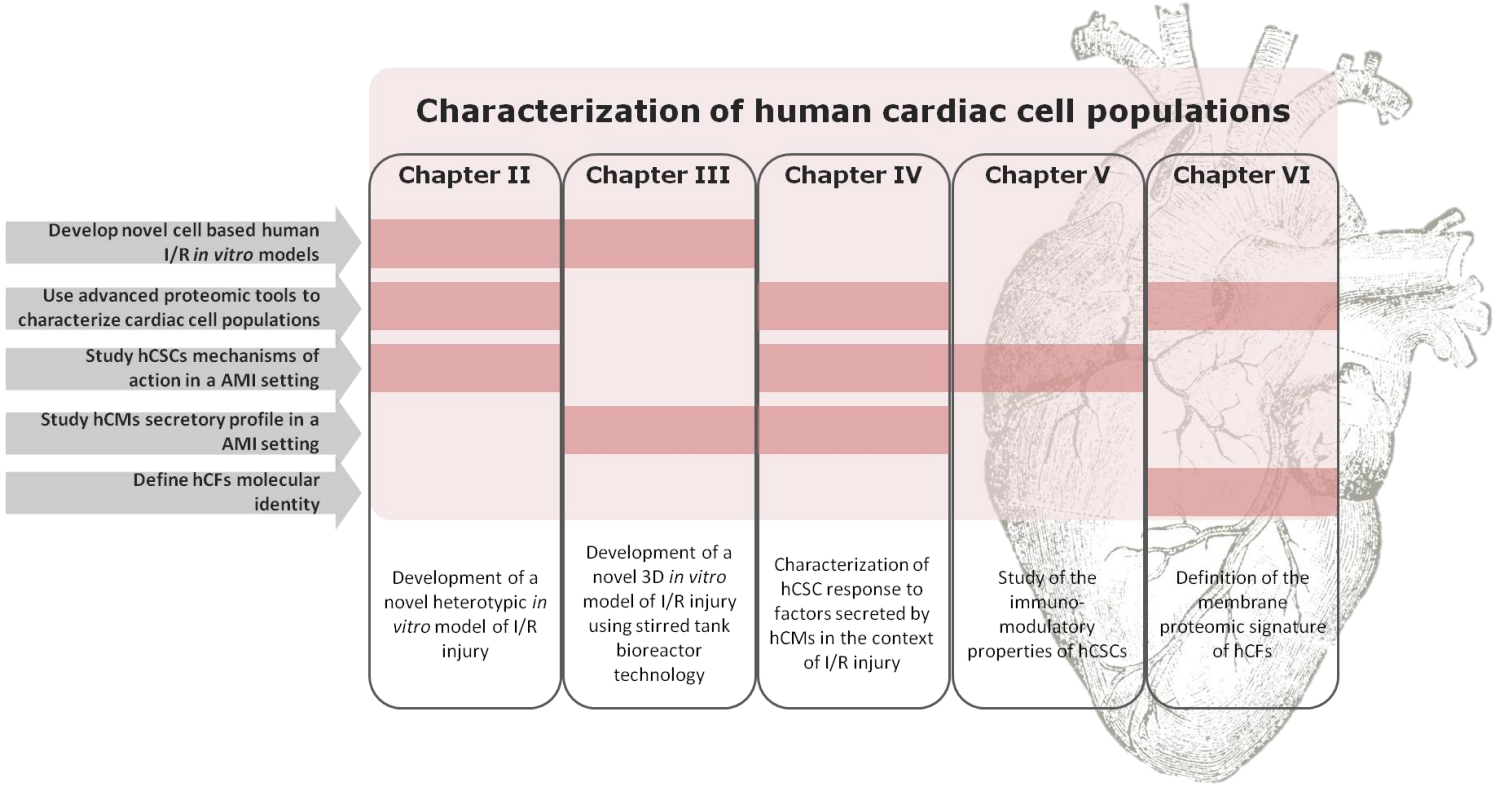

Figure 1.6. Thesis overview and main aims. Dark pink stripes represent the association of each main aim (on the left) in each chapter. I/R: Ischemia/Reperfusion; AMI: acute myocardial infarction; hCSCs: human cardiac stem cells; hCMs: human cardiomyocytes; hCFs: human cardiac fibroblasts.

The development of an *in vitro* cell model of myocardial I/R injury using a heterotypic co-culture approach of hiPSC-CMs and hCSCs to study hCSC response in an AMI context was the aim in **Chapter II**. A MS-based protocol was explored for the characterization of hCSC whole proteome upon injury, to provide valuable insights on how an insult situation readjusts cellular pathways, directing hCSCs to modulate proteins involved in several pathways such as cell cycle regulation, proliferation through EGF signaling and reactive oxygen species detoxification.

Another I/R injury *in* vitro model is described in **Chapter III**, we take advantage of stirred-tank bioreactor technology to fully monitor and control environmental parameters such as pH and dissolved oxygen during an I/R setup. In this model, the physiological relevance is addressed by hiPSC-CM 3D culture strategy. The conditioned medium from these experiments was then used in the culture of hCSCs, and their response to hiPSC-CM spheroid secreted paracrine factors was analyzed in terms of whole-proteome quantitative profiling using quantitative SWATH technology **(Chapter IV**). In **Chapter V**, a different aspect of hCSC biology is studied, namely the immunomodulatory capacity of these cells, important in the context of hCSC-transplantation strategies upon AMI. **Chapter VI** focused on hCFs. In this chapter, a characterization of hCFs membrane proteins was performed, with the aim of creating a dataset that could be the basis for the identification of novel hCF specific markers. Finally, in **Chapter VII**, the results obtained along Chapters II-VI were discussed and contextualized with the literature.

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Chapter II

Modeling acute myocardial infarction *in vitro*: using a 2D co-culture system to study human cardiac stem cells activation and regeneration mechanisms

This Chapter was adapted from:

Sebastião, M.J., Pereira, R., Palacios, I., Serra, M., Gomes-Alves, P., Alves, P.M. “Myocardial ischemia/reperfusion injury human *in vitro* model to study human cardiac stem cells activation and regeneration mechanisms.” (*submitted*).

Author Contribution

Maria João Sebastião participated in the experimental setup and design, performed the experiments, data analysis and wrote the chapter.

Abstract

Human cardiac stem/progenitor cells (hCSCs) have a recognized role in myocardial repair upon ischemia/ reperfusion (I/R) injury, mainly through auto/paracrine signaling. However, the mechanisms underlying this response are still poorly understood. To further investigate hCSC regenerative process, we established the first *in vitro* human heterotypic model of myocardial I/R injury using hCSCs and human induced pluripotent cell-derived cardiomyocytes (hiPSC-CMs). This model recapitulates hallmarks of I/R, namely hiPSC-CM death upon insult, protective effect of hCSCs on hiPSC-CM viability (37.6% higher vs hiPSC-CM mono-culture), and hCSC proliferation (approximately 3-fold increase vs hCSCs mono-culture), emphasizing the importance of paracrine communication between these two populations. In particular, in co-culture supernatant upon injury, we report higher angiogenic functionality as well as a significant increase in CXCL6 secretion rate, suggesting an important role of this chemokine in myocardial regeneration. hCSCs response to I/R was further characterized using mass-spectrometry tools allowing us to propose new pathways in the hCSCs-mediated regenerative process, including cell cycle regulation, proliferation through EGF signaling, and reactive oxygen species detoxification.

This work contributes with new insights into hCSC biology in response to I/R, and the model established constitutes an important tool to study the molecular mechanisms involved in the myocardial regenerative process.

Contents

1. Introduction

Acute Myocardial Infarction (AMI), is still a major cause of death in the world (Benjamin *et al.*, 2017). AMI consists on the cessation of blood flow, causing oxygen and nutrient supply imbalance, leading to myocardial tissue damage, with loss of cardiomyocytes (CMs). For AMI patients, the intervention of choice is immediate myocardial reperfusion with restauration of blood flow. However, this process can aggravate the damage, as the increase of molecular oxygen levels occurs at a toxic rate (Ischemia Reperfusion I/R Injury), contributing to up to 50% of the final scar tissue size (Hausenloy and Yellon, 2013). Current treatments are successful in reducing immediate mortality but do not avoid the subsequent scarring and degeneration of myocardium tissue with loss of contractile function, often leading to Chronic Heart Failure (CHF), a highly fatal condition in which the only available clinic option is heart transplant (Lund *et al.*, 2014).

Regenerative medicine based strategies for infarcted myocardium include autologous and allogeneic cell therapies. Several cell types were already applied in clinical trials, including bone-marrow derived mesenchymal stromal cells (BM-MSCs) (e.g. REPAIR-ACS-NCT00711542, BOOST-NCT00224536), adipose tissue derived mesenchymal stromal cells (AT-MSCs) (e.g. ADVANCE- NCT01216995, and APOLLO- NCT00442806), and cardiac stem/progenitor cells (CSCs) (e.g. SCIPIO- NCT00474461, CADUCEUS - NCT00893360, and CAREMI- NCT02439398). For all cell types, clinical trials have demonstrated some physiological improvements but very low cell retention after some weeks, suggesting that the overall beneficial effect of transplanted cells is due to paracrine modulation rather than differentiation and functional integration in the tissue (Madonna *et al.*, 2016). In fact, novel strategies focused on the induction of the

endogenous heart regenerative potential, such as direct growth factor administration, have shown to induce tissue regeneration by reduction of fibrosis, induction of angiogenesis, inhibition of apoptotic processes and recruitment of endogenous CSCs (Ellison *et al.*, 2011; Koudstaal *et al.*, 2014). Although in low percentages, with about one stem cell per 8000-20000 CMs (Anversa *et al.*, 2006), endogenous CSCs seem to play an important regenerative role in cardiac homeostasis and in response to physiological stress and I/R injury. While the physiological alterations undergone by CMs during I/R have been extensively covered, the mechanisms by which CSCs exert their protective role are still not well defined. Upon injury, there are increased levels of signaling growth factors and cytokines released in the myocardium (Torella *et al.*, 2007; Li *et al.*, 2014). Such signals have already been proposed to trigger CSCs proliferation, differentiation, migration to the site of injury and growth factor secretion that together have an effect on cardiomyocyte protection, reduction of inflammation and reduction of scar tissue size. Although some doubts and controversy still exist regarding the ability of CSCs to generate new CMs upon injury (Nadal-Ginard, Ellison and Torella, 2014; van Berlo *et al.*, 2014), the regenerative paracrine potential of CSCs has already been well documented in several animal studies (Urbanek *et al.*, 2005; Crisostomo *et al.*, 2015).

However, mainly due to the lack of relevant human models, the role of CSCs in I/R injury has been studied *in vitro* with murine cells. There are relevant gaps between murine and human cardiac physiology both *in vitro* and *in vivo*, such as different functionality of CMs ion channels, and a higher tolerability to drugs in mice and mice cells. Such differences can cause misinterpretation of the results and have already been pointed as one of the causes of high drug attrition rates (Denning *et al.*, 2016).

Aiming at filling the gap between murine and human *in vitro* models, in this work we have established the first *in vitro* human cell based myocardial I/R injury model, using human CSCs (hCSCs) and human induced pluripotent stem cell-derived CMs (hiPSC-CMs). The goal of our work is to use this co-culture model as a tool to characterize hCSC response to I/R injury, and its effect on CM death and survival upon injury. Our model was able to recapitulate important features of AMI, namely CM death, a paracrine protective effect of CSCs in CMs survival and CSC proliferation activation. For the first time, CXCL6, a cytokine with documented angiogenic properties, already identified as having an important regenerative role in both mesenteric and myocardial infarction was found to be highly secreted by hCSCs upon I/R injury in the co-culture condition. Moreover, we also demonstrated higher angiogenic potential of the co-culture supernatant upon ischemia. Human CSC whole proteome analysis showed that upon injury, and in the presence of hiPSC-CMs, there was an enrichment in proteins involved in pathways and functions related with cell proliferation, paracrine signaling, stress response and regeneration processes when comparing to control and mono-culture conditions.

2. Materials and Methods

2.1. Cell culture

CSCs were obtained from human right atria appendage myocardial tissue, isolated and characterized as described elsewhere (Lauden *et al.*, 2013). Cells were cultured at 37°C in humidified incubators (5% CO_2, 3% O_2) in CM001-R medium composed by DMEM:F12: Neurobasal medium (1:1), supplemented with 1% penicillin streptomycin, 10% fetal bovine serum embryonic stem cell-qualified, N2 supplement (1X), B27

supplement (1X), 0.9 mM l-glutamine, 50 µM β-mercaptoethanol (Sigma), insulin transferrin selenium (0.5X), 10 ng/mL bFGF, 20 ng/mL EGF-I and 30 ng/mL IGF-II (Prepotech), (all percentages in v/v). Medium was replaced by 50% every 3 days. Cells were subcultured when about 80% confluent using Tryple™ Select Enzyme for 5 minutes at 37°C. All cell culture reagents were purchased from Gibco, Life Technologies unless otherwise stated.

Human iPSC (DF19-9-11T.H, WiCell) were cultured and differentiated to CMs (hiPSC-CMs) as previously described (Correia *et al.*, 2016, 2018). Using this protocol, monolayer cultures composed of >90% of hiPSC-CMs were obtained after 15 days. To further improve maturation state of this cell population, hiPSC-CMs were cultured for additional 10 days in Pluricyte Medium (NCardia), as described elsewhere (Ribeiro *et al.*, 2015). Cells were maintained at 37°C in humidified incubators (5% CO_2, 95% air).

2.2. Ischemia/ Reperfusion Injury setup

I/R experiments were performed with: mono-cultures of hCSCs (plated at $2x10^4$ hCSCs/cm^2); mono-cultures of hiPSC-CMs (plated at 1-1.5$x10^5$ hiPSC-CMs/cm^2); and co-cultures of the two cell types (hCSCs:hiPSC-CMs 1:10 - 1:20) using Transwell® permeable insert supports (0.4 µm pore size, Corning). The insert supports are semi permeable polyester membranes separating both cell types allowing paracrine interaction. hCSCs were seeded in transwell supports (1.2 $x10^4$ hCSCs/ insert and positioned above hiPSC-CM culture dishes (1.2-2.4$x10^5$ hiPSC-CMs/ well) at the beginning of the ischemia phase, allowing a paracrine interaction between the two cell types during all the sequence of I/R injury. All cell populations were cultured in CM001-R at 3% O_2 (myocardial physiologic normoxia) in humidified incubators (5% CO_2,

95% air) at least 15 h before I/R experiments. I/R injury experimental setup is illustrated in figure 2.1.

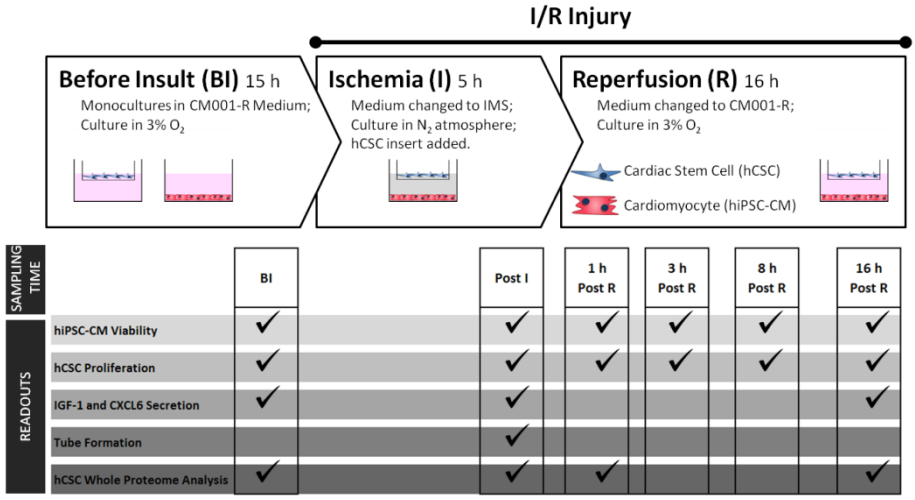

Figure 2.1. Schematic representation of Ischemia/Reperfusion injury experimental setup. Ischemia was mimicked by replacing expansion medium by Ischemic Mimetic Solution (IMS) and by culturing cells in a N_2 gaseous environment. After 5 h of ischemia, reperfusion was mimicked by re-establishing normoxic culture conditions (CM001-R at 3% O_2). I/R setup was performed using mono-cultures of hiPSC-CMs, mono-cultures of hCSCs and co-cultures of the two cell types using transwell permeable inserts. The impact of the I/R was evaluated regarding hCSC proliferation, hiPSC-CM viability, hCSC whole proteome and secretion of IGF-1 and CXCL6 factors in different time points: (BI- Before injury; Post I – Post Ischemia; Post R – Post Reperfusion). Control cultures were done in parallel (using normoxic conditions).

Briefly, ischemia was mimicked by replacing CM001-R medium by Ischemic Mimetic Solution (IMS; in mM: NaCl, 135; KCl, 8; $MgCl_2$, 0.5; NaH_2PO_4, 0.33; HEPES, 5.0; $CaCl_2$, 1.8; Na^+-lactate, 20; pH 6.8) (Zhao *et al.*, 2013) and by placing cells in a N_2 gaseous environment at 37°C. After 5 h of ischemia, reperfusion was mimicked by re-establishing control culture conditions (CM001-R at 3% O_2). Control cultures for all culture setups were maintained in parallel (CM001-R at 3% O_2). The impact of I/R injury was evaluated regarding hCSC proliferation, hiPSC-CM viability, secretion of growth factors and hCSC whole proteome analysis.

2.3. Total cell number

hCSC number was accessed by nuclei count with crystal violet solution staining. Briefly, cell were resuspended in lysis buffer (0.1% Triton X-100 in 0.1M citric acid) directly in culture wells /transwells and incubated at 37°C for at least 48 h. Nuclei were stained with crystal violet dye (0.1% v/v in lysis buffer) and the total number of nuclei counted in a Fuchs-Rosenthal hemocytometer chamber. Fold increase in hCSC number was calculated as the ratio between the cell number at the experimental time point assayed and cell number before I/R injury.

2.4. Cell viability

hiPSC-CM viability was assessed by cell membrane integrity analysis: cell monolayers were incubated with 20 µg/mL fluorescein diacetate (FDA), that stains viable cells, and 10 µg/mL propidium iodide (PI), a membrane impermeable DNA-dye that stains non-viable cells, in DPBS for 2-5 minutes. Samples were then observed under a fluorescence microscope (DMI 6000, Leica Microsystems GmbH).

hiPSC-CM viability was further assessed using the metabolic indicator PrestoBlue® Cell Viability Reagent (Life Technologies), according to the manufacturer's recommendation. Briefly, cells were incubated with CM001-R containing 10% (v/v) PrestoBlue®, for 1 h. Supernatant's fluorescence was measured in 96-well plates using a microwell plate fluorescence reader (Infinite 200 PRO NanoQuant TECAN). Values obtained were normalized by the initial values obtained before I/R injury.

2.5. Immunofluorescence microscopy

Cell monolayers were washed with DPBS and fixed in 4% (w/v) paraformaldehyde (PFA) and 4% (w/v) sucrose in DPBS for 20 min. Afterwards, cells were permeabilized for 10 min in 0.1% (v/v) Triton X-100 in DPBS and blocked with 0.2% (v/v) Fish Skin Gelatin (FSG) in DPBS for 30 min, at room temperature (RT, 18-20°C). Cells were then incubated with primary antibodies diluted in 0.125% (v/v) FSG, 0.1% (v/v) Triton X-100 for 2 h at RT. Cells were washed with DPBS and incubated with secondary antibodies diluted in 0.125% (v/v) FSG, 0.1% (v/v) Triton X-100 for 1 h at RT in the dark. The following primary antibodies were used: α-sarcomeric actinin (1:200, Sigma), CD26 (1:80, Thermo Fisher) and Ki-67 (1:200, Abcam).

2.6. Flow cytometry

After a 5 min dissociation step with TrypLE^{TM}Select at 37°C, cells were washed with DPBS by centrifugation and a total of 2-3x10^5 cells were used per analysis. For membrane markers, cells were incubated with the primary antibody for 1 h and with the secondary antibody for 30 min at 4°C in DPBS with 5% (v/v) FBS. For intracellular markers, cells were permeabilized using the Inside Stain Kit (Miltenyi Biotec) according with manufacturer‘s instructions. The following primary antibodies were used: SirPα/β (1:20, CD172a/b-PE, BioLegend), Troponin T (1:200, TnT, ThermoScientific), CD105 (1:20,BD Pharmingen), CD166 (1:20,BD Pharmingen), CD44 (1:5, eBiosciences), CD11b (1:10, AbDSerotec), CD34 (1:5, BD Pharmingen), CD45 (1:5, BD Pharmingen) and isotype controls mouse igG1k (1:5, BD Pharmingen), mouse IgG1 (1:2.5,Santa Cruz Biotechnologies) and rat IgG2b (1:5, eBiosciences) . Cells were analyzed in a CyFlow® space (Partec GmbH) instrument, registering at least 10,000 events/sample.

2.7. Quantification of growth factors

Quantification of growth factors CXCL6 and IGF-1 in cell's conditioned medium was performed by ELISA Human Quantikine ELISA kit (R&D Systems), according to manufacturer's instructions. Optical density was measured in 96-well plates using a microwell plate reader (Infinite 200 PRO NanoQuant TECAN). The specific rate of growth factor secretion was estimated according to the following equation:

$$q_{GF} = \frac{\Delta C_{GF}}{C_{cell} \times \Delta t}$$

Where ΔC_{GF} (g/L) is the variation in growth factor concentration during the time period Δt (h) and C_{cell} the concentration of cells (cell/L).

2.8. HUVECS culture and tube-formation assay

Human Umbilical Vein Endothelial Cells (HUVECs, Lonza ref 2517A) were cultured at 37°C in humidified incubators (5% CO_2, 3% O_2), in 0.1% gelatin-coated plates with Endothelial Cell Growth medium 2 (ECGM2, PromoCell). Medium was replaced every 3 days. Cells were subcultured when about 90% confluent using 0.5% Trypsin-EDTA for 7 minutes at 37°C.

Tube formation assay was performed according to Pedroso et al., 2011. Briefly, ice-cold undiluted Matrigel (Growth factor Reduced, BD Biosciences) (1.97 mg/cm^2) was used to coat 96-well plates and incubated for 40 min at 37°C to allow the Matrigel to solidify. HUVECs were seeded at a density of 5.5x10^4 cells/cm^2 and incubated with the conditioned mediums from I/R experiments. ECGM2 was used as positive control for tube formation. At least four independent images were acquired per condition after 4h of incubation and the morphological

aspects of the tube network were quantified using the ImageJ angiogenesis analyzer plugin, including total branching length (sum of length of the trees composed from segments and branches), total segment length (sum of length of the segments) and number of nodes (Carpentier, 2012).

All cell culture reagents were purchased from Gibco, Life Technologies unless otherwise stated.

2.9. Whole proteome analysis

hCSC cells were harvested, and washed twice with DPBS by centrifugation. Supernatants were discarded and cell pellets were placed at −80°C until further analysis. Proteins were extracted, quantified and processed from cell pellets as described elsewhere (Abecasis *et al.*, 2017). Two biological replicates with 3 technical replicates per time point were run. Protein samples were analyzed by NanoLC–MS/MS using TripleTOF 6600 (ABSciex). External calibration was performed using beta-galactosidase digest (ABSciex). The 40 most intense precursor ions from the MS spectra were selected for MS/MS analysis. Data were acquired with the Analyst software TF 1.7 (ABSciex). The raw MS and MS/MS data were analyzed using Protein Pilot Software v.5.0 (ABSciex) for protein identification. The search was performed against Swissprot protein database with taxonomic restriction to *Homo sapiens*. Protein identification was considered when unused scores were greater than 1.3 (95% confidence). Analysis of the protein lists was performed using Venny 2.1 (*http://bioinfogp.cnb.csic.es/tools/venny/*), and Ingenuity Pathway Analysis (IPA, Quiagen). Statistically significant representation of biological functions and canonical pathways was identified based on IPA p-value. This probability score is calculated taking into account the total number of proteins known to be associated with a given function or

pathway, and their representation in the experimental dataset. IPA's calculated p-value is displayed as –log (p-value). All proteomic data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD008156.

2.10. Statistical analysis

Statistical analyses were performed with GraphPad Prism6 (GraphPad Software Inc.). All data are shown as mean with standard deviation. Differences in hCSC fold increases (n=3/5), differences in hiPSC-CM viability percentages (n=2/3), differences in specific growth rate secretion of CXCL6 and IGF-1 (n=3) and differences in tube formation (n=3/4) were analyzed by parametric One Way ANOVA Tukey test. Individual p-values for each comparison were obtained using the multiplicity adjusted p-value test. Differences for ki67 expression percentage were analyzed by parametric Student's T test with Welch's correction. P-values below 0.05 were considered significant.

3. Results

In this study, we developed an *in vitro* I/R injury co-culture model with hCSCs and hiPSC-CMs, using transwell inserts to allow paracrine communication between the two cell types.

Identity of hCSCs was demonstrated by the expression of hCSCs cell-surface specific markers by flow cytometry. As demonstrated before (Gomes-Alves *et al.*, 2016), this cell population is negative for CD34, CD45, CD11b, and for CM marker cTnT, and displays a high percentage of cells positive for adult stem cell markers CD44 (97.2% ± 2.2), CD105

(95.5% ± 1.7) and CD166 (75.1% ± 13.3) (Supporting information figure S2.1A). The expression of CD26 (DPP4), recently found to be selectively upregulated in hCSCs when compared to human mesenchymal stem cells and human dermal fibroblasts (Torán *et al.*, 2018) was also detected by immunostaining (Supporting information figure. S2.1B).

During the establishment of the I/R injury setup, two different types of hiPSC-CMs were tested: i) hiPSC-CMs differentiated as previously described (Correia *et al.*, 2016, 2018), and ii) the same cells with an extra maturation step (Ribeiro *et al.*, 2015) (please see material and methods section for details). With the first hiPSC-CMs tested (without the extra maturation step) cell viability was not affected by the injury setup neither in mono-culture or in co-culture with hCSCs conditions (Supporting Information figure S2.2). These results suggest that these cells were not metabolically mature enough, not reflecting the phenotype of adult CMs found in the heart. Such results are in accordance with findings that point to a relevant role of CMs maturation stage on cell survival upon I/R injury. In fact, primary embryonic human CMs are resistant to hypoxia, while primary adult human CMs are highly dependent on an adequate oxygen supply, which might be related with the different metabolic phenotypes between the different developmental stages (Robertson, Tran and George, 2013; Hidalgo *et al.*, 2018). hiPSC-CMs with an extra maturation step were therefore used in following studies, since they better reflected the typical loss of cell viability during AMI.

hiPSC-CMs typical cardiac markers were not altered in our assay conditions (CM001-R at 3% O_2, representing myocardial physiological normoxia (Khan et al., 2010)) when compared to the cell's maturation culture conditions (Pluricyte® CM medium, 21% O_2), presenting a high percentage of cells positive for the cardiac specific proteins SIRPA (72.9% ± 5.2) and cTnT (92.6% ± 2.9) (Supporting information Fig. S3A)

and the characteristic striated pattern of α-sarcomeric actinin (Supporting information Fig. S3B).

The heterotypic model was subjected to I/R injury (figure 1) and analyzed in terms of hCSC proliferation, hiPSC-CM viability, secretion of key growth factors, angiogenic potential and hCSC whole proteome. Co-cultures were compared with mono-cultures of the two cell types and controls without injury.

3.1. hCSCs proliferation is activated upon I/R in presence of hiPSC-CMs

hCSC proliferation was evaluated in control (CTL) and injury conditions in mono- and co-culture with hiPSC-CMs. hCSCs in co-culture with hiPSC-CMs showed a significant higher fold increase at 16 h post reperfusion when compared to the respective co-culture control (2.7± 0.6 vs 1.8 ± 0.1, respectively, p=0.0396) and when compared with hCSCs in mono-culture in injury condition (2.7± 0.6 vs 1.4 ± 0.3, respectively, p=0.0009) (figure. 2.2A, B).

To further confirm the differences observed in proliferation profile between hCSCs subjected to injury in both mono- and co-culture conditions, hCSCs were labeled with Ki-67, a marker specific for active proliferating cells. Although for all conditions tested the percentage of Ki-67^+ hCSCs was between 60-80%, there was a significant decrease of Ki-67^+ hCSCs at 1 h post reperfusion in mono-culture in comparison with co-culture conditions (59.3% ± 3.14 vs 68.8% ± 2.3, respectively, p=0.0163) (figure 2.2C,D), which might explain the lower cell proliferation observed in this condition. These findings suggest that injury diminishes hCSC proliferation capacity, especially in early reperfusion period. Such effect is, however, prevented when the co-culture model was used.

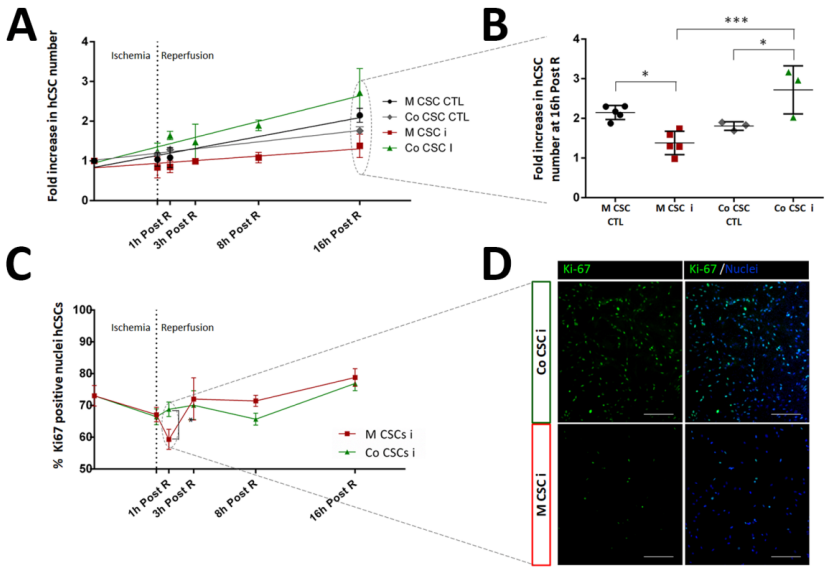

Figure 2.2. **Impact of I/R injury conditions on hCSCs proliferation.** (A) Fold increase in hCSC cell number (B) Fold increase in hCSC cell number at 16 h Post Reperfusion; (C) Quantification of Ki67^{+} hCSCs by immunostaing; (D) Representative culture imaging of Co CSC i and M CSC i labelled with Ki67 at 1h Post R. Black circles: mono-culture CTL (M CSC CTL); Grey Diamonds: co-culture CTL (Co CSC CTL); Red squares: mono-culture hCSCs insult (M CSC i); Green triangles: co-culture hCSCs insult (Co CSC i). Post R: Post Reperfusion. *P<0.05; **P<0.01; ***P<0.005.Fold increase was calculated as the ratio between the cell number at the experimental time point assayed and cell number before injury.

3.2. hCSCs exert a paracrine protective effect on hiPSC-CMs upon I/R injury

The impact of I/R injury conditions in hiPSC-CM viability was evaluated for mono- and co-cultures conditions. A decrease in viability was observed for hiPSC-CM upon injury when compared to CTL, triggered after the first hour of reperfusion (figure 2.3A). hCSCs exerted a paracrine protective effect on hiPSC-CMs in the co-culture condition, with a higher viability ratio across all reperfusion time points assayed when compared with the mono-culture condition, as confirmed by PrestoBlue® Cell Viability Reagent results (figure 2.3A,B) and by cell staining with

live/dead cell dyes (figure 2.3C). At 16 h post reperfusion, hiPSC-CM subjected to injury in co-culture conditions presented significantly higher viability when compared to hiPSC-CM in mono-culture (68.4% ± 3.0 vs 30.8% ± 17.9,respectively, p=0.008) (figure 2.3B). When comparing to respective controls, at 16 h post reperfusion, hiPSC-CMs in mono-culture showed a significant decrease of 62.8% in viability, while in co-culture, hiPSC-CMs viability dropped only by 21.9%.

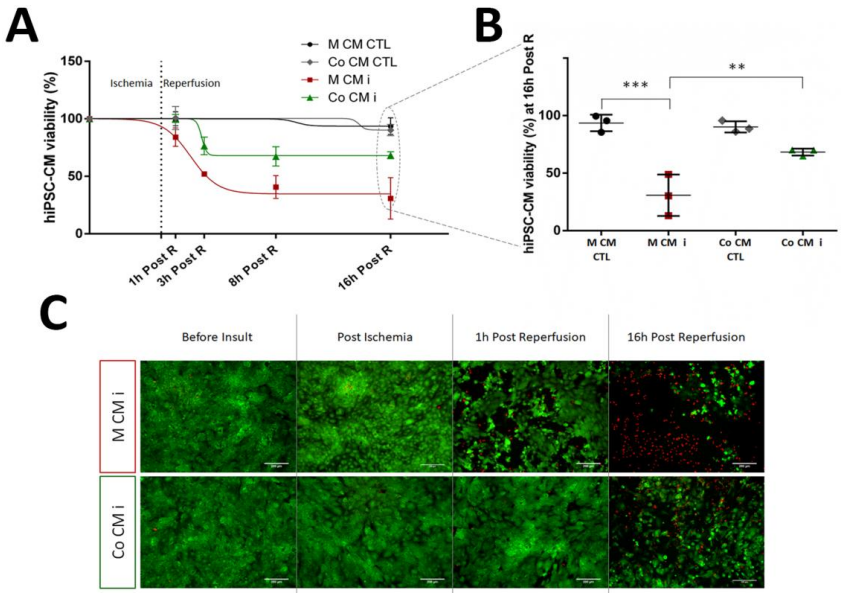

Figure 2.3. Effect of I/R injury on hiPSC-CM viability. hiPS-CM viability was assessed by PrestoBlue® assay (A,B) and by cell staining with FDA (live cells, green) and PI (dead cells, red), scale bars 200 µm (C). Black circles: mono-culture hiPSC-CMs CTL (M CM CTL); Grey diamonds: co-culture hiPSC-CMs CTL (Co CM CTL); Red squares: mono-culture hiPSC-CMs insult (M CM i); Green triangles: co-culture hiPSC-CMs insult (Co CM i). Post R: Post Reperfusion. *P<0.05; **P<0.01; ***P<0.005.

3.3. Secretion of key growth factors is up-regulated in I/R

To further access the paracrine effect observed in the co-culture condition, the secretion of two key proteins with documented cardiac regenerative properties was quantified in our I/R system: Chemokine

ligand 6 (CXCL6 or GPC-2) and Insulin-like growth factor 1 (IGF-1) (Kawaguchi *et al.*, 2010; Miyamoto *et al.*, 2010; Kim *et al.*, 2012; Koudstaal *et al.*, 2014).

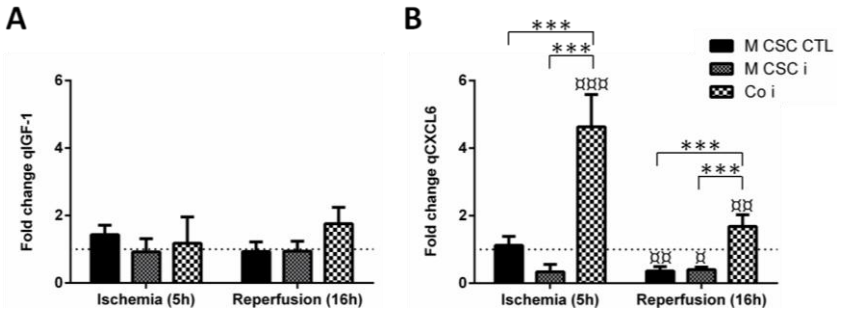

Figure 2.4. Specific rates of CXCL6 and IGF-1 secretion during ischemia and reperfusion. (A) qIGF-1 measured in conditioned media of mono-culture hCSCs CTL (M CSC CTL); mono-culture hCSCs insult (M CSC i) and co-culture hCSCs:hiPSC-CMs insult (Co i). (B) qCXCL6 measured in conditioned media of mono-culture hCSCs CTL (M CSC CTL); mono-culture hCSCs insult (M CSC i) and co-culture hCSCs:hiPSC-CMs insult (Co i). Specific growth factors secretion rates were normalized to the values before insult (dashed line). *P<0.05; **P<0.01; ***P<0.005; ¤P<0.05; ¤¤P<0.01; ¤¤¤P<0.005; ¤¤¤¤P<0.0001 vs before insult.

Regarding IGF-1, our data shows that both hCSCs and hiPSC-CMs secrete IGF-1 either in mono- and co-culture conditions (data not shown for hiPSC-CMs mono-cultures). IGF-1 is secreted and recognized by both cell types (Kawaguchi *et al.*, 2010; Ellison *et al.*, 2011; Valiente-Alandi *et al.*, 2016), therefore it was not possible to isolate and quantify how much IGF-1 each cell population secreted in the co-culture condition, neither if there was an uptake of IGF-1 by hiPSC-CM or hCSC. We could not detect significant changes in IGF-1 secretion upon injury neither in hCSCs mono-cultures nor in co-cultures with hiPSC-CMs (figure 2.4A).

CXCL6 seems to be selectively secreted by hCSC, as it was not identified in either control or injury conditioned medium of mono-cultures of hiPSC-CMs (data not shown). However, in the co-culture condition,

there is a significant increase in CXCL6 cell specific secretion rate in both phases of injury, more pronounced during ischemia (figure 2.4B). In hCSCs mono-culture conditions, we observed some alterations in the CXCL6 cell specific secretion rates during injury, but such rates do not differ between control and injury (figure 2.4B).

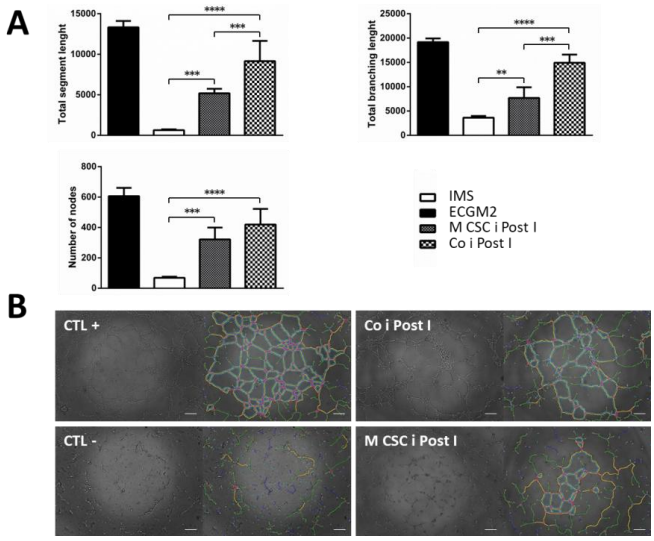

Figure 2.5. Angiogenenic functional evaluation of I/R conditioned medium. Ischemic Mimetic Solution (IMS), Endothelial Cell Growth medium 2 (ECGM2), conditioned media of hCSC mono-culture post ischemia (M CSC i Post I) and of co-culture control post ischemia (Coi Post i) conditions were tested for angiogenic potential by HUVECS tube formation assay. (A) Total segment length, total branching length and number of nodes are represented. ¤¤P<0.01; ¤¤¤P<0.005; ¤¤¤¤P<0.0001 vs IMS. (B) Representative original images (left) and quantification by Angiogenesis analyser are shown. In the right image, there is an indication of master junctions (pink dots), master segments (yellow), meshes (light blue), branches (green) and isolated segments (blue). Scale bars 200 µm.

CXCL6 is described as being an angiogenic chemokine secreted by CSCs (Torán *et al.*, 2017). In order to access if the I/R conditioned medium has a pro-angiogenic paracrine profile, the *in vitro* tube formation assay was performed. HUVECs were incubated with conditioned media from co-cultures and mono-cultures of hCSCs upon ischemic injury. As shown in figure 2.5, the angiogenic potential of conditioned medium from

the co-culture condition post ischemia is higher when comparing to the mono-culture medium, represented by a significant increase in total segment length and total branching length as well as by a higher number of nodes (figure 2.5A).

3.4. Proteomic analysis of hCSCs reveals enrichment in key hallmark pathways and functions upon injury

To further understand the regenerative response of hCSCs to the I/R injury, whole proteome analysis of hCSCs was performed in all culture conditions. More than 3800 proteins were identified in all samples (figure 2.6). Biological canonical pathway and functions (terms) enrichment analysis was performed using IPA software (full list of scores of canonical pathways and functions analysis in Supporting file 2.1). Our analysis was focused on terms with relevant described roles in AMI, associated with the following categories: cell proliferation, cytoskeleton organization, maintenance of cell viability, cell death, oxidative stress, paracrine signaling, regeneration, stress response, and metabolism.

Proteins exclusively identified in hCSCs subjected to I/R injury in mono- (M CSC i) and co-culture (Co CSC i), (n=690) were analyzed. Within this subset, from the 10 top terms with highest number of proteins, 8 were associated with cell death and viability (figure 2.6), suggesting that the I/R injury setup elicited an hCSCs a physiological response involving activation of mechanisms of cell survival.

Approximately 9% of the identified proteins (n=354) were exclusively identified in hCSCs subjected to injury in co-culture conditions (Co CSC i). Within this subset, there was a high enrichment in proteins associated with cell proliferation (n=83), cell viability (n=32), organization of cytoplasm (n=27), organization of cytoskeleton (n=22), and metabolism of protein (n=20), all included in the 10 top terms (Fig. 6). By exploring in

more detail this subgroup of proteins (Supporting information Fig. S4), we can highlight the identification of: APOD and APOH, proteins involved in CM viability and opsonization (Tsukamoto *et al.*, 2013), proteins associated with CXCR4 signaling (RHOB, PIK3R1, GNAQ, PRKCA, RPS6KB2), proteins associated with IGF-1 signalling (JAK1,PIK3R1,RPS6KB2, SFN, RASA1, IGFBP2), and SERPINB3, a protease inhibitor activated by hypoxia (Cannito *et al.*, 2015) with described roles in protection from oxidative damage (Ciscato *et al.*, 2014), cell proliferation (Quarta *et al.*, 2010), IL-6 signalling, and unfolded protein response (Sheshadri *et al.*, 2014).

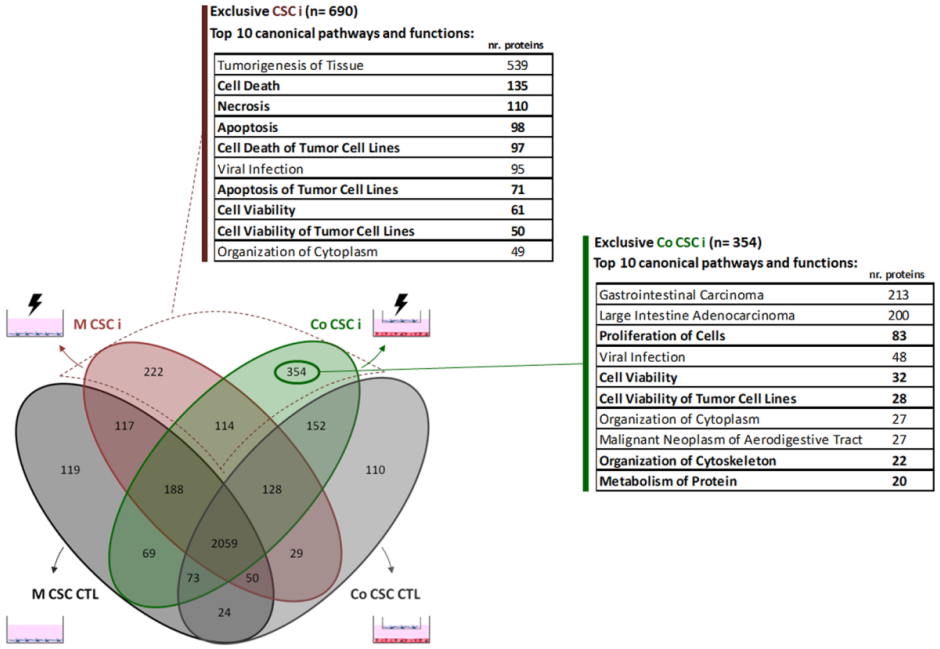

Figure 2.6. **hCSC Whole proteome analysis.** Venn diagram illustrates the overlap between proteins identified in hCSCs in: mono-culture control (M CSC CTL); co-culture control (Co CSC CTL); mono-culture insult (M CSC i), and co-culture insult (Co CSC i) conditions. Top 10 canonical pathways and functions with -log(p-value)≥ 1.3 and highest number of identified proteins are highlighted for the subsets of proteins exclusively identified in hCSCs subjected to I/R injury in both mono- and co-culture conditions (CSC i, red) and for the subset of proteins exclusively identified in Co CSC i proteome (green) .

Moreover, several other pathways and functions relevant in I/R response were enriched in Co CSC i proteome when compared to control (Co CSC CTL) (Supporting Information table S1) and M CSC i (Supporting Information table S2.2), including cell proliferation via EGF-mediated pathways, pathways associated with actin filaments, with Reactive Oxygen Species (ROS) metabolism, corticotropicoid releasing hormone signaling (described has playing a major role in cell adaptation under stressful conditions (Hillhouse *et al.*, 2002)), and glycolysis. Paracrine signaling terms more enriched in Co CSC i proteome included IGF-1, HGF, VEGF, Oncostatin M, Neuregulin, Netrin, PDGF, IL-1, IL-2, IL-3, and IL-6 signaling pathways (Supporting information Table S2.1 and S2.2), all with documented roles in myocardial I/R (Saini *et al.*, 2005; Ellison *et al.*, 2012; Valiente-Alandi *et al.*, 2015; C. Y. Park *et al.*, 2016).

Following this first comparative analysis, a more exhaustive proteomics characterization of hCSCs in the co-culture condition was carried out. Terms enrichment was compared between different time points, namely in control situations (Co CSC CTL), immediately post ischemia period (Co CSC Post i), 1h post reperfusion (Co CSC 1h Post R), and 16h post reperfusion (Co CSC 16h Post R) (table 2.1)

Table 2.1. Canonical pathways and functions differentially enriched in Co CSC CTL and Co CSC throughout injury.
log (p-value)≤1.3 were considered as non significant (n.s.) (less than 95% confidence).

Category	Canonical Pathway/ Function	-log (p-value) 1,3 40 n.s.			
		Co CSC CTL	Co CSC Post I	Co CSC 1h Post R	Co CSC 16h Post R
1. Cell Proliferation	Cell Cycle Progression	11.82	8.58	7.41	9.96
	Cytokinesis	n.s.	4.35	n.s.	n.s.
	Mitosis	7.39	4.97	n.s.	6.88
	Arrest in Mitosis	4.78	n.s.	n.s.	5.38
	Role of CHK Proteins in Cell Cycle Checkpoint Control	2.33	n.s.	n.s.	1.74
	Cell Cycle: G1/S Checkpoint Regulation	1.44	n.s.	n.s.	1.91
	Cell Cycle Regulation by BTG Family Proteins	1.43	n.s.	n.s.	2.16
	ERK5 Signaling	n.s.	1.49	2.13	2.67
	EGF Signaling	n.s.	n.s.	1.48	2.67
	Cholecystokinin/Gastrin-mediated Signaling	1.60	2.01	2.50	3.21
	FLT3 Signaling in Hematopoietic Progenitor Cells	n.s.	n.s.	n.s.	1.46
2. Cytoskeleton Organization	Cell Movement of Embryonic Cell Lines	n.s.	n.s.	5.83	n.s.
	Cell Movement of Epithelial Cell Lines	n.s.	n.s.	4.85	n.s.
	Cell Movement of Kidney Cell Lines	n.s.	n.s.	5.02	n.s.
	Extension of Cells	n.s.	3.89	n.s.	n.s.
	Invasion of Tumor	n.s.	4.59	n.s.	n.s.
	Invasion of Tumor Cells	n.s.	3.98	n.s.	n.s.
	CCR3 Signaling in Eonisophils	2.67	3.53	2.09	2.33
3. Maintenance of Cell Integrity / Cell Death	Apoptosis	35.06	26.94	29.56	36.25
	Necrosis	36.61	28.54	33.19	36.68
	Autophagy	5.85	4.04	5.01	5.75
	Cell Survival	18.01	12.04	15.00	18.82
	Cell Viability	17.77	11.82	15.53	18.72
	Death Receptor Signaling	2.79	1.52	3.56	2.48
	Repair of DNA	5.70	n.s.	5.69	7.07
	Nucleotide Excision Repair Pathway	1.43	n.s.	n.s.	n.s.
	Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.60	n.s.	1.60	2.20
	Mismatch Repair in Eukaryotes	3.32	n.s.	2.54	3.16
	BER Pathway	2.59	n.s.	1.83	2.47

Table 2.1. (Continued)

4. Oxidative Stress	Hypoxia Signaling in the Cardiovascular System	2.94	3.14	3.40	3.56
	Synthesis of ROS	7.82	7.27	6.09	7.19
	Metabolism of ROS	9.06	7.97	7.24	8.34
	Superoxide Radicals Degradation	1.85	2.20	2.78	2.67
	Metabolism of H_2O_2	n.s.	4.42	n.s.	n.s.
	Catabolism of H_2O_2	n.s.	4.12	n.s.	n.s.
	HIF1a Signaling	n.s.	1.53	n.s.	n.s.
5. Paracrine Signaling / Regeneration	IGF-1 Signaling	4.00	6.43	5.78	5.73
	GM-CSF Signaling	1.84	2.43	1.53	1.63
	HGF Signaling	3.06	2.72	2.10	3.03
	VEGF Signaling	5.92	7.04	5.92	7.46
	IL-2 Signaling	n.s.	1.79	n.s.	1.84
	IL-3 Signaling	2.25	2.61	1.92	3.06
	IL-8 Signaling	5.45	4.76	3.78	3.55
	IL-15 Signaling	n.s.	1.54	1.38	1.77
	IL-22 Signaling	n.s.	n.s.	n.s.	1.37
	Role of JAK family kinases in IL-6-type Cytokine Signaling	n.s.	n.s.	n.s.	1.75
	JAK/Stat Signaling	1.34	n.s.	1.61	1.99
	PDGF Signaling	2.50	2.58	3.24	4.12
	Neuregulin Signaling	3.99	3.24	2.40	4.50
	Oncostatin M Signaling	4.25	3.92	3.00	5.39
	Vasculogenesis	n.s.	n.s.	n.s.	4.94
	Angiogenesis	7.06	5.55	6.28	7.35
	Differentiation of Cells	n.s.	4.25	4.73	6.45
6. Stress Response	Hypersensitive Reaction	7.83	10.84	7.50	8.03
	Acute Phase Response Signaling	3.47	3.77	4.11	2.73
	Stress Response of Cells	n.s.	n.s.	4.64	n.s.
	Endoplasmic Reticulum Stress Pathway	n.s.	1.72	n.s.	1.68
	Unfolded Protein Response	2.50	3.00	1.70	1.88
	Cardiac ß-adrenergic Signaling	1.66	2.65	n.s.	n.s.
	Corticotropin Releasing Hormone Signaling	n.s.	n.s.	n.s.	1.62
	HIPPO signaling	5.54	4.23	5.03	5.08

Table 2.1 (Continued)

7. Metabolism	Glutathione Redox Reactions I	3.06	3.03	6.52	3.61
	Glutathione-mediated Detoxification	1.99	2.60	3.95	2.42
	Cysteine Biosynthesis III (mammalia)	2.20	3.57	2.20	2.74
	Methionine Degradation I (to Homocysteine)	1.86	2.35	1.86	2.41
	Cysteine Biosynthesis/Homocysteine Degradation	n.s.	1.95	n.s.	n.s.
	Glutamate Biosynthesis II	n.s.	n.s.	1.75	1.70
	Glutamate Degradation X	n.s.	n.s.	1.75	1.70
	Glycolysis I	5.60	7.79	5.60	7.21
	Glycolysis of cells	n.s.	4.07	n.s.	n.s.
	Consumption of oxygen	n.s.	4.30	n.s.	n.s.

By analyzing the different time points, terms related with cell proliferation via EGF (EGF, Cholecystokinin/Gastrin-mediated and ERK5 Signaling) were found as having an increasing representation along injury (table 2.1, Category 1). FLT3 signaling was also identified as more enriched at 16h post reperfusion, while cytokinesis (table 2.1, Category 1) and IGF-1 signaling, also involved in cell proliferation activation (Waring *et al.*, 2014) (table 2.1, Category 5), were found as more enriched post ischemia. Regarding terms associated with cytoskeleton organization (table 2.1, Category 2); cell invasion, extension, and CCR3 signaling (involved in eosinophils recruitment to inflammation sites (Bonaros *et al.*, 2008)), were enriched after the ischemic period, while terms associated with cell movement were enriched at 1h post reperfusion, suggesting an activation of hCSC migration and homing to injury in these early injury time points. Importantly, although several terms related with cell death and survival were found as less enriched in hCSCs upon ischemia, the same returned to their control levels during reperfusion period (table 2.1, Category 3).

It is worth to mention that cell-cycle regulation pathways (table 2.1, Category 1) and DNA repair mechanisms, including BER pathway (table 2.1, Category 3) representation decreased on early injury time points,

and recovered to values closer to control at 16h post reperfusion. Regarding oxidative stress, terms related with degradation and metabolism of H_2O_2 and HIF-1α signaling were found as more enriched in hCSCs post ischemia (table 2.1, Category 4).

IGF-1 and GM-CSF signaling was also more represented upon ischemia (table 2.1, Category 5), while other pathways associated with cytokine/growth factors paracrine signaling and cardiac regeneration were found as more enriched in hCSCs after 16h of reperfusion, including VEGF, Neuregulin, Oncostatin M, Jak/Stat, PDGF, IL-2, IL-3, IL-6, IL-15, and IL-22 signaling (table 2.1, Category 5).

Stress response-associated functions such as hypersensitive reaction, endoplasmic reticulum stress (ERS), unfolded protein response (UPR), and cardiac β-adrenergic signaling were more enriched upon ischemia (table 2.1, Category 6), while acute phase response signaling and stress response of cells representation was higher at 1h post reperfusion. Importantly, cardiac regeneration associated functions such as cell differentiation, vasculogenesis and angiogenesis were also found as more enriched after 16 hours of reperfusion (table 2.1, Category 6). Cystein Biosynthesis and gluthatione-mediated detoxification terms, both important for protection from oxidative stress (Giordano, 2005), were found as more enriched post ischemia (table 2.1, Category 7). Glycolysis and oxygen consumption related pathways was also found to be more represented upon ischemia (table 2.1, Category 7), which might point to a compensatory mechanism to lack of oxygen availability during this phase of injury.

4. Discussion

In this study, we developed an *in vitro* human cellular model of myocardial I/R injury with hCSCs and hiPSC-CMs that enabled us to further decipher the action mechanisms of hCSCs upon injury. The co-culture model developed recapitulates important hallmarks of I/R injury, namely CM death, CSC proliferation activation upon insult and the protective role of CSCs on CMs. New players on hCSC regeneration response upon I/R including activation of pathways related with cell proliferation, cytoskeleton organization, maintenance of cell integrity, stress response, paracrine signaling, cardiac regeneration and metabolism were identified based on proteome hCSC data (figure 2.7). We also showed, for the first time, an increase in CXCL6 secretion rate by hCSCs in an I/R setting.

During the ischemic phase of injury, cells were cultured in conditions mimicking the myocardial pathophysiological state of ischemia including lack of oxygen availability, nutrient deficiency, acidosis, lactate accumulation, and hyperosmosis (Zhao *et al.*, 2013). hCSC proteomic analysis results point to a down-regulation of cell-cycle regulation pathways, DNA repair mechanisms, and cell repair mechanisms during the ischemic phase of injury (figure 2.7A). In fact, down-regulation of cell cycle checkpoint control and DNA repair processes has been reported as a process involved in activation of several types of quiescent adult stem cells (Cheung and Rando, 2013). Proteome analysis also indicates an activation of endoplasmic reticulum stress pathways, such as ERS and UPR, which have been previously associated with cellular adaptation to glucose deprivation and hypoxic stimuli (Groenendyk, Agellon and Michalak, 2013). Proteins associated with glycolysis and consumption of oxygen were also enriched in this time point, which might point to an

adaptative response of hCSCs to the lack of O_2, via an increase the expression of protein machinery associated with energy production.

The ability of CSCs to differentiate into *de novo* CMs upon AMI is still under active discussion, with different authors defending contrary results (Ellison *et al.*, 2013; Nadal-Ginard, Ellison and Torella, 2014; van Berlo *et al.*, 2014). In our model, hCSCs did not label for Nkx2.5, an early marker of cardiac differentiation (results not shown) neither in CTL nor in injury conditions, although an enrichment in proteins related with cell differentiation was found in hCSCs upon the ischemic phase of injury.

Although such controversy still exists regarding cardiac differentiation potential of CSCs upon injury, there is a general consensus in the field that CSCs exert a protective effect on hCMs under stress mainly due to paracrine mechanisms (Kawaguchi *et al.*, 2010; C.-Y. Park *et al.*, 2016), supported by extensive pre-clinical and clinical data indicating that transplanted CSCs do not survive neither engraft in the myocardium while physiological improvement is still registered (Madonna *et al.*, 2016). In order to further understand the paracrine cross-talk between hCSCs and hiPSC-CMs in our model, we quantified the secretion of two key proteins: CXCL6 and IGF-1, both with documented cardiac regenerative properties. CXCL6 is an angiogenic chemokine shown to improve heart function in mice (Kim *et al.*, 2012; Torán *et al.*, 2017) and identified as up-regulated during mesenteric (intestinal) I/R injury (Jawa *et al.*, 2013). This chemokine was also recently identified in the secretome of hCSCs, where addition of an anti-CXCL6 antibody inhibited the migration and angiogenic properties of CSC conditioned medium, proving the importance of this chemokine in key paracrine regenerative potential features of these cells. We show for the first time an increase in hCSC CXCL6 secretion specific rate in a myocardial I/R injury setting, which was more pronounced during ischemia, but still significant after 16h of

reperfusion (figure 2.7A,C). These results support previous findings that show that this chemokine is upregulated in ischemic conditions via HIF-1α signaling (Tian *et al.*, 2014). Cardiac stem cells have been shown to home and migrate to the site of injury, a process activated by HIF-1α transcription factor and SDF-1 chemokine in response to ischemia (Ceradini *et al.*, 2004; Rota *et al.*, 2008). Although CXCL6 was not identified by proteome analysis, an enrichment in proteins related to HIF-1α signaling, and CCR3 signaling, a pathway important for cell homing to inflammation and injury sites (Bonaros *et al.*, 2008), was also found in hCSCs upon ischemia (figure 2.7A). Moreover, we were able to identify proteins associated with CXCR4 signalling, a pathway activated by SDF-1 with documented roles in cell motility and chemotactic response (Penn *et al.*, 2012). Altogether, our data and previous reports regarding CSC secretome, namely CXCL6 function, point to an effect of this chemokine in hCSC migration and angiogenesis. In fact, we also demonstrate pro-angiogenic properties of conditioned medium from the ischemic phase of injury.

IGF-1 is one of the key proteins shown to be up-regulated upon injury or stress by CSCs (Smith *et al.*, 2010; Valiente-Alandi *et al.*, 2016) and CMs (Ellison *et al.*, 2012; Waring *et al.*, 2014) and has also been shown to have a role in the activation of hCSC regenerative potential (Kawaguchi *et al.*, 2010; C.-Y. Park *et al.*, 2016) and proliferation (Ellison *et al.*, 2012; Waring *et al.*, 2014) *in vitro* and *in vivo*. Although no significant increase in IGF-1 secretion was detected by ELISA, we registered an enrichment in IGF-signaling associated proteins in hCSC proteome upon ischemia and reperfusion time points (figure 2.7A,C). Several other proteins associated with other cytokine/growth factor paracrine signaling pathways were also found to be more represented in hCSC proteome upon ischemia, including VEGF, IL-2, IL-3, IL-15, and GM-CSF signaling,

all with documented paracrine roles acting in CM physiology during I/R injury (Saini *et al.*, 2005; Sesti *et al.*, 2005; Turillazzi *et al.*, 2014).

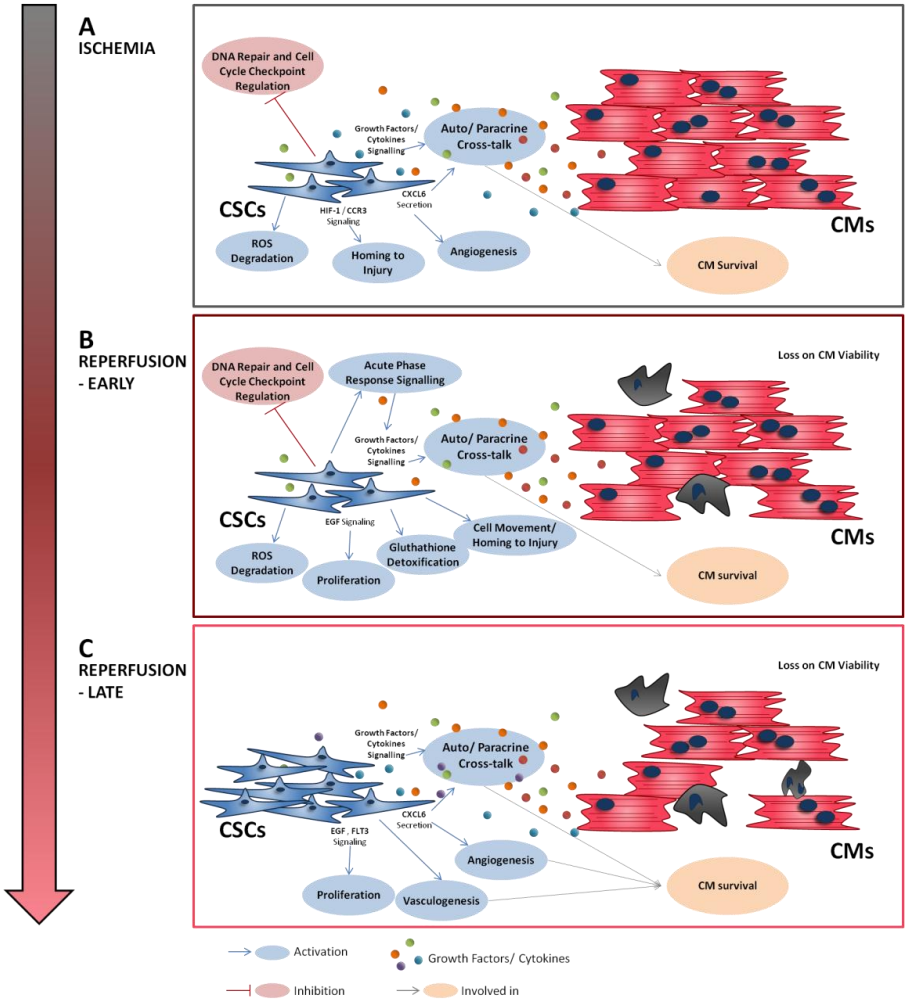

Figure 2.7. Schematic representation of proposed mechanisms involved in hCSCs response to the I/R injury model, during (A) ischemia, (B) early (1h Post Reperfusion) and (C) late (16h Post Reperfusion) reperfusion phases of I/R injury.

hCSC proteome upon ischemia was also enriched in terms related with ROS metabolism, cysteine synthesis, glutamate metabolism and gluthatione-mediated detoxification (figure 2.7B). L-cysteine is one of the main precursors for gluthathione, a molecule that protects cells from

oxidative stress (Giordano, 2005). In fact, glutaminolysis, a pathway that leads to the production of gluthatione via L-cysteine and glutamate, was found as a key energy source for proliferating mouse CSCs (Salabei *et al.*, 2015). ROS formation and oxidative stress has been described as caused by the sudden oxygen elevation in the early reperfusion phase of injury (Hausenloy and Yellon, 2013), rather than during ischemia. However, during the preparation of the samples for proteomic analysis upon ischemia, hCSCs were briefly subjected to atmospheric oxygen concentrations, which could induce such oxidative stress responses. Together, these data point to a mechanistic response of hCSCs to ischemia phase of injury focused on a down-regulation of cell cycle checkpoint and DNA repair processes in favor of an enrichment in proteins associated with stress coping mechanisms, induction of cell homing and motility, and activation of pathways associated with paracrine communication including CXCL6 secretion (figure 2.7A). Such paracrine signaling activation is described as having a beneficial effect on CM protection. hCSC paracrine protection on hiPSC-CM was recapitulated in our model. Viability of hiPSC-CMs was mainly affected in the early reperfusion phase of the injury, which is consistent with the described *in vivo* pathophysiology of CM death during I/R, where the first minutes of reperfusion are also the main trigger for CM death and tissue damage due to the oxidative stress, calcium overload, mitochondrial permeability pore (MPTP) opening and hypercontracture (Hausenloy and Yellon, 2013).

Several authors have reported an increase in the number of resident CSC upon AMI in mice (Ellison *et al.*, 2013; Valiente-Alandi *et al.*, 2016) and human (Urbanek *et al.*, 2005) hearts. Stastna *et al.*, in 2010, also showed that connective tissue growth factor, a pro-fibrosis factor released by CMs upon AMI, induces rat CSC proliferation *in vitro*, and

atrial natriuretic peptide, a vasodilator molecule also secreted by CMs, had an opposite effect, decreasing rat CSC proliferation (Stastna and Van Eyk, 2012). These results suggest that CSC proliferation is regulated upon injury by paracrine factors secreted by CMs. In fact, in our co-culture I/R injury model, we were able to observe a significant increase in hCSC proliferation upon reperfusion (figure 2.7B,C). Our hCSC whole-proteome results also showed higher enrichment of pathways related with cell proliferation via EGF signaling after reperfusion, which points to EGF as one of the key actuator signals in CSC proliferation activation upon AMI (figure 2.7B,C). In fact, EGF has been reported as having a positive effect on cardiosphere-derived hCSCs proliferation and migration (Aghila Rani and Kartha, 2010), as being one of the main factors secreted in mice CSC conditioned medium (C.-Y. Park *et al.*, 2016), as up-regulated in mice CSCs upon injury (Valiente-Alandi *et al.*, 2016), and EGF receptor has also been identified in a hCSCs receptome characterization study by our group (Gomes-Alves *et al.*, 2015). Other signaling pathways described to be related with cell proliferation were also found to be more enriched at 16 hours post reperfusion, such as FLT3 signaling (figure 2.7C), a pathway described as important for proliferation in hematopoietic progenitor cells (Kim *et al.*, 2015), cytokinesis, and IGF-1 pathway.

Collectively, our data suggests an hCSC response to early reperfusion through activation of paracrine signaling mechanisms (including acute phase response signaling), cell proliferation, glutathione-mediated ROS detoxification, and cell movement, while still inhibiting cell cycle repair mechanisms (figure 2.7B). Later in reperfusion, hCSCs proteome also demonstrated an enrichment in several pathways associated with cytokine/ growth factors paracrine signaling, all with documented paracrine roles acting in CM physiology during I/R injury, including

CXCL6 secretion, a recovery of cell cycle repair mechanisms and activation of angiogenesis and vasculogenesis related pathways (figure 2.7C). These results are in line with the concept that hCSCs regenerative capacity is mainly centered on their paracrine potential, being CXCL6 one of its critical players.

Our data support the importance of a paracrine cross-talk between CSCs and CMs during AMI, and the idea that heterotypic cell models better recapitulate *in vivo* features of I/R when compared to monotypic models.

5. Conclusion

The I/R injury model developed recapitulates important hallmarks of AMI. The use of robust advanced analytical technologies such as LC-MS/MS whole cell proteome analysis, combined with more classical and targeted methodologies (ELISA growth factor quantification, viability assays) and functional assays (tube formation assay) enabled us to further understand the response of hCSC to I/R injury, shedding new insights on the possible mechanisms involved. We show, for the first time, an increase in CXCL6 secretion by hCSCs in a myocardial I/R setting, reinforcing the described role of CXCL6 in hCSC regenerative and pro-angiogenic potential and suggesting the importance of this chemokine in the paracrine-mediated protective effect of CSCs in myocardium upon AMI. The results reported herein strengthen the importance of studying physiological processes using more complex human co-culture *in vitro* models leading to a better recapitulation of the *in vivo* paracrine signaling and a more relevant model for future applications such as drug discovery.

This work provides new insights and raises new questions related with hCSC biology in acute myocardial infarction. We believe that our model provides an important tool towards a better understanding of hCSC action mechanisms upon AMI, which will enable the development of novel therapies focused on activation, recruitment and improvement of the endogenous heart regeneration capacity.

6. Acknowledgments

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I. Palacios is employee of Coretherapix (Tigenix Group). The remaining authors declare no conflict of interest.

7. Supporting information

Supporting Information File 2.1 available at:

https://www.dropbox.com/sh/3kemuejjxov0mar/AADZG8V1Bg0tAEeoekCN05_Za?dl=0

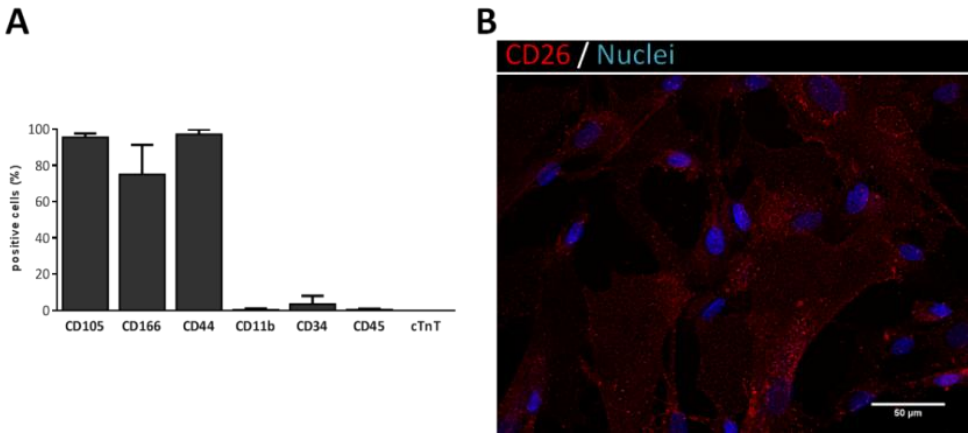


Figure S2.1. Phenotypic characterization of hCSCs. hCSCs were characterized using specific cell markers by flow cytometry (A) and immunostaining (B). Scale bars: 50 µm. Error bars represent SD of n=3.

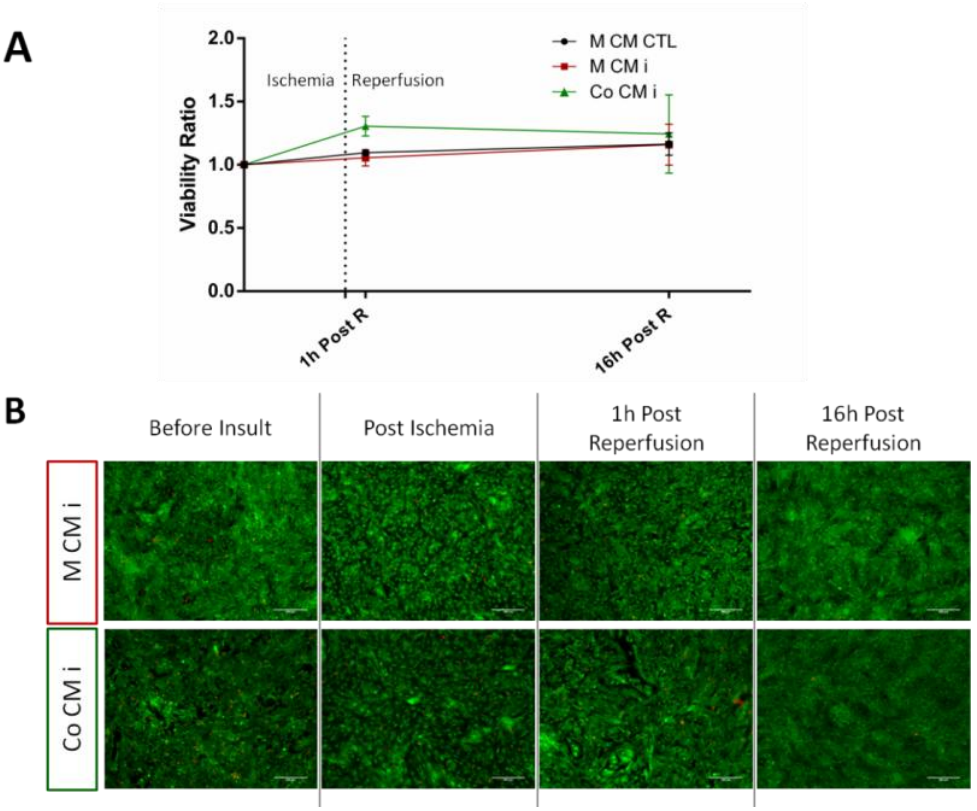


Figure S2.2. Effect of I/R injury on hiPSCs-hCMs without maturation step. Viability of hiPSC-CMs after 15 days of differentiation (without maturation step) was assessed by PrestoBlue® assay (A) and by cell staining with FDA (live cells, green) and PI (dead cells, red), scale bars 200 µm (B). Black circles: mono-culture hiPSC-CMs CTL (M CM CTL); Red squares: mono-culture hiPSC-CMs insult (M CM i); Green triangles: co-culture hiPSC-CMs insult (Co CM i). Post R: Post Reperfusion.

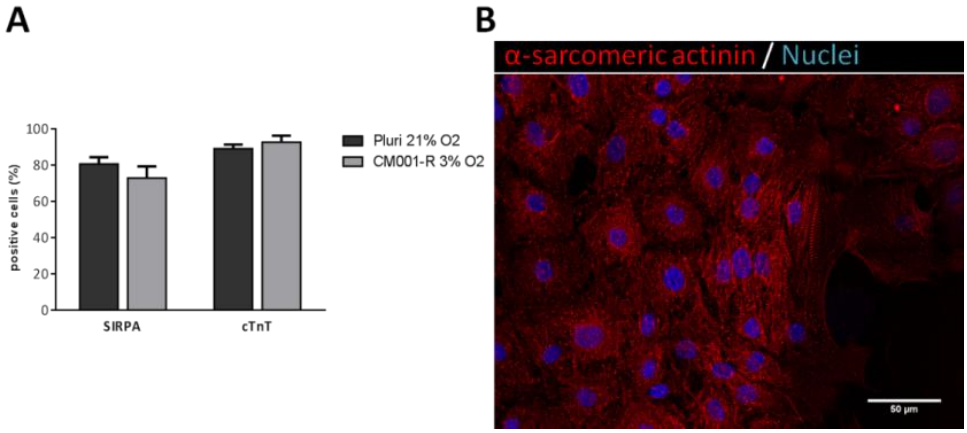
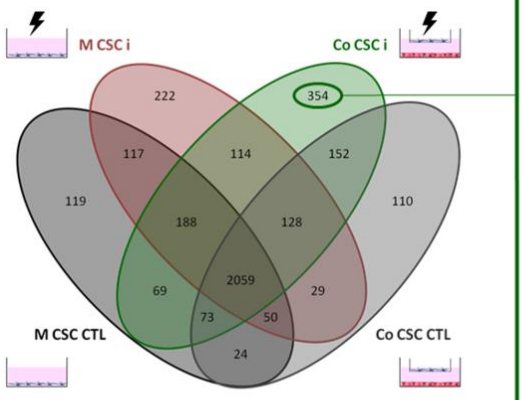


Figure S2.3. Phenotypic characterization of hiPSC-CMs. hiPSC-CMs were characterized using specific cell markers by flow cytometry (A) and immunostaining (B). hiPSC-CMs retain their cardiomyocyte markers expression after 2 days in assay conditions (CM001-R at 3% O_2: light grey bars) comparing to the hiPSC-CM maturation culture conditions (Pluricyte® CM medium at 21% O_2: dark grey bars). Scale bars: 50 µm. Error bars represent SD of n=3 (Unpaired t test) .



Proteins associated with:

PROLIFERATION (N=83)		CYTOSKELETON ORGANIZATION (N= 39)	MAINTENANCE OF CELL INTEGRITY/ CELL DEATH (N=38)	OXIDATIVE STRESS (N=11)	PARACRINE SIGNALING/ REGENERATION (N=20)	STRESS RESPONSE (N=16)	METABOLISM (N=10)
AATF	MEF2D	ADNP	APOD	APOA2	APOA2	CASP8	AHCYL2
AFF1	MIER1	ATXN3	ARID1A	APOD	APOD	CRB1	BPGM
APOD	MINA	CDC42SE1	ASF1A	GPX7	APOH	GNAQ	CTH
APOH	NDUFAF2	CHD3	CASP8	JAK1	CASP8	GPX7	GAPDHS
ARID1A	NFKB2	CHGA	CCNH	NFKB2	CRADD	IGF2	GPX3
ARMC10	NUBP1	CHMP1B	CUX1	PIK3R1	FGF1	JAK1	GPX7
ASPSCR1	PDP1	CYTH2	DPF2	PPP1R10	GNAQ	MEF2D	MGST2
BRAP	PI4KA	DLC1	DPH2	PRKCA	HLA-B	MGST2	MUT
CASP8	PIK3R1	EPB41	DUSP14	RHOB	IGFBP2	NDUFAF2	PGAM4
CCNH	PKD2	EXOC8	FGF1	UBE2J1	JAK1	PANX1	PRMT3
CCPG1	PKP1	FGF1	FIP1L1	UBE2L6	NFKB2	PPP1R10	
CDKN2AIP	POLR2K	FNBP1L	GPR180		PIK3R1	PRKCA	
CHGA	PPP1R10	GNAQ	IGF2		PRKCA	SFN	
CHMP1B	PRG4	HLA-B	IGFBP2		RASA1	TAOK3	
CNOT7	PRKCA	IQGAP2	JAG1		RPS6KB2	UQCR11	
CTH	PTPN9	KIF20B	JAK1		SERPINF1	VNN1	
CUX1	PTPRR	KIF5A	KIF1A		SFN		
DAPK3	RACGAP1	KRT13	KIF5A		SPHK1		
DDX41	RASA1	KRT4	LAMTOR3		STAT5A		
DLC1	RHOB	KRT71	MSH5		VAV2		
DPF2	RPS6KB2	KSR2	NFKB2				
EPPK1	SERPINB3	NFKB2	NSA2				
EXOC8	SERPINE2	PI4KA	PDP1				
FDFT1	SERPINF1	PIK3R1	PIK3R1				
FGF1	SFN	PRKCA	POLR2K				
FIP1L1	SNW1	PRSS3	PRKCA				
GNAQ	SPHK1	RACGAP1	PTPN9				
GPN3	STAT5A	RASA1	RASA1				
GPX3	STEAP3	RHOB	RPS6KB2				
IGF2	SUPV3L1	RPS6KB2	SERPINE2				
IGFBP2	SYMPK	SERPINF1	SFN				
IGHG1	TOP2B	SPHK1	SGPP1				
IGKC	UBE2J1	STAT5A	SNW1				
IQGAP2	UBE2L6	STMN2	SPHK1				
JAG1	USP16	TNN	SYMPK				
JAK1	UTP20	VAMP7	TAOK3				
KIF1A	VAMP7	VAV2	TOP2B				
KIF20B	VAV2	WDR5	VPS11				
KLC2	VMP1	WNK1					
LAMB2	WDR5						
LGALS7	WNK1						
MBD2							

Figure S2.4. Proteins identified in hCSCs. Venn diagram illustrates the overlap between proteins identified in hCSCs in: mono-culture control (M CSC CTL); co-culture control (Co CSC CTL); mono-culture insult (M CSC i), and co-culture insult (Co CSC i) conditions. Proteins related with cell proliferation, cytoskeleton organization, maintenance of cell integrity, cell death, paracrine signalling, regeneration, stress response, and metabolism are highlighted for the subset of proteins identified exclusively in Co CSC i proteome.

Table S2.1. Canonical pathways and functions enriched in Co CSC I vs Co CSC CTL. –log (p-value)≤1.3 were considered as non significant (n.s.) (less than 95% confidence). Pathway/ function terms were only selected for analysis when –log (p-value) ratio between the two conditions ≥1.2.

Category	Canonical Pathway/ Function	-log (p-value) Co CSC CTL < Co CSC I
Cell Proliferation	Cholecystokinin/Gastrin-mediated Signaling	1.60 / 3.09
	EGF Signaling	n.s. / 2.82
	Renin-Angiotensin Signaling	1.61 / 2.52
	ERK5 Signaling	n.s. / 2.48
	FLT3 Signaling in Hematopoietic Progenitor Cells	n.s. / 1.80
Cytoskeleton Organization	Quantity of actin stress fibers	n.s. / 5.32
	Quantity of actin filaments	n.s. / 5.19
	Quantity of filaments	n.s. / 5.08
Maintenance of Cell Integrity / Cell Death	DNA Methylation and Transcriptional Repression Signaling	2.55 / 4.17
	Death Receptor Signaling	2.79 / 3.54
	Myc Mediated Apoptosis Signaling	2.39 / 3.04
	Autophagy	n.s. / 1.53
Oxidative Stress	Hypoxia Signaling in the Cardiovascular System	2.94 / 4.25
	Superoxide Radicals Degradation	1.85 / 2.43
Paracrine Signaling / Regeneration	VEGF Signaling	5.92 / 7.14
	Differentiation of Cells	n.s. / 5.60
	Oncostatin M Signaling	4.25 / 5.41
	Vasculogenesis	n.s. / 5.10
	PDGF Signaling	2.50 / 4.04
	IL-3 Signaling	2.25 / 3.07
	IL-2 Signaling	n.s. / 2.03
	JAK/Stat Signaling	1.34 / 2.70
	PEDF Signaling	n.s. / 1.42
	Role of JAK family kinases in IL-6-type Cytokine Signaling	n.s. / 1.46
	IL-1 Signaling	1.52 / 1.85

Table S2.2. (Continued)

Stress Response	Endoplasmic reticulum stress response	10.35	12.49
	Tec Kinase Signaling	2.59	4.31
	PDGF Signaling	2.50	4.04
	AMPK Signaling	3.03	3.93
	Acute Myeloid Leukemia Signaling	n.s.	1.91
	Corticotropin Releasing Hormone Signaling	n.s.	1.32
Metabolism	Glutathione Redox Reactions I	3.06	5.63
	Glutathione-mediated Detoxification	1.99	3.27
	Cysteine Biosynthesis/Homocysteine Degradation	n.s.	1.60
	Cysteine Biosynthesis III (mammalia)	2.20	3.89
	Methionine Degradation I (to Homocysteine)	1.86	2.80

Table S2.2. Canonical pathways and functions enriched in Co CSC I vs Mono CSC i. –log (p-value)≤1.3 were considered as non significant (n.s.) (less than 95% confidence). Pathway/ function terms were only selected for analysis when –log (p-value) ratio between the two conditions ≥1.2.

Category	**Canonical Pathway/ Function**	**-log (p-value)**	
		Mono CSC i	**Co CSC i**
		<	
Cell Proliferation	Mitosis	5.34	7.49
	Cholecystokinin/Gastrin-mediated Signaling	1.99	3.09
	EGF Signaling	1.46	2.82
	Renin-Angiotensin Signaling	1.90	2.52
	ERK5 Signaling	n.s.	2.48
	Prolactin Signaling	n.s.	1.47
Cytoskeleton Organization	Morphology of Cells	18.76	23.54
	Cdc42 Signaling	6.64	9.00
	Quantity of Actin Stress Fibers	4.42	5.32
	Quantity of Actin Filaments	3.97	5.19
	PTEN Signaling	2.76	3.48
Maintenance of Cell Integrity/ Cell Death	Myc Mediated Apoptosis Signaling	2.32	3.04
	BER pathway	n.s.	2.20

Table S2.2. (Continued)

Oxidative Stress	Metabolism of ROS	5.45	7.63
	Synthesis of ROS	4.83	6.65
	Superoxide Radicals Degradation	1.70	2.43
Paracrine Signaling / Regeneration	IGF-1 Signaling	4.06	6.87
	Oncostatin M Signaling	4.45	5.41
	vasculogenesis	n.s.	5.10
	Chemokine Signaling	3.74	4.63
	Neuregulin Signaling	2.26	4.44
	p70S6K Signaling	3.36	4.04
	PDGF Signaling	2.67	4.04
	IL-3 Signaling	1.81	3.07
	HGF Signaling	1.88	2.84
	JAK/Stat Signaling	1.52	2.70
	IL-2 Signaling	1.38	2.03
	IL-1 Signaling	1.41	1.85
	Netrin Signaling	n.s.	1.82
	CNTF Signaling	1.31	1.64
	TNFR1 Signaling	n.s.	1.55
	Role of JAK family kinases in IL-6-type Cytokine Signaling	n.s.	1.46
	PEDF Signaling	n.s.	1.42
Stress Response	Corticotropin Releasing Hormone Signaling	n.s.	1.32
	Acute Phase Response Signaling	2.95	3.99
Metabolism	Cysteine Biosynthesis/Homocysteine Degradation	n.s.	1.60
	Cysteine Biosynthesis III (mammalia)	1.98	3.89
	Methionine Degradation I (to Homocysteine)	1.67	2.80
	Glycolysis I	5.14	7.48
	Glycolysis of cells	n.s.	5.17

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Chapter III

Merging bioreactor technology with 3D culture approaches: a novel myocardial I/R *in vitro* model

Author Contribution

Maria João Sebastião participated in the experimental setup and design, performed the experiments, participated in data analysis and wrote the chapter.

Abstract

During acute myocardial infarction (AMI) Ischemia/Reperfusion (I/R) injury causes cardiomyocyte (CM) death and loss of heart tissue function, making AMI one of the major causes of death worldwide. Cell based *in vitro* models of I/R injury have been increasingly used as a complementary approach to *in vivo* preclinical research. However, most *in vitro* I/R studies use murine cells in two-dimensional (2D) setups, which are not able to properly recapitulate human cellular physiology cellular interactions as well as nutrient and gas gradients that occur in the human heart. In this work we established a novel human *in vitro* model of myocardial I/R injury using CMs derived from human induced pluripotent stem cells (hiPSC-CMs). hiPSC-CMs were cultured as three-dimensional (3D) aggregates in stirred tank bioreactors. Using this experimental setup, we were able to recapitulate important hallmarks of CM response to AMI, including loss of viability and disruption of sarcomere and mitochondria ultra-structure. We also show higher angiogenic functionality and increased secretion of key pro-angiogenic and pro-inflammatory cytokines in culture supernatant from I/R injury condition.

Contents

1. Introduction

Acute myocardial infarction (AMI) consists on the cessation of blood flow to an isolated region of the heart, causing oxygen and nutrient depletion. Coronary intervention with restauration of blood flow remains the clinical intervention of choice for AMI patients: although necessary for the replenishment of blood to the affected area, this process causes increased myocardial tissue damage (Ischemia/ Reperfusion, I/R injury) with loss of cardiomyocytes (CMs) causing up to 50% of the final damaged tissue size (Hausenloy and Yellon, 2013).

During AMI, CMs in stress have been reported to activate the expression and secrete growth factors and cytokines associated with inflammation, remodeling of extracellular matrix (ECM), cell adhesion, recruitment of endogenous cardiac stem/progenitor cells (CSCs) and angiogenesis, (Li *et al.*, 2014; Ong *et al.*, 2015). However, such findings and most research associated with CM physiology during myocardial I/R injury is mainly based in murine animal models and murine cell populations *in vitro*, including primary neonatal and adult CMs, mouse atrial HL-1 cells and rat ventricular H9c2 cell lines. Although central to the study of I/R pathophysiology, murine systems hold several disadvantages: murine and human cell physiology differ at a tissue and cell level, including relevant differences in ion channels (Davis *et al.*, 2011) and microRNAs with documented roles in cardiovascular processes (Roux, Gonzàlez-Porta and Robinson-Rechavi, 2012). Such differences have already been pointed as one of the causes of the poor translational success from preclinical to clinical trials: about 90% of candidate therapies that showed efficacy in preclinical studies fail in human trials (Olson *et al.*, 2000), generating massive costs in the drug development chain.

Therefore, there is an increasing need for novel relevant human *in vitro* cellular models that can effectively recapitulate human CM response to

I/R injury. These models can be further used as tools for preclinical research, contributing to excel the drug developmental process.

The advent of induced pluripotent stem cell (iPSC) research has accelerated human cell biology research *in vitro*, and several reports show that human iPSC-derived CMs (hiPSC-CMs) are effective in toxicological safe screenings, being already used for preclinical *in vitro* testing in the pharmaceutical industry (Denning *et al.*, 2016). Moreover, several strategies were already developed to improve the maturation state and physiological relevance of hiPSC-CMs, including hormone supplementation (Yang *et al.*, 2014; Ribeiro *et al.*, 2015) and culture in three-dimensional (3D) aggregate configuration (Correia *et al.*, 2018). In fact, several studies show that culturing in 3D recapitulates adult CM physiological electrical and contractile function when comparing to planar culture systems (Zhang *et al.*, 2013; Daily *et al.*, 2015; Correia *et al.*, 2018). Besides improving the maturation state of cells, 3D *in vitro* cardiac models allow a higher resemblance with *in vivo* tissue microenvironment and architecture, enabling enhanced cell-cell and cell-ECM contacts (Serra *et al.*, 2012; Ryan *et al.*, 2016).

To overcome the drawbacks of the existent *in vitro* models of I/R and aiming at filling the gap between these and *in vivo* animal research, in this work we developed and characterized a novel *in vitro* I/R system using hiPSC-CM 3D spheroids and stirred tank bioreactor technology. Bioreactors allow for a fine control and monitoring of critical process parameters to mimic the extracellular microenvironment of the ischemia and reperfusion phases of AMI, including pO_2 and pH. Moreover, bioreactors allow for non-destructive sampling for culture characterization at different stages of I/R injury. Using this experimental setup, we were able to efficiently show several hallmarks of CM response to AMI, including loss of viability, the characteristic disorganization sarcomeres

and mitochondria ultra-structure, and higher secretion of several key pro-inflammatory and pro-angiogenic molecules during both phases of injury.

2. Materials and Methods

2.1. Cell culture

2.1.1. Directed differentiation of hiPSC-CMs

Human iPSC (DF19-9-11T.H, WiCell) were cultured in Matrigel® (Corning) coated plates in mTESR1 medium (STEMCELL Technologies) and subcultured when about 80-90% confluent using Versene (Gibco) for 7 minutes at 37°C. Differentiation to CMs was initiated when cell confluence reached about 90%, as described elsewhere (Lian *et al.*, 2013; Correia *et al.*, 2016).

At day 7 (when a high proportion of beating zones is observed), cells were dissociated by incubation with TrypLE Select (ThermoFisher Scientific) for 5 minutes and replated in AggreWell™400Ex plates (Stem cell technologies) at density of 1500 cell/microwell, as previously described for aggregation of hPSC-CMs (Nguyen et al., 2014): cells were centrifuged at 100 g during 3 min, and cultured in RPMI Medium supplemented with B27 (without insulin). Medium was exchanged after 24 hours, and aggregates were transferred to orbital suspension cultures (agitation rate of 90 rpm) and maintained for additional 8 days. Using this protocol, aggregates composed of >90% of cTnT positive cells (confirmed by flow cytometry) were obtained after 15 days (Correia *et al*., 2018). To further improve maturation state of this cell population, hiPSC-CMs were cultured for additional 10 days in a commercial triiodothyronine (T3) hormone rich medium (Pluricyte Medium, NCardia) as described in Chapter II and elsewhere (Ribeiro et al., 2015). Medium

was exchanged every two days. Cell cultures were performed in at 37ºC an humidified atmosphere (5% CO_2, 3% O_2).

2.1.2. Ischemia/Reperfusion injury setup

After the differentiation and maturation phase (day 25), hiPSC-CMs aggregates were inoculated ($1.3x10^5$ cell/mL) in small-scale flat-bottom stirred-tank bioreactors equipped with trapezoid shaped paddle impellers with long arms (DASGIP® cellferm-pre bioreactor system Eppendorf AG) in 230 mL of CM001-R media composed by DMEM:F12: Neurobasal medium (1:1), supplemented with 1% penicillin streptomycin, 3% fetal bovine serum (FBS) embryonic stem cell-qualified, N2 supplement (1X), B27 supplement (1X), 0.9 mM l-glutamine, 50 µM β-mercaptoethanol (Sigma) (all percentages in v/v). hiPSC-CM aggregates were incubated in CM001-R media at least 15 h before I/R experiments. Data acquisition and process control were performed using DASGIP® Control Software 4.0 (Eppendorf AG). Dissolved oxygen, pH and temperature were monitored with pH and oxygen electrodes (Mettler-Toledo) and a temperature sensor, respectively. Electrodes and temperature sensor were calibrated as described by the manufacturer. Cells were cultivated under defined and controlled culture conditions: temperature 37°C, surface aeration rate 0.1 vols (vvm); 3% O_2 (15% pO2 air saturation), corresponding to myocardial physiologic normoxia (Khan *et al.*, 2010). Stirring rate was set to 70 rpm.

I/R experimental setup is depicted in figure 3.1. Ischemia was mimicked by replacing CM001-R medium with Ischemic Mimetic Solution (IMS; in mM: NaCl, 135; KCl, 8; $MgCl_2$, 0.5; NaH_2PO_4, 0.33; HEPES, 5.0; $CaCl_2$, 1.8; Na+-lactate, 20; pH 6.8) (Zhao *et al.*, 2013), and by decreasing O_2 to values below 0.4%. After 5 h of ischemia, reperfusion was mimicked by re-establishing control culture conditions (CM001-R without FBS at a O_2

of 3%) for an additional 16 hours. A CTL bioreactor was maintained in parallel (CM001-R without FBS at O_2 of 3%), respecting the same medium exchange intervals.

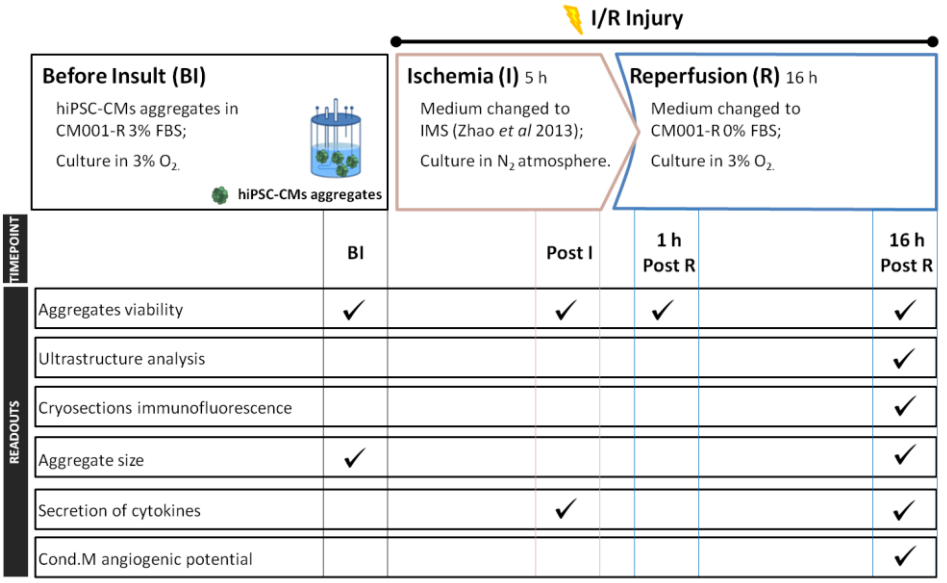

Figure 3.1. Schematic representation of Ischemia/Reperfusion injury (I/R) bioreactor-based experimental setup. Ischemia was mimicked by replacing CM001-R medium with 3% FBS by Ischemic Mimetic Solution (IMS) and by culturing cells in a N_2 gaseous environment. After 5 h of ischemia, reperfusion was mimicked by re-establishing normoxic culture conditions (CM001-R without FBS at 3% O_2). I/R setup was performed using aggregates of hiPSC-CMs in stirred tank bioreactors. The effects of the I/R were evaluated regarding hiPSC-CMs viability, hiPSC-CMs structure and ultrastructure, hiPSC-CMs aggregate size, hiPSC-CMs secretory profile and angiogenic potential of conditioned (Cond.) media at different time points: (BI- Before injury; Post I – Post Ischemia; Post R – Post Reperfusion). Control cultures were done in parallel (using normoxic conditions).

2.2. Culture characterization

2.2.1. Cell viability

The impact of I/R injury in hiPSC-CMs viability was evaluated by cell membrane integrity analysis: aggregates were incubated with 20 µg/mL fluorescein diacetate (FDA), that stains viable cells, and 10 µg/mL propidium iodide (PI), a membrane impermeable DNA-dye that stains

non-viable cells, in DPBS for 2-5 minutes. Samples were then observed under a fluorescence microscope (DMI 6000, Leica Microsystems GmbH) and representative images were taken. Viability of 3D hiPSC-CM cultures was further assessed using NucView® 488 caspase-3 substrate (Biotium), according to the manufacturer's recommendations. Briefly, aggregates were incubated for 1 hour with fresh medium containing NucView® 488 reagent (1:200). Samples were then observed under a fluorescence microscope (DMI 6000, Leica Microsystems GmbH) and representative images were taken.

2.2.2. Aggregate size measurement

Aggregates were imaged in a fluorescence microscope (DMI6000, Leica Microsystems GmbH, Wetzlar, Germany) and Ferret diameter was measured using ImageJ open source software (Rasband, WS, ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2012).

2.3. Phenotypic characterization

2.3.1. Immunofluorescence microscopy

hiPSC-CMs aggregates were washed with DPBS, fixed in 4% (w/v) paraformaldehyde (PFA) and 4% (w/v) sucrose in DPBS for 20 min, and dehydrated in 30% (w/v) sucrose overnight. Samples were then embedded in Tissue-Tek® O.C.T. (Sakura) and frozen at −80°C for cryosectioning. Frozen samples were sliced with a thickness of 10 µm in a cryostat (Cryostat CM 3050 S, Leica). Afterwards, sections were permeabilized for 10 min in 0.1% (v/v) Triton X-100 in DPBS and blocked with 0.2% (v/v) Fish Skin Gelatin (FSG) in DPBS for 30 min, at room temperature (RT, 18-20°C). Sections were then incubated with primary antibodies diluted in 0.125% (v/v) FSG, 0.1% (v/v) Triton X-100 for 2 h at

RT. Cells were washed with DPBS and incubated with secondary antibodies diluted in the same solution for 1 h at RT in the dark. The following primary antibodies were used: α-sarcomeric actinin (1:200, Sigma) and troponin T (1:100, Millipore). Alexa Fluor 488 phalloidin was used to stain F-actin (1:100, Invitrogen).

2.3.2. Transmission electron microscopy

hiPSC-CMs aggregates were washed with DPBS and fixed in 4% (w/v) paraformaldehyde (PFA) and 4% (w/v) sucrose in DPBS for 20 minutes. Samples were stored in DPBS at 4°C until further processing. Aggregates were fixed in a mixture 2% PFA and 2% glutaraldehyde in 0.1 M Phosphate Buffer for 1 hour on ice. Subsequently, samples were washed 3 times with 0.1 M Phosphate Buffer and embedded in 2% low melting point agarose. Agarose was allowed to solidify on ice prior to post-fixation with osmium tetroxide (1% v/v in 0.1 M Phosphate Buffer, 30 minutes on ice protected from light). After several washes in distilled water, the samples were contrasted in 1% Tannic Acid for 20 minutes on ice, washed several times in distilled water, dehydrated in a graded series of ethanol and infiltrated in epon resin (epon: ethanol mixtures: 1:3, 1:1, 3:1, 1 hour and 30 minutes each, pure epon overnight) and embedded in flat embedding molds. Ultrathin sections were cut in a Reichert Ultramicrotome using a diamond knife. Samples were visualized in a H-7650 Transmission Electron Microscope (Hitachi) and representative images were taken.

2.3.3. Cytokine array

Conditioned medium from ischemic and reperfusion phases of both CTL and injury bioreactors were collected, centrifuged at 1,000 xg for 5 min at

RT to remove dead cells and debris and stored at -20°C until further use. Cytokine detection was performed according to manufacturer's instructions (Ab133997, Abcam) and included the screening for the following 42 targets: ENA-78, GCSF, GM-CSF, GRO, GRO-α, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/p70, IL-13, IL-15, IFN-γ, MCP-1, MCP-2, MCP-3, MCSF, MDC, CXCL9, MIP-1δ, CCL5, SCF, SDF-1, CCL17, TGF-β1, TNF-α, TNF-β, EGF, IGF-I, Angiogenin, Oncostatin M, Thrombopoietin, VEGF-A, PDGF BB and Leptin. The intensity of chemiluminescent signal is proportional to the amount of cytokine bound and was determined using the Image LabTM software version 5.0 (Bio-Rad). Spot densities were normalized according to the manufacturer instructions. Mean signal density of each spot was also subtracted with the mean spot densities of basal medium alone (CM001-R medium). IMS-based conditioned medium dismissed similar normalization, since IMS is a DPBS-based medium, where no proteins were quantified using Microplate BCA Protein Assay Kit (Thermo Scientific).

2.3.4. Quantification of growth factors by ELISA

Growth factors HGF and IGF-1 were also quantified in hiPSC-CM aggregates conditioned medium by ELISA (Human Quantikine ELISA kits, R&D Systems), according to manufacturer's instructions. Optical density was measured in 96-well plates using a microwell plate fluorescence reader (Infinite 200 PRO NanoQuant, TECAN).

2.3.5. HUVECS culture and tube-formation assay

Human Umbilical Vein Endothelial Cells (HUVECs, catalog No. 2517A, Lonza) were cultured at 37°C in humidified incubators (5% CO_2, 3% O_2), in 0.1% gelatin-coated plates with Endothelial Cell Growth medium 2

(ECGM2, PromoCell). Medium was replaced every 3 days. Cells were subcultured when about 90% confluent using 0.5% Trypsin-EDTA for 7 minutes at 37°C.

Tube formation assay was performed according to Pedroso et al., 2011. Briefly, ice-cold undiluted Matrigel (Growth factor Reduced, BD Biosciences) (1.97 mg/cm^2) was used to coat 96-well plates and incubated for 40 min at 37°C to allow the Matrigel to solidify. HUVECs were seeded at a density of 5.5x10^4 cell/cm^2 and incubated with the conditioned media from I/R experiments. ECGM2 and DMEM media were used as positive and negative controls for tube formation, respectively. At least four independent images were acquired per condition after 4h of incubation and the morphological aspects of the tube network were quantified using the ImageJ angiogenesis analyzer plugin (Carpentier G. Angiogenesis analyzer. Image J News (2012). http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ), including total master segment length (sum of the length of the detected master segments in the analyzed area), total segment length (sum of length of the segments) total branching length (sum of length of the trees composed from segments and branches), number of nodes and number of junctions (Carpentier, 2012). All cell culture reagents were purchased from Gibco, Life Technologies unless otherwise stated.

2.4. Statistical analysis

Statistical analyses were performed with GraphPad Prism6 (GraphPad Software Inc.). All data are shown as mean with standard deviation. Data were analyzed by One Way ANOVA Tukey test or student t-test (tube formation analysis, cytokine and ELISA results). P-values below 0.05 were considered significant.

3. Results

A 5 h period of acidosis, hyperosmosis and oxygen and nutrient deprivation was performed to mimic the ischemic period. Reperfusion was mimicked by re-establishing control culture conditions, with nutrient-rich medium (without serum) and 3% O_2, corresponding to myocardial physiologic normoxia (Khan *et al.*, 2010). Bioreactor time-profiles of pO_2 and pH throughout the experiment are depicted in figure 3.2.

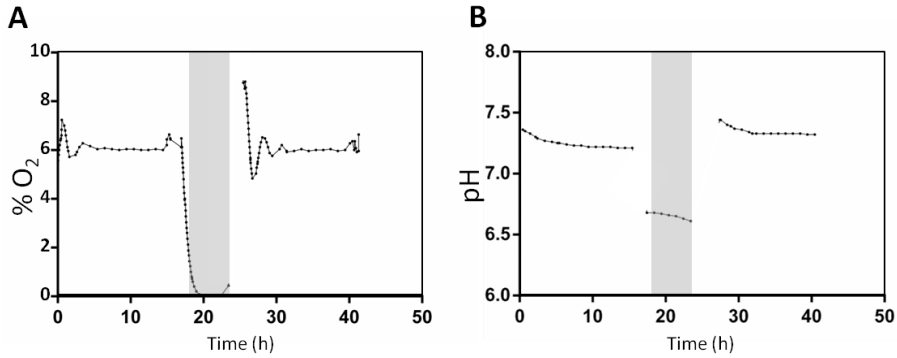

Figure 3.2. Time-profiles of I/R bioreactors. (A) O_2 percentage in the culture medium; (B) pH in the culture medium. The ischemic phase is represented by a grey rectangle. Zero hours correspond to cell inoculation in the bioreactors.

hiPSC-CM aggregates response to the I/R injury was evaluated in terms of cell viability, aggregate structure, cell ultra-structure, protein secretion profile and angiogenic capacity of the resultant conditioned medium.

3.1. Effect of I/R injury on hiPSC-CM aggregates viability

A decrease in viability was observed in hiPSC-CM aggregates upon injury when compared to CTL condition. Caspase 3, an early marker of apoptosis was detected after the ischemic period (figure 3.3A), while a

burst of apoptotic cells was detected by PI staining after the first hour of reperfusion (figure 3.3B). Aggregates maintained in CTL conditions kept high viability throughout the experiment (figure 3.3A,B).

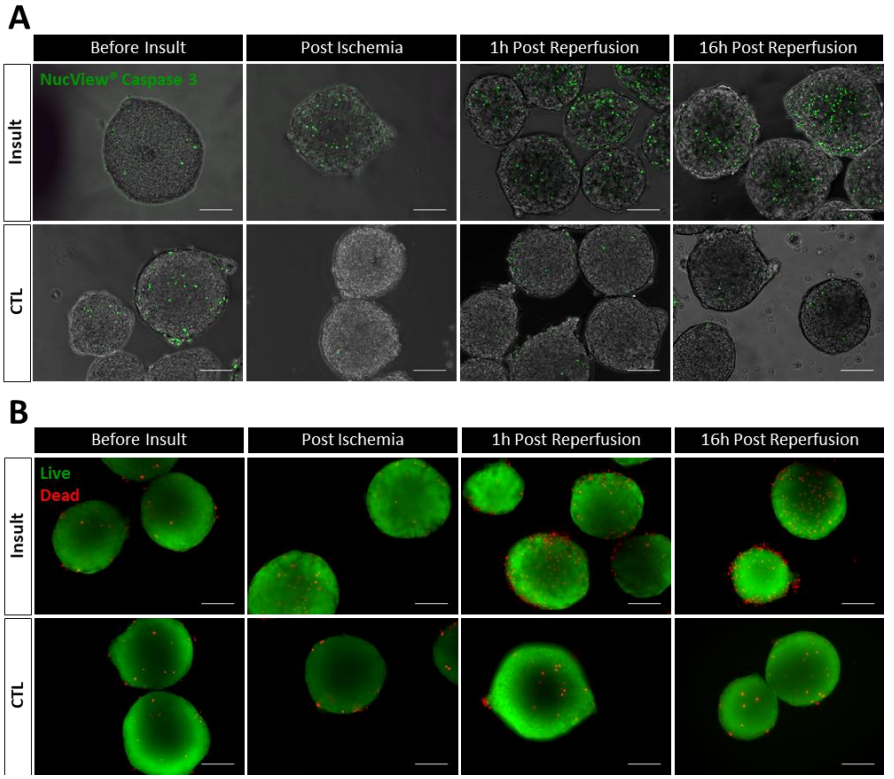

Figure 3.3. Effect of I/R injury in hiPSC-CM aggregates viability. hiPSC-CM aggregates viability was assessed by NucView Staining (green) (A) and by cell staining with FDA (live cells, green) and PI (dead cells, red) (B), before insult, post ischemia, and at 1 hour and 16 hours post reperfusion. Scale bars 200 µm.

hiPSC-CM aggregates average diameter was determined before I/R injury in the bioreactor (260.8 ± 33.1 µm). Neither CTL nor I/R aggregates significantly changed their average diameter during the bioreactor culture (262.9 ± 32.6 and 255.0 ± 30.6 µm, respectively) (figure 3.4).

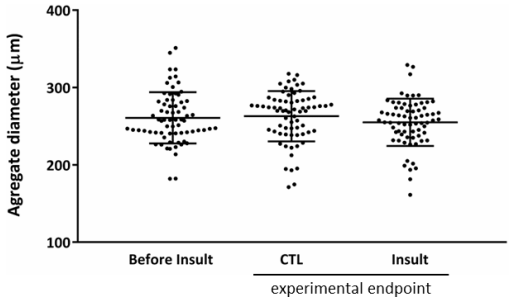

Figure 3.4. hiPSC-CM aggregate size. Average aggregate diameters (A) were measured before and after I/R bioreactor setup experiments (experimental endpoint, corresponding to 16 h after reperfusion).

3.2. Effect of I/R injury on phenotype and ultra-structure of hiPSC-CM

Aggregates exposed to I/R injury presented hollow lacunae in their core (figure 3.5), probably related with the high cell death in this condition (figure 3.3). Nevertheless, both CTL and Insult aggregates maintained the expression of the typical cardiomyocyte phenotypic markers Troponin-T and α-sarcomeric actinin (figure 3.5).

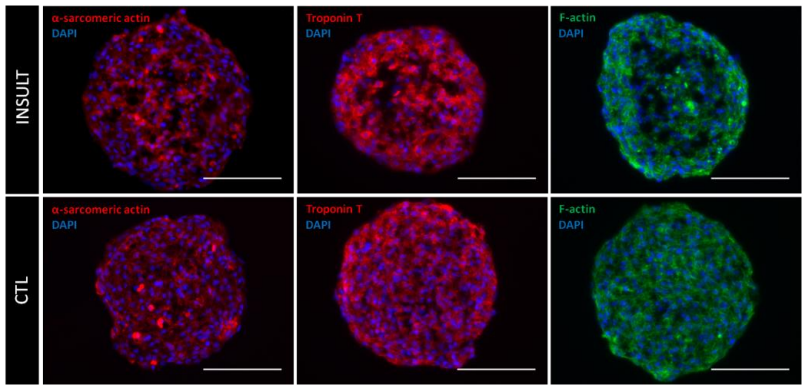

Figure 3.5. hiPSC-CMs aggregates cryosections were characterized using specific cell markers at 16 hours post reperfusion (insult) and in control (CTL) conditions. Although retaining their cardiomyocyte specific markers (α-sarcomeric actinin and Troponin T), hiPSC-CM aggregates upon injury presented hollow lacunae in their structure. Scale bars 100 µm.

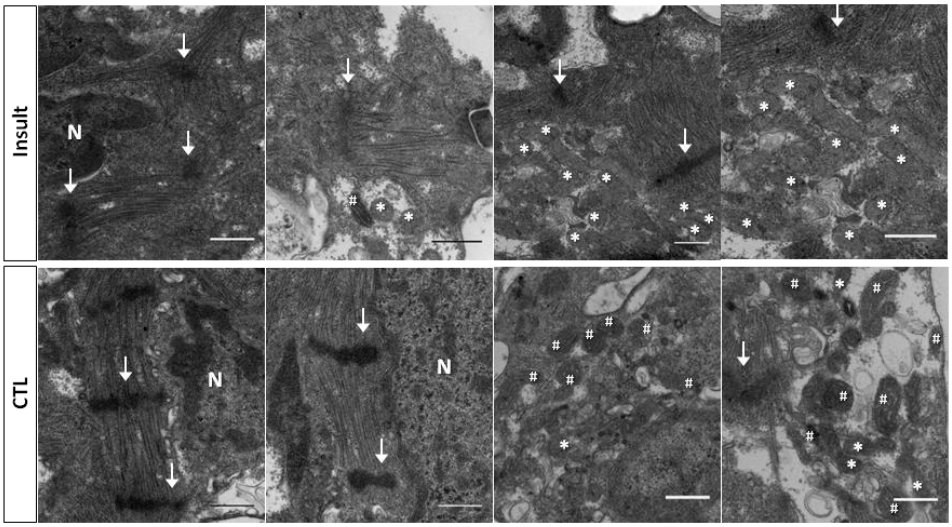

Figure 3.6. Characterization of hiPSC-CM aggregate ultrastructure. Transmission electron microscopy (TEM) images of hiPSC-CMs aggregates in control (CTL) and at 16 hours Post Reperfusion (Insult) conditions. Z-disks (↓), nuclei (N), mithocondria with visible cristae (#) and mithocondria with disorganized cristae structure (*) are highlighted. Scale bars: 500 nm.

TEM analysis was conducted to further characterize the effect of the I/R injury bioreactor setup on the hiPSC-CMs. When compared to the CTL condition, cells exposed to I/R exhibited: i) disruption of sarcomeres myofilament and Z band organization and; ii) higher number of mitochondria with disorganized cristae and membrane rupture (figure 3.6).

3.3. Impact of I/R injury on the secretion profile of hiPSC-CM

In order to evaluate the hiPSC-CM aggregate secretory profile in response to the I/R injury, we collected the conditioned media after the ischemic and reperfusion phases of the injury and analyzed its content regarding key cytokines and growth factors by cytokine array. Several proteins were identified as differentially expressed between CTL and ischemic conditioned media (figure 3.7) and between CTL and reperfusion conditioned media (figure 3.8).

hiPSC-CM aggregate subjected to ischemia showed increased secretion of molecules with key roles in inflammation (MCSF, MDC, Oncostatin-M, GRO, GRO-α, IL-1β, IL-4, IL-5, IL-8, IL-12 p40/p70, IL-15, CCL5, CCL17, CXCL9, TNF-β, VEGF, MCP-1, MCP-2, MCP-3, MIP-1, and ENA-78), angiogenesis (Thrombopoietin, PDGF-BB, GM-CSF, and ENA-78) and migration (GRO, GRO-α, IL-8, SDF-1, IGF-1, VEGF, MCP-1, MCP-2, ENA-78, and SCF) (figure 3.7A). IGF-1 was further validated by ELISA assay, confirming the increased secretion of this growth factor by ischemic hiPSC-CM aggregates vs CTL (figure 3.7B).

Upon reperfusion phase of injury, hiPSC-CM aggregates subjected to I/R injury showed increased secretion of pro-inflammatory cytokines (I-309, CCL5, TNF-α) as well as two pro-angiogenic proteins (Angiogenin and GCSF) (figure 3.8A). Moreover, HGF, a growth factor also previously shown to be upregulated in damaged CMs (Ong *et al.*, 2015) was quantified by ELISA, also showing an increased secretion in reperfusion injury conditions (figure 3.8B).

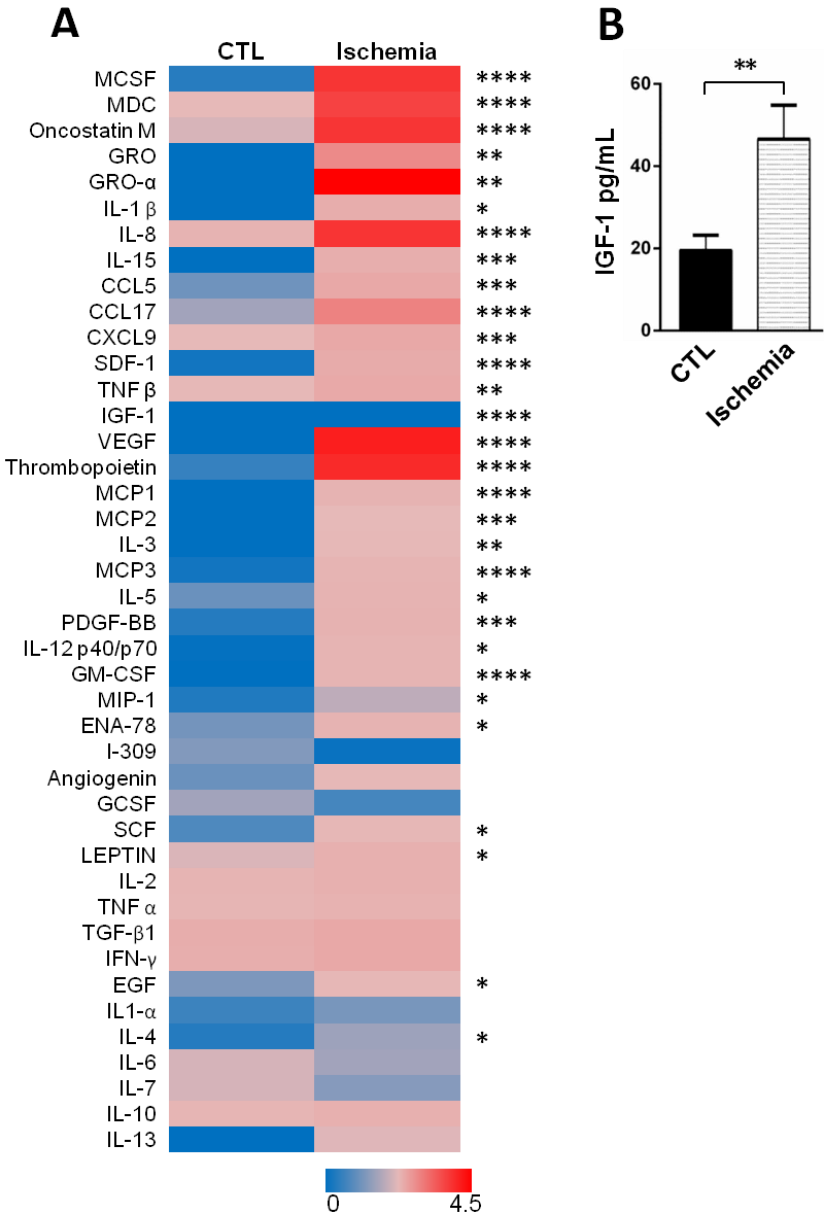

Figure 3.7. Secretion of cytokines and growth factors in hiPSC-CMs aggregates upon ischemia phase of I/R injury. hiPSC-CM aggregates conditioned medium from control (CTL) and upon Ischemia was profiled using a cytokine array. Color scale from 0 (blue) to 4.5 (red) (units in mean normalized spot density) (A). IGF-1 concentration was validated by ELISA assay (B). *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001 (student t-test, n= 2).

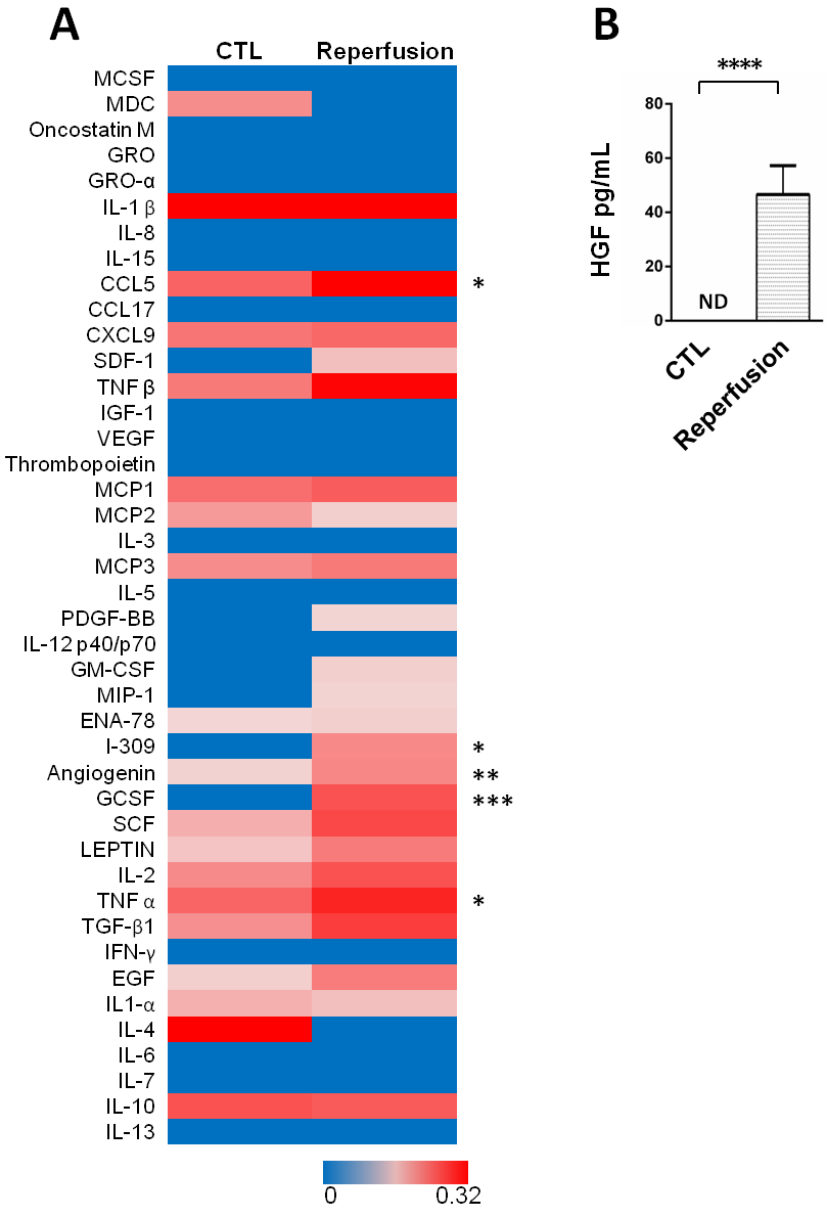

Figure 3.8. Secretion of cytokines and growth factors in hiPSC-CMs aggregates upon reperfusion phase of I/R injury. hiPSC-CM aggregates conditioned medium from control (CTL) and upon Ischemia was profiled using a cytokine array. Color scale from 0 (blue) to 0.32 (red) (units in mean normalized spot density) (A). HGF concentration was measured by ELISA assay (B). *P≤0.05; **P≤0.01; ***P≤0.001; ND: not detected (student t-test, n= 2).

Upon identification of three key angiogenic cytokines in post reperfusion injury supernatant (I-309, Angiogenin and GCSF) (Figure 3.8A), the increased pro-angiogenic potential of this medium vs CTL was tested by *in vitro* tube formation assay. HUVECs were incubated with conditioned media from hiPSC-CM aggregates in CTL condition and after 16h of reperfusion. As shown in figure 3.9, the angiogenic potential of the conditioned medium from the hiPSC-CM aggregates is increased when comparing to CTL conditions, represented by a significant increase in total master segment length (figure 3.9A), total segment length (figure 3.9B) and number of nodes (figure 3.9D). Although not significant, other parameters also show higher values in conditioned medium upon injury, such as total branching length (figure 3.9C) and number of junctions (figure 3.9E).

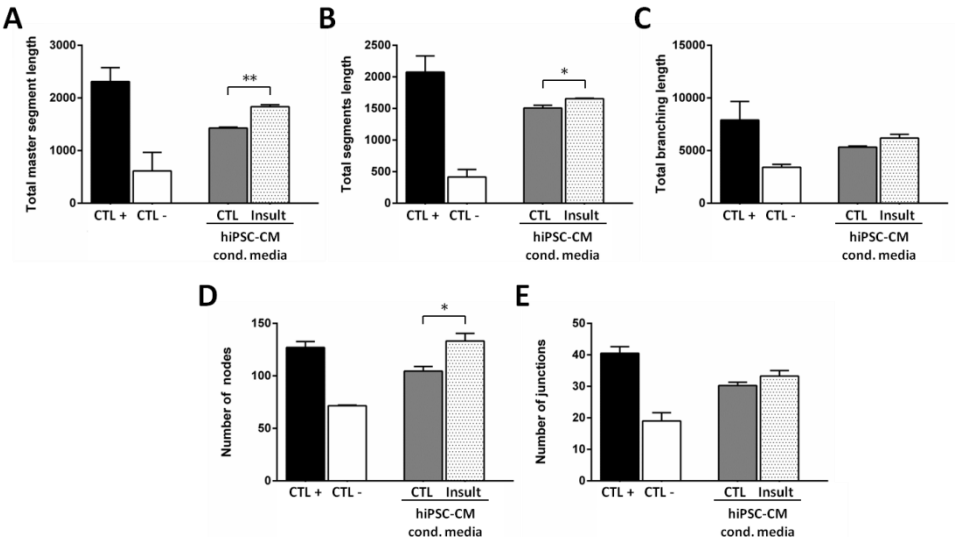

Figure 3.9. Angiogenic functional evaluation of I/R bioreactors conditioned medium. Conditioned media of hiPSC-CMs aggregates in control (CTL, grey bars) and at 16 hours Post Reperfusion (Insult, black and white dotted bars) conditions were tested for angiogenic potential by HUVECS tube formation assay. (A) Total master segment length, (B) total segment length, (C) total branching length, (D) number of nodes and (E) number of junctions quantification by angiogenesis analyzer software are represented. $^{*}P<0.05$; $^{**}P<0.01$ (student t-test, n=2). ECGM2 and DMEM were used as positive (black bars) and negative (white bars) control, respectively.

4. Discussion

In this work we have developed a novel human I/R injury *in vitro* model using hiPSC-CM 3D cultures and bioreactor technology. In order to improve the physiological relevance of the model, several strategies were applied, including: i) cells were submitted to a 10-day maturation period with T3 hormone-rich commercial media optimized for hiPSC-CM myocardial maturation (Ribeiro *et al.*, 2015); ii) hiPSC-CMs were cultured in 3D aggregates; iii) and in defined oxygen and pH conditions.

Besides the addition of a hormone-based maturation process, cells were cultivated in 3D, which has also been reported to improve the functional maturation level of hCMs (Zhang *et al.*, 2013; Daily *et al.*, 2015; Correia *et al.*, 2018), as well as allowing for a higher recapitulation of the native *in vivo* microenvironment, with higher cell-cell and cell-ECM interactions. Culture in 3D also results in the creation of oxygen, metabolite and nutrient gradients along the spheroid structure, which does not occur in a monolayer system. In fact, in the core of spheroids exposed to I/R setup, we observed hollow lacunae, probably left behind by dead cells.

The I/R injury bioreactor setup was successful in mimicking several hallmarks of CM response to AMI, including CM death, cellular ultra-structure modifications and secretion of key cytokines and growth factors in the different phases of injury.

hiPSC-CM spheroids showed a higher concentration of dead cells upon the first hour of reperfusion, which is consistent with the described *in vivo* pathophysiology of CM death during AMI, where reactive oxygen species (ROS) accumulation, calcium overload and mitochondrial permeability pore (MPTP) opening during the first moments after reperfusion cause a burst of CM death due to mitochondria swelling (with loss of typical cristae folding organization) and ultimately rupture, with release of

cytochrome c and several apoptotic factors, causing activation of apoptotic pathways by caspase 3 and 9 activation. (Hausenloy and Yellon, 2013). High caspase 3 levels were detected in Post Ischemia time-point; however it is important to take into account that caspase 3 staining was performed for 1 h in reperfusion conditions (3% pO_2, in CM001-R media). During AMI, upon reperfusion, the intracellular calcium overload, pH normalization and ROS accumulation activate calpain system mechanisms (Hernando *et al.*, 2010), leading in turn to myofibrillar protein degradation and inherent disruption of sarcomere structure (Portbury, Willis and Patterson, 2011). In our model we observe ultrastructural hallmarks of CM death upon AMI: analysis by TEM revealed morphological changes characteristic of I/R injury, including loss of organized mitochondria cristae folding structure as well as loss of sarcomere organization pattern.

Using cytokine array and ELISA assays, we were also able to characterize hiPSC-CM aggregates secretory profile in both phases of injury, comparing CTL with I/R injury conditions. Secretion of inflammatory cytokines by CMs is an hallmark of AMI pathophysiology, resulting in the recruitment of immune cells to the injury site (reviewed in Frangogiannis, 2014). Besides pro-inflammatory, CM signals released upon injury have also been reported to be associated with angiogenesis, remodeling of ECM as well as recruitment and activation of resident CSCs (Li *et al.*, 2014; Ong *et al.*, 2015). hiPSC-CM subjected to ischemia showed increased secretion of molecules with key roles in inflammation, several of which previously identified as up-regulated in ischemic heart tissue such as: MCSF (Frangogiannis *et al.*, 2003), Oncostatin-M (Gwechenberger *et al.*, 2004), IL-1β (Saparov *et al.*, 2013), IL-8 (Saini *et al.*, 2005; Vandervelde *et al.*, 2005), IL-15 (Turillazzi *et al.*, 2014), CCL5 (Montecucco *et al.*, 2012), SDF-1, GM-CSF (Vandervelde *et al.*, 2005;

Ong *et al.*, 2015), VEGF (Vandervelde *et al.*, 2005; Ong *et al.*, 2015), PDF-BB, SCF, EGF, MCP-2, MCP-3 (Ong *et al.*, 2015), MCP-1 (Kakio *et al.*, 2000), and IL-5 (Saini *et al.*, 2005). Other molecules have also been previously associated to angiogenesis (PDGF-BB, GM-CSF, IL1-β) as well as migration (GRO, GRO-α, ENA-78 Boucek *et al.*, 2015), (SDF-1, Renko *et al.*, 2018) and activation (IGF-1, Waring *et al.*, 2014) of CSCs.

Conditioned media of hiPSC-CM aggregates exposed to reperfusion showed increased levels of pro-inflammatory (CCL5, I-309, TNF-α) as well as pro-angiogenic (I-309, Angiogenin and GCSF) proteins. Again, most of the proteins identified as differentially expressed have been previously identified as increased upon AMI in *in vivo* and *in vitro* setups including: CCL5 (Montecucco *et al.*, 2012), and TNF-α (Vandervelde *et al.*, 2005; Ong *et al.*, 2015; Awada, Hwang and Wang, 2017; Shi *et al.*, 2017). HGF, a protein associated with activation of resident stem cell migration (Aghila Rani and Kartha, 2010; Awada, Hwang and Wang, 2017) was also identified as more secreted upon reperfusion. The increased pro-angiogenic capacity of hiPSC-CM spheroid conditioned media was also validated by tube formation assay.

Interestingly, different sets of cytokines (with the exception of CCL5, found in both phases) were found increased in post ischemia and post reperfusion phases. Moreover, we detected a more acute response regarding number of cytokines and average cytokine signal intensity upon ischemia when comparing to post 16h of reperfusion. Such results emphasize the distinct pathophysiology of the two phases of injury, and the importance to recapitulate both when studying AMI *in vitro*. To the best of our knowledge, most studies focused on cytokine release in AMI contexts do not study ischemia and reperfusion phases separately.

5. Conclusion

In summary, we successfully established a novel and relevant human I/R injury *in vitro* model, with recapitulation of important hallmarks of CM response to AMI. Culture of hiPSC-CMs as 3D aggregates and the application of stirred tank controlled bioreactors allowed us to recapitulate key CM hallmark responses to AMI as well as the extracellular microenvironment physico-chemical parameters of both phases of I/R injury. Moreover, we provide a characterization of hiPSC-CM cytokine secretion upon the different phases of injury.

The cell model developed herein represents a step closer to *in vivo* human CM milieu, and can be employed as an alternative to planar (2D), human immature and murine cell based I/R models. This model has the potential to serve as a platform for further studies aimed at interrogating hCM mechanisms of action upon AMI. Moreover, the use of hiPSC-CMs allows investigation of hCMs with different genetic backgrounds. Adding to mechanistic studies, we also envision our model as relevant for *in vitro* drug screening assays. In future studies, we also plan to increase the complexity and representation of *in vivo* tissue by adding other myocardium human cell populations such as endothelial cells, hCFs and smooth muscle cells, all with key roles in AMI physiology.

6. Acknowledgments

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Chapter IV

Using advanced quantitative proteomic tools to unveil hCSCs response to hCM I/R injury paracrine factors

Author Contribution

Maria João Sebastião participated in the experimental setup and design, performed the experiments, participated in data analysis and wrote the chapter.

Abstract

Human Cardiac Stem Cells (hCSCs) transplantation is arising as a novel therapy option for acute myocardial infarction (AMI) patients. The majority of the studies reported point to paracrine signaling as the main effector in hCSCs activation and regenerative capacity. However, the mechanisms of action of these cells in response to AMI paracrine context are still poorly understood. In the present study, a quantitative proteomics analysis was employed to investigate hCSC response to paracrine factors released by human cardiomyocytes (hCMs) in response to an *in* vitro AMI Ischemia/Reperfusion (I/R) injury. A total of 714 proteins were quantified, from which 86 were differentially expressed, including 63 proteins up-regulated and 23 down-regulated in hCSCs exposed to hCM I/R injury conditioned media vs hCSCs exposed to control hCM conditioned media. Bioinformatics functional analysis revealed up-regulation of proteins involved with endocytosis, paracrine signaling, proliferation, migration and stress response. This work provides an insight into hCSC biology in response to paracrine cues derived from hCMs upon I/R injury, and the data generated herein constitutes a rich resource for further studies aiming at potentiating the regenerative properties of these cells.

Contents

1. Introduction

Acute myocardial infarction (AMI) and the inherent tissue damage caused by Ischemia/Reperfusion (I/R) injury is one of the biggest causes of death in developed countries (Benjamin *et al.*, 2017). In response to the I/R stress, cardiomyocytes (CMs), the main functional cellular unit of the myocardium (Pinto *et al.*, 2016; Zhou and Pu, 2016) secrete an array of signaling vesicles (Gupta and Knowlton, 2007; Yu *et al.*, 2012; Ribeiro-Rodrigues *et al.*, 2017; Loyer *et al.*, 2018), cytokines, chemokines and growth factors (Torella *et al.*, 2007; Li *et al.*, 2014; Ong *et al.*, 2015) which together with the produced reactive oxygen species and I/R injury physico-chemical changes recruit immune cells to the site of injury. Such molecules have also been proposed to activate resident cardiac stem/ progenitor cell (CSC) populations.

Since their discovery by Beltrami and colleagues (Beltrami *et al.*, 2003), CSCs have been increasingly receiving more and more attention due to their regenerative properties. CSCs have been reported to proliferate and migrate to the site of injury upon I/R, releasing paracrine signals with beneficial effects on CM survival and reduction of inflammation. Such properties have been demonstrated in several preclinical trials focused on endogenous CSC activation with growth factors (Urbanek, Rota, *et al.*, 2005a; Ellison *et al.*, 2011; Koudstaal *et al.*, 2014; O'Neill *et al.*, 2016; Blanco Blazquez *et al.*, 2017) and transplantation of CSCs in animal models of AMI (meta-analysis in Zwetsloot *et al.*, 2016). However, so far clinical studies have not demonstrated the same level of functional results, still lacking a robust benefit over standard-of-care. One of the reasons pointed to the lack of success in the translation from animal studies to the clinic is the limited understanding of human CSCs (hCSCs) regenerative mechanisms of action (Mathur *et al.*, 2017).

Several studies have been conducted to better understand CSC regenerative biology (Stastna *et al.*, 2010; Albulescu *et al.*, 2015; Sharma *et al.*, 2015; Park *et al.*, 2016; Torán *et al.*, 2017). However, while paracrine communication is considered as the main player in the activation and response of this cell population, most studies do not evaluate the important contribution of the paracrine milieu resultant from injured CMs upon AMI.

In order to investigate the effect of human CM secretome upon I/R injury on hCSCs, we incubated hCSCs with conditioned medium from humsn pluripotent stem cell derived CMs (hiPSC-CMs) that were cultured as three-dimensional (3D) aggregates in stirred-tank bioreactors and subjected to an *in vitro* I/R injury setup (**Chapter III**). hCSC proteome was characterized by a non-targeted label-free quantitative proteomics approach (SWATH-MS, sequential window acquisition of all theoretical mass spectra mass spectrometry). In total, 714 proteins were quantified, including 86 proteins differentially expressed between hCSCs incubated with control and hiPSC-CM I/R injury conditioned medium. Functional analysis revealed an upregulation of proteins associated with migration, proliferation, paracrine signaling and endocytosis.

2. Material and Methods

2.1. Generation of hiPSC-CM I/R Conditioned Media

hiPSCs were differentiated to 3D hiPSC-CM aggregates and subjected to I/R injury in a bioreactor setup as previously described (please see **Chapter III** for details). Conditioned medium (serum free) from both insult and control bioreactors were collected at the end of reperfusion phase, centrifuged (1,000xg, 5 min) to remove dead cells and debris, and stored at -20°C until further use.

2.2. hCSC cell culture

CSCs were obtained from human right atria appendage myocardial tissue, isolated and characterized as described elsewhere (Lauden *et al.*, 2013). Cells were cultured at 37 °C in humidified atmosphere (5% CO_2, 3% O_2) in CM001-R medium composed by DMEM:F12: Neurobasal medium (1:1), supplemented with 1% penicillin streptomycin, 10% fetal bovine serum (FBS) embryonic stem cell-qualified, N2 supplement (1X), B27 supplement (1X), 0.9 mM l-glutamine, 50 μM β-mercaptoethanol (Sigma), insulin transferrin selenium (0.5X), 10 ng/mL bFGF, 20 ng/mL EGF-I and 30 ng/mL IGF-II (Prepotech), (all percentages in v/v). Medium was replaced by 50% every 3 days. Cells were subcultured when about 80% confluent using Tryple™ Select Enzyme for 5 minutes at 37 °C. Cells were used at passages 7–8. Three different donors were analyzed: hCPC1 (57 years old, male), hCPC03 (78 years old, mal) and hCPC04 (17 years old, female). All cell culture reagents were purchased from Gibco, Life Technologies unless otherwise stated.

2.3. hCSC proliferation

hCSCs were plated at $1{,}5x10^4$ cell/cm^2 in complete CM001-R medium. After 24h, medium was exchanged by CM001-R medium without FBS. hCSC number was accessed by nuclei count with crystal violet solution staining after 3 days of incubation. Briefly, cells were resuspended in lysis buffer (0.1% Triton X-100 in 0.1M citric acid) directly in culture wells and incubated at 37°C for at least 48 h. Nuclei were stained with crystal violet dye (0.1% v/v in lysis buffer) and the total number of nuclei counted in a Fuchs-Rosenthal hemocytometer chamber. Fold increase in hCSC number was calculated as the ratio between the cell number at the experimental time point assayed and inoculated number of cells.

2.4. Quantification of growth factors

hCSCs were plated at 1,5x10^4 cell/cm^2 in complete CM001-R medium. After 24h, medium was exchanged by CM001-R medium without FBS. After 3 days of incubation, quantification of growth factors CXCL6 and IGF-1 in hCSCs conditioned medium was performed by ELISA. Human Quantikine ELISA kit (R&D Systems) were used according to manufacturer's instructions. Optical density was measured in 96-well plates using a microwell plate reader (Infinite 200 PRO NanoQuant TECAN).

2.5. Incubation of hCSCs with I/R Conditioned Media

hCSCs were plated at 1,5x10^4 cell/cm^2 in complete CM001-R medium. After 24h, medium was exchanged by hiPSC-CM conditioned media. A control with non conditioned basal CM001-R (without FBS) was maintained in parallel. After 3 days in culture, the impact of hiPSC-CM conditioned media was evaluated regarding hCSC whole proteome. Cells were cultured at 37°C in humidified incubators (5% CO_2, 3% O_2). The experimental workflow is illustrated in figure 4.1.

2.6. Quantitative whole proteome analysis

After 3 days in culture with hiPSC-CM conditioned media, hCSCs were harvested (Tryple™ Select Enzyme for 5 minutes at 37°C) and washed twice with DPBS by centrifugation. Cell pellets were placed at −80 °C until further proteomic analysis. Proteins were extracted, quantified and processed from cell pellets as described elsewhere (Abecasis et al., 2017). Briefly, cell pellets were resuspended in lysis buffer [50 mM Tris (pH 7.8); 250 mM Sucrose; 2 mM EDTA] with protease inhibitors and incubated on ice for 10 min. Cells were lysed with 30 passes through the 301/2 Gauge needle at 4 °C. The cell debris, unbroken nuclei, and other

membrane proteins were pelleted and removed by centrifugation at 1,000 x *g* for 10 min at 4 °C and total protein amount in the supernatant was quantified using a Microplate BCA Protein Assay Kit (Thermo Scientific).

Proteins were digested in gel as described elsewhere (Soares *et al.*, 2016). Briefly, protein bands were distained with 50% (v/V) acetonitrile, reduced with 10mM DTT, alkylated ith iodoacetamide 55mM, and digested at 37°C with 6.7 µg/mL trypsin.

2.6.1. Generation of the spectral reference library

Each sample (2.5 µg) was used for information-dependent acquisition (IDA) analysis by NanoLC-MS using TripleTOF 6600 (ABSciex). A reversed phase nanoLC with a trap and elution configuration, using a Nano cHiPLC Trap column (200 µm × 0.5 mm ChromXP C18-CL 3 µm 120 Å) and nano column (75 µm × 15 cm ChromXP C18-CL 3 µm 120 Å) was performed. Water with 0.1% (v/v) formic acid (solvent A) and acetonitrile with 0.1% (v/v) formic acid (solvent B) were used. Samples were loaded in the trap column at a flow rate of 2 µL/min for 10 min using 100% (v/v) solvent A. Peptide separation was performed in the nano column at a flow rate of 300 µL/min applying a 90 min linear gradient of 5% to 30% (v/v) of solvent B. IDA scanning full spectra (400–2000 m/z) for 250 ms. The top 40 ions were selected for subsequent MS/MS scans (150–1800 m/z for 50 ms each) using a total cycle time of 2.3 s. The selection criteria for parent ions included a charge state between +2 and +5, and counts above a minimum threshold of 125 counts per second. Ions were excluded from further MS/MS analysis for 12 s. Fragmentation was performed using rolling collision energy with a collision energy spread of 5. The spectral library was created by combining all IDA raw files using ProteinPilot™ software (v5.0 ABSciex) with the Paragon algorithm and with the following search parameters: search against

Homo sapiens from Uniprot/SwissProt database (version 03/2018); trypsin digestion; iodoacetamide cysteine alkylation; through identification efforts. After a false discovery rate (FDR) analysis, only FDR<1% were considered. The output of these searches, in the form of a group file was used as the reference spectral library.

2.6.2. SWATH-MS analysis and targeted data extraction

For quantitative analysis, 2.5 µg of each sample were subjected to three SWATH runs. Similar chromatographic conditions to the previously described IDA run were used. The mass spectrometer was operated in a cyclic product ion data independent acquisition (DIA). A variable windows calculator (SWATH Variable Window Calculator_V1.0, AB SCIEX) and SWATH acquisition method editor (AB SCIEX) were used to setup the SWATH acquisition. A set of 32 overlapping windows (containing 1 m/z for the window overlap) was constructed, covering the precursor mass range of 400 – 1200 m/z. A 50 ms survey scan was acquired at the beginning of each cycle, and SWATH MS/MS spectra were collected for 96 ms resulting in a cycle time of 3.172 s. Rolling collision energy with a collision energy spread of 15 was used. The spectral alignment and targeted data extraction of DIA samples were performed using PeakView v.2.1 (AB SCIEX; Framingham, US) with the reference spectral library. For data extraction the following parameters were used: six peptides/protein, six transitions/peptide, peptide confidence level of >99%, FDR threshold of 1%, excluded shared peptides, and extracted ion chromatogram (XIC) window of 10 min and width set at 20 ppm, as previously described (Cunha *et al.*, 2017; Simão *et al.*, 2018). A total of 714 proteins were quantified under these conditions. The full list of quantified proteins can be accessed in supporting information file 4.1.

2.6.3. Proteomic data analysis

To identify differentially expressed proteins, student's T test analysis was performed using GraphPad Prism6 (GraphPad Software Inc., California, USA). Resulting p values and fold changes were used to define up- and down- regulated proteins. Differentially expressed proteins were defined as those which showed a fold change greater than 1.5 (up-regulated) or lower than 0.67 (down-regulated) and p values lower or equal to 0.05. Hierarchical clustering heat map analysis was performed using Perseus software environment (Tyanova et al., 2016). Pathway analysis was performed using Ingenuity Pathway Analysis software (IPA, Quiagen, Germany) by uploading the entire quantified protein list (only proteins with p value≤0.05) and the respective fold change. Statistically significant representation of biological functions and canonical pathways was identified based on IPA p-value, displayed as –log(p-value). This probability score is calculated taking into account the total number of proteins known to be associated with a given function or pathway, and their representation in the experimental dataset. Prediction of inhibition and activation of biological functions and canonical pathways was based on IPA z-score, a statistical measure of the match between expected relationship direction and observed protein expression resulting in activation (z≥2) or inhibition (z≤-2) of the respective pathway. All pathway figures were constructed using Servier medical art images, licensed under a Creative Common Attribution 3.0 Generic License http://smart.servier.com/.

3. Results

In this study we characterized the effect of the secretome of injured hCMs in hCSCs protein expression profile. hiPSC-CM 3D aggregate cultures were exposed to a stirred-tank bioreactor-based *in vitro* I/R injury model, which demonstrated success to recapitulate several hallmarks of hCM response to AMI, including loss of hCM viability, disruption of sarcomere and mitochondria ultra-structure and the secretion of several pro-inflammatory, pro-migratory and pro-angiogenic cytokines (**Chapter III**).

hCSCs were incubated with either basal medium or conditioned medium from control hiPSC-CM aggregates (CTL) or from hiPSC-CM aggregates I/R injury (Insult) cultures. After 3 days of incubation, hCSCs were harvested and analyzed by label-free quantitative unlabeled proteomic analysis (SWATH-MS). In total, 714 proteins were quantified in all samples (full list of proteins in supporting information file 4.1). Biological canonical pathway and functions enrichment analysis was performed using IPA software. The experimental workflow is summarized in figure 4.1.

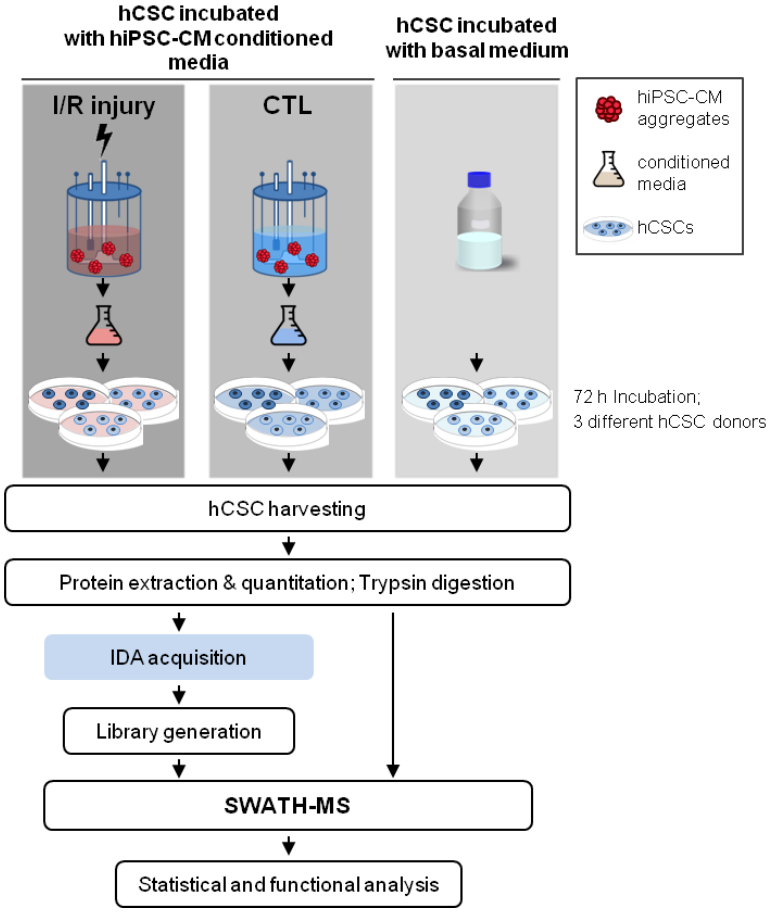


Figure 4.1. Experimental design and quantitative proteomic workflow. Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) conditioned media (pool of 2 biological replicates) from Ischemia/Reperfusion (I/R) injury and control (CTL) bioreactor setups were incubated with cultures of human cardiac stem/progenitor cells.

Upon SWATH, we verified by Principal Component Analysis (PCA) (figure 4.2) that protein expression profile of one of the three biological replicates analyzed (corresponding to hCSCs isolated from three different donors) did not cluster correctly. Therefore, we decided to exclude this donor (donor hCPC1) from all the functional proteomic analysis done. Moreover, we observed a decreased proliferation rate as well as a lower IGF-1 and CXCL6 secretion of this donor (figure 4.3).

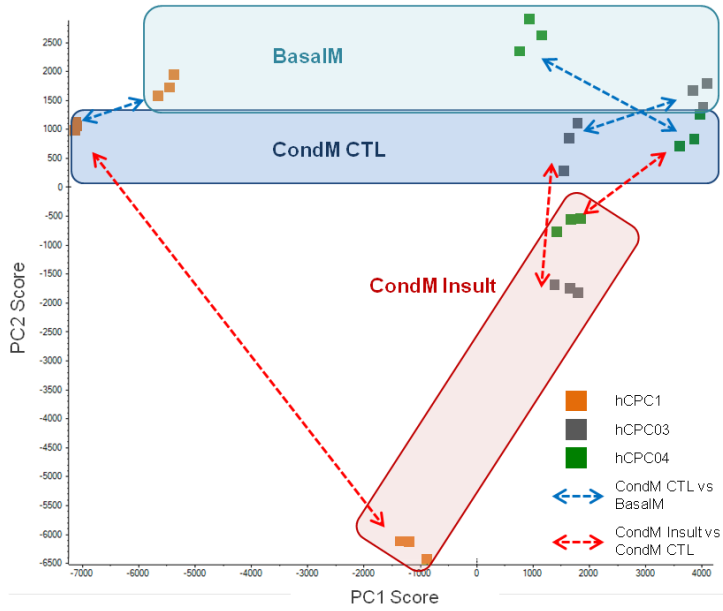


Figure 4.2. Principal Component Analysis (PCA) of all samples, including technical and biological replicates (PCA settings: Unsupervised; Weighting – None; Scaling – Pareto). Scores for PC1 (39.4%) versus PC2 (18.8%) are displayed. Samples are grouped according to the experimental condition (Light Blue: hCSCs incubated with basal medium, BasalM; Dark Blue: hCSCs incubated with control hiPSC-CM conditioned medium, CondM CTL; Red: hCSCs incubated with I/R injury hiPSC-CM conditioned medium, CondM Insult). The effect of CondM CTL vs BasalM (blue arrows) and CondM Insult vs CondM CTL (red arrows) are indicated.

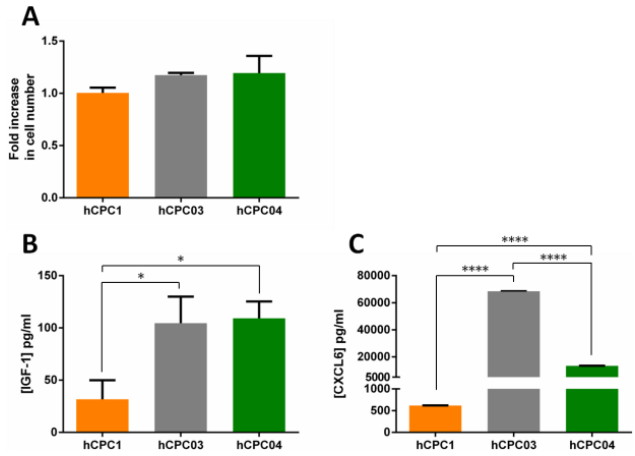
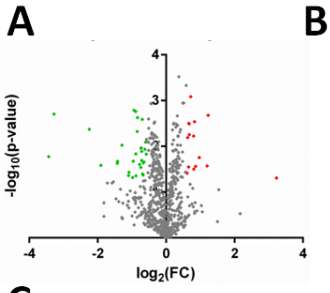


Figure 4.3. hCSC donors analyzed present different characteristics. (A) Fold increase in hCSC cell number; (B) IGF-1 and (C) CXCL6 concentration measured in hCSC conditioned media. *P<0.05; ****P<0.0001, ANOVA Fisher's LSD test. Error bars represent SD of 2 technical replicates.

3.1. Differentially proteome profile in hCSCs exposed to hiPSC-CM conditioned media (control conditions)

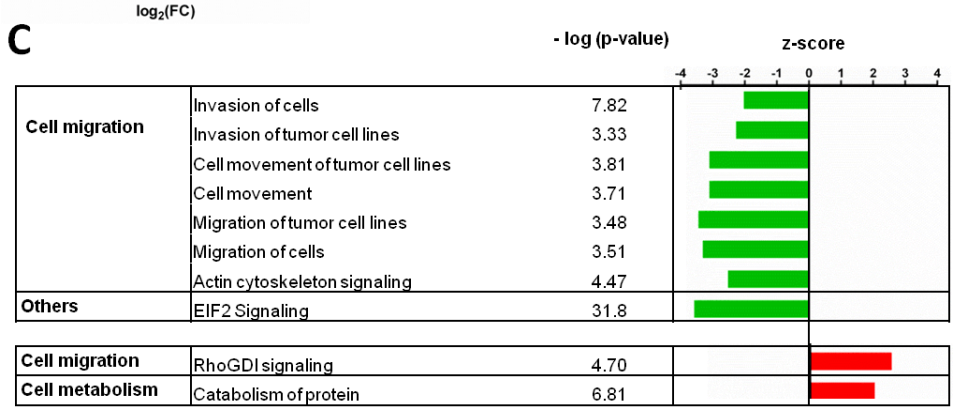
Firstly, to evaluate the impact of hiPSC-CM secretome (control conditions) on hCSC protein expression, we compared the proteome of hCSCs incubated in hiPSC-CM CTL conditioned medium vs hCSCs incubated in basal medium (figure 4.4). From the 714 proteins quantified, 31 were classified as down-regulated (fold change≤0.67, p-value≤0.05) and 15 were classified as up-regulated (fold change≤1.5, p-value≤0.05) in hCSCs exposed to hiPSC-CM CTL conditioned medium (figure 4.4B, supporting file 4.2).



B

Down-regulated						Up-regulated			
4F2	H4	LAMP1	RL6	SURF4	XPO1	1433E	KI16B	SFXN3	
ARC1B	HEXA	LAMP2	RS28	TBB2B		1433Z	LDHB	TCPE	
CD276	HNRPC	OSTC	SC24D	TBB6		ADT3	MOGS	UBE2K	
DYHC1	HS90A	RAN	SFPQ	TOIP1		AK1C1	RCN3		
EIF3L	IPO7	RHOC	SMD1	UAP1		HEXB	RS17		
FUBP2	ITB1	RL14	ST1A2	VATE1		IFT46	SEPT7		

C



Cell migration	Invasion of cells	7.82
	Invasion of tumor cell lines	3.33
	Cell movement of tumor cell lines	3.81
	Cell movement	3.71
	Migration of tumor cell lines	3.48
	Migration of cells	3.51
	Actin cytoskeleton signaling	4.47
Others	EIF2 Signaling	31.8
Cell migration	RhoGDI signaling	4.70
Cell metabolism	Catabolism of protein	6.81

Figure 4.4. Quantitative proteomic analysis of hCSC incubated with hiPSC-CM control conditioned medium in comparison with hCSCs incubated with basal medium. (A) Vulcano plot representing the proteins identified and quantified by SWATH-MS. This analysis enabled the identification and quantification of 714 proteins, including differentially expressed proteins (-log (p-value) ≤1.3): 31 down-regulated (log_2(FC)≤-0.58, green) and 15 up-regulated (log_2(FC)≥0.58, red) in hCSCs incubated with hiPSC-CM CTL conditioned medium. (B) list of down- and up-regulated proteins. (C) Canonical pathways and functions inhibition (z-score≤-2, green) and activation (z-score≥2, red) scores. Only pathways and functions with p-value≤0.05 (-log p -value ≥1.3) were considered. FC: fold change.

Functional analysis (figure 4.4C) showed a significant inhibition of several canonical functions and pathways associated with cell movement and migration, as well as inhibition of EIF2 signaling, a pathway associated with endoplasmic reticulum stress response (Groenendyk, Agellon and Michalak, 2013). RhoGDI signaling, a pathway associated with suppression of cancer cell migration (Gildea *et al.*, 2002; Xiao *et al.*, 2014) was also up-regulated. On the other hand, one canonical function related with higher cell metabolism was identified as activated by IPA analysis (figure 4.4C) (full list of scores and z-scores of canonical pathways and disease functions in supporting information file 4.3).

3.2. Differential proteome profile in hCSCs exposed to hiPSC-CM I/R injury paracrine factors

Following this first comparative analysis, a more exhaustive proteomics characterization of hCSC incubated with hiPSC-CM Insult and CTL conditioned medium was carried out.

Hierarchical clustering of proteomic data revealed that biological replicates clustered together in function of the experimental condition (Insult vs CTL conditioned medium), demonstrating experimental reproducibility (figure 4.5A) and that culture conditions had more impact in hCSC proteome changes than variability between the two donors. From the total 714 proteins identified, 63 were classified as up-regulated (fold change≥1.5, p-value≥0.05) and 23 as down-regulated (fold change≥0.67, p-value≥0.05) in hCSCs incubated with Insult conditioned media (figure 4.5B,C, supporting file 4.4).

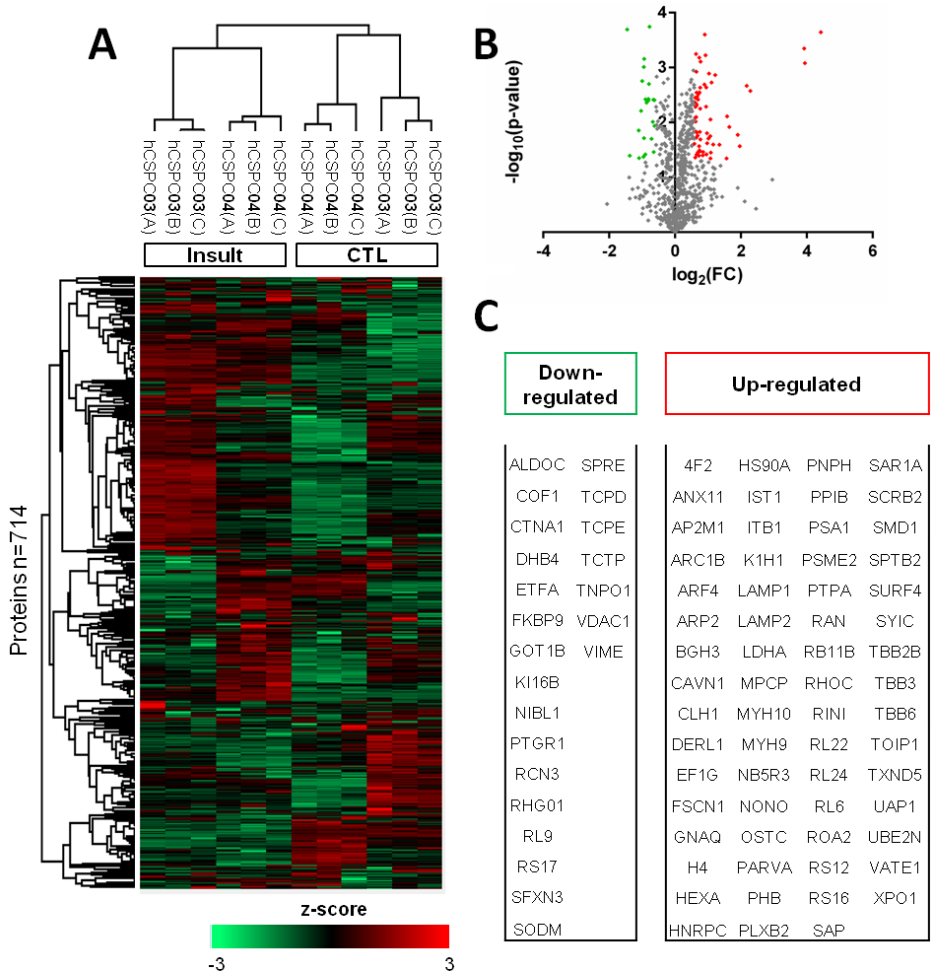


Figure 4.5. Quantitative proteomic analysis between hCSCs incubated with Ischemia/Reperfusion injury (insult) conditioned media and hiPSC-CM control (CTL) conditioned media. (A) Hierarchical clustering (Pearson correlation) heatmap of intensities of proteins (n=714). Perseus z-score values were color coded from green (down-regulation) to red (up-regulation).Heat map analysis was performed for the biological (hCPC03 and hCPC04) and technical (A, B, C) replicates. (B) Vulcano plot representing the proteins identified and quantified by SWATH-MS. This analysis enabled the identification and quantification of 714 proteins, including differentially expressed proteins (-log(p-value)≤1.3) between control and Insult condition: 23 down-regulated (log_2(FC)≤-0.58, green) and 63 up-regulated (log_2(FC)≥0.58, red). (C) List of down- and up-regulated proteins. FC: fold change.

Functional analysis revealed that several biological functions and canonical pathways were enriched (z-score≥2), including terms associated with cell migration and proliferation (table 4.1, figure 4.6), paracrine signaling (table 4.1, figure 4.6, 4.7) and stress response (table 4.1, figure 4.7). On the other hand, RhoGDI signaling, reported to have a suppressive role in metastasis of bladder (Gildea *et al.*, 2002) and breast cancer (Xiao *et al.*, 2014) was identified with a z-score of -2.71, indicating the inhibition of this pathway (table 4.1, figure 4.6), (full list of z-scores of canonical pathways and disease functions in supporting information file 4.5).

Table 4.1. Canonical pathways and functions inhibition (z-score≤-2, green) and activation (z-score≥2, red). Only functions and pathways with –log(p-value)≥1.3 are represented.

		- log (p-value)	z-score -4 -3 -2 -1 0 1 2 3 4
Cell migration	RhoGDI Signaling	5.21	
Cell migration	Actin Cytoskeleton Signaling	6.96	
	Migration of cells	5.66	
	Cell movement of tumor cell lines	5.34	
	Cell movement	6.20	
	Migration of tumor cell lines	4.60	
	Ephrin Receptor Signaling	4.45	
	Rac Signaling	5.13	
	Regulation of Actin-based Motility by Rho	6.53	
	CXCR4 Signaling	1.52	
Cell proliferation	Thrombin Signaling	1.60	
	Cdc42 Signaling	4.12	
	PAK Signaling	2.46	
	p70S6K Signaling	5.50	
Paracrine signaling	Chemokine Signaling	2.45	
	PAK signaling	2.46	
	Neuregulin Signaling	3.55	
	VEGF signaling	2.46	
Stress response	EIF2 Signaling	34.00	
	Apoptosis	7.76	
	NRF2- mediated oxidative stress	4.74	

From the subset of up-regulated proteins, we also identify 2 proteins associated with hypoxia induced HIF1-α signaling (HSP90A, and LDHA) (figure 4.7, supporting information file 4.4); 6 proteins associated with remodeling of cell-cell junctions (ARP2, ARC1B, TBB3, TBB6, and TUBB2B) (supporting information file 4.4); and 7 proteins associated with

endocytosis (ARP2, AP2M1, ARC1B, CLH1, ITB1, RB11B, and RB11B) (figure 4.8, supporting information file 4.4).

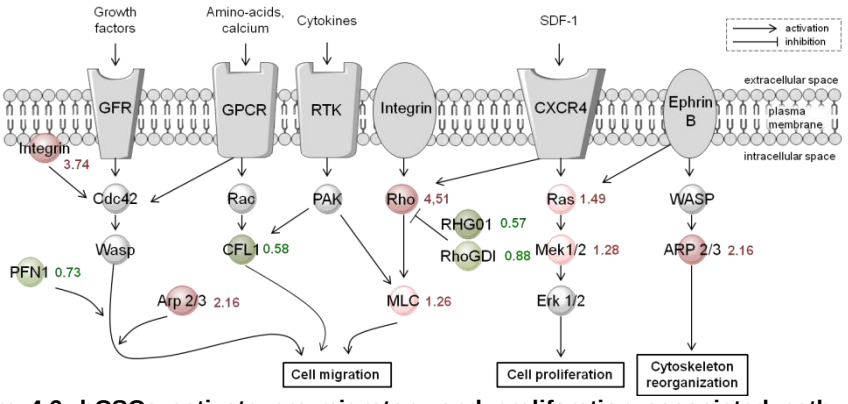


Figure 4.6. hCSCs activate pro-migratory and proliferation associated pathways. Proteomic analysis revealed that Cdc42, Rac, PAK, Rho, CXCR4, and Ephrin Receptor pathways are activated in hCSCs exposed to hiPSC-CM I/R injury conditioned media, while RhoDGI signaling is inhibited. Proteins are depicted as green (negative fold change), red (positive fold change) and grey (not quantified in our analysis). Only proteins with p-value≤0.05 were used in the analysis.

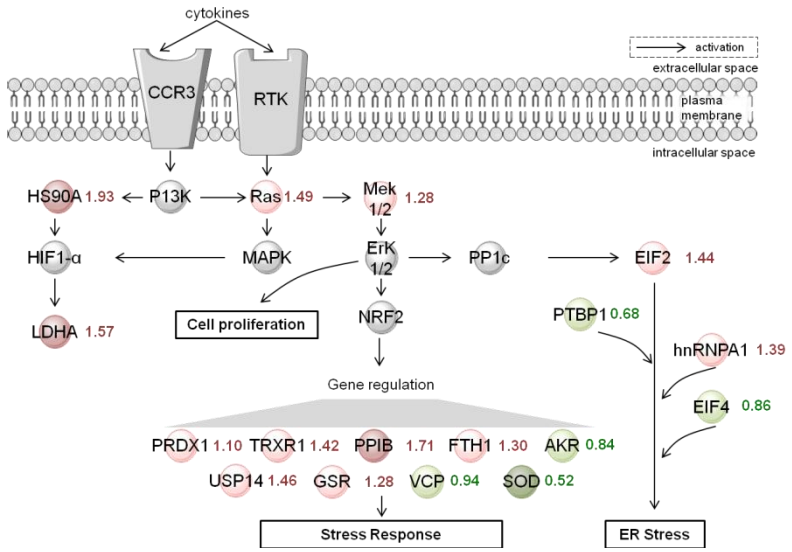


Figure 4.7. hCSCs activate stress response and proliferation associated pathways. Proteomic analysis revealed that HIF1-α, EIF2 and NFR2 pathways are activated in hCSCs exposed to hiPSC-CM I/R injury conditioned media. Proteins are depicted as green (negative fold change), red (positive fold change) and grey (not quantified in our analysis). Only proteins with p-value≤0.05 were used in the analysis.

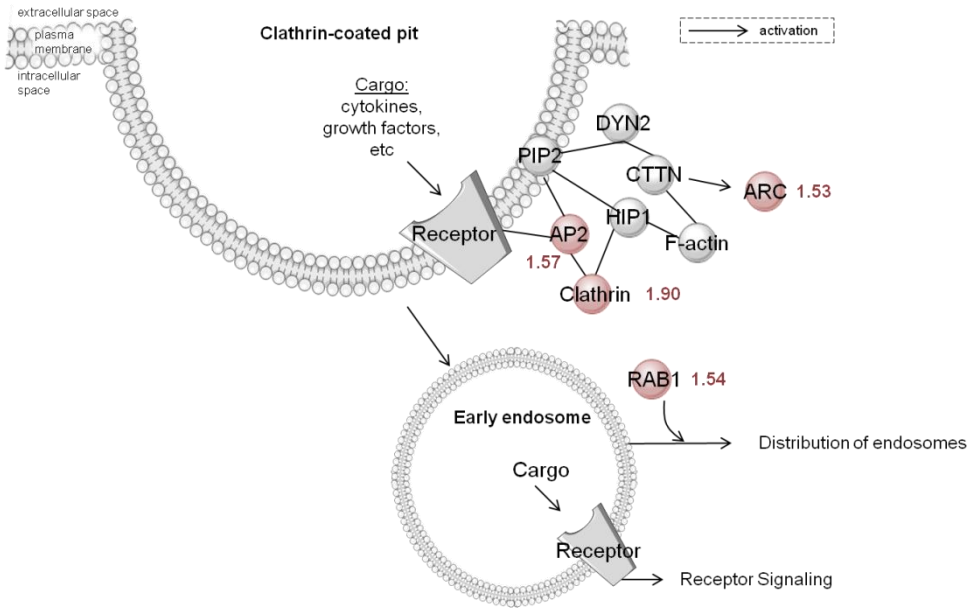


Figure 4.8. hCSCs activate clathrin-mediated endocytosis mechanisms. Proteins are depicted as red (positive fold change) and grey (not quantified in our analysis). Only proteins with p-value≤0.05 were used in the analysis.

Upstream regulator analysis (figure 4.9) also indicated activation of stress response HSP90B protein.

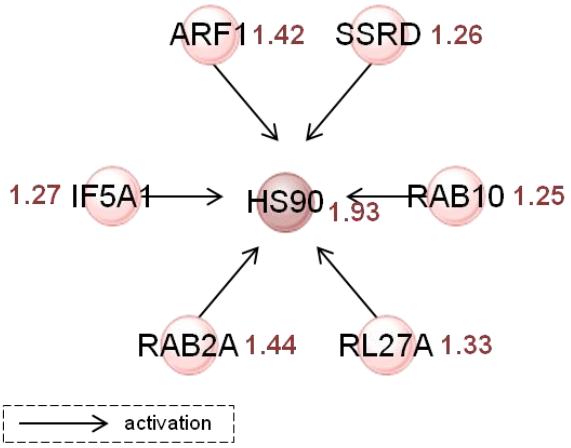


Figure 4.9. Upstream regulator analysis revealed activation of stress response regulator HSP90. Proteins are depicted as red (positive fold change) and grey (not quantified in our analysis). Only proteins with p-value≤0.05 were used in the analysis.

4. Discussion

hCSCs regenerative properties have been extensively described in several AMI animal models (meta-analysis in Zwetsloot *et al.*, 2016). Such findings have already been used and translated into several clinical trials (reviewed in **Chapter I**). Although with promising results, several factors still hamper more robust and beneficial effects of CSC transplantation and endogenous CSC activation based strategies, including low survival and low tissue engraftment of transplanted cells as well as lack of proliferation and migration of endogenous CSCs.

In this study we applied SWATH-MS technology to characterize and quantify proteomic changes in hCSCs upon incubation with hiPSC-CM conditioned medium obtained in a context of I/R injury, in order to expand the knowledge on how hCSC respond to the milieu of growth factors, chemokines and extracellular vesicles released in the heart tissue upon AMI.

Upon SWATH analysis, we verified by PCA that protein expression profile of one of the three biological replicates analyzed did not cluster correctly. Moreover, we also observed a decreased proliferation rate as well as lower IGF-1 and CXCL6 (pro-migratory and pro-angiogenic cytokine) (Torán *et al.*, 2017) secretion of this donor. Such event is probably related with inherent donor to donor variability. In fact, several studies demonstrate that donor dependent factors such as genetic background, co-morbidities and age affect hCSCs response as well as regenerative potential, including secretory profile, proliferation, and migration capacity (Dimmeler and Leri, 2008; Wu *et al.*, 2016; Agarwal *et al.*, 2017; Sharma *et al.*, 2017).

Characterization of hiPSC-CM aggregates secretory profile upon I/R injury (reperfusion conditioned medium, **Chapter III**) revealed that hiPSC-CMs secreted pro-inflammatory, pro-angiogenic and pro-migratory proteins, some previously identified as increased upon AMI *in vivo* and *in vitro* setups. In fact, by incubating hCSCs in this hiPSC-CM conditioned medium resulting from I/R injury setup, we registered the activation of several paracrine signaling pathways in hCSCs, including chemokine and neuregulin signaling, previously shown to be activated in hCSCs upon physiological stress (Torella *et al.*, 2007). Moreover, several proteins associated with clathrin-mediated endocytosis were found as up-regulated, indicating that hCSCs may use this pathway to increase the rate of paracrine signals internalization when exposed to the injury derived conditioned medium. Also, stress response has been associated with regulation of endocytic pathways (reviewed in Di Fiore and von Zastrow, 2014).

hCSCs response also translated into upregulation of proteins associated with activation of proliferation, migration and stress response pathways. Proliferation of endogenous CSCs has been previously shown to increase upon AMI in adult mice (Ellison *et al.*, 2013; Valiente-Alandi *et al.*, 2016) and human (Urbanek, Torella, *et al.*, 2005) hearts. Proteomics functional analysis revealed activation of several pro-proliferation pathways, including Thrombin (Fabrizi *et al.*, 2011) and receptor tyrosine kinases (RTK) associated PAK signaling. Moreover, EIF2 and CXCR4 signaling, also related with activation of cell proliferation were identified as upregulated as well. CXCR4-Rac signaling is also a key pathway involved in cell migration and homing to injury of several cell types (Shen *et al.*, 2011; Penn *et al.*, 2012) including CSCs (Sanada *et al.*, 2014). Other pathways related with cell motility and migration were also found as activated including Rho, Cdc42 as well Ephrin signaling, described to

be associated with hCSC motility (Goichberg *et al.*, 2013). Moreover, several proteins associated with remodeling of cell-cell junctions were also quantified as up-regulated. Activation and migration of cardiac stem cells in response to AMI has been reported by several authors in response to several molecules secreted by CMs, including CTGF (Stastna and Van Eyk, 2012), SDF-1 (Ceradini *et al.*, 2004; Rota *et al.*, 2008), TNF (Al-Lamki *et al.*, 2013), EGF and HGF (Urbanek, Rota, *et al.*, 2005b; Aghila Rani and Kartha, 2010; Boucek *et al.*, 2015). In fact, we identified HGF and TNF-α as significantly upregulated in hiPSC-CM insult conditioned media (**Chapter III**). Moreover, CCL5, a chemokine previously associated with migration of adult stem cells (Bonaros *et al.*, 2008; Phi *et al.*, 2017), and GCSF, known for bone marrow stem cells mobilization (Deindl *et al.*, 2006) were also identified as secreted by hCMs upon I/R injury (**Chapter III**), reinforcing the pro-migratory profile of the hiPSC-CM conditioned media and its effect on hCSCs.

Proteins associated with several stress response pathways including EIF2, NFR2 signaling and activation of heat shock protein 90 (HSP90, a regulator of HIF1-α signaling) were also identified as upregulated in hCSCs exposed to I/R injury paracrine factors. HIF-1α signaling has been shown to be activated in hCSCs in response to I/R injury (Ceradini *et al.*, 2004; Rota *et al.*, 2008).

While in hCSCs exposed to hiPSC-CM insult conditioned medium we see an activation of cell migration, proliferation and stress response pathways, when comparing hCSCs exposed to basal medium with hCSCs exposed to CTL hiPSC-CMs conditioned medium an opposite trend was registered. Namely, we observed inhibition of migration and stress-associated EIF2 signaling. Inhibition of migration is associated with quiescence and stemness state maintenance (Cheung and Rando, 2013; Rumman, Dhawan and Kassem, 2015). We also registered

increase in catabolism of protein pathway that also relates with regulation of cellular quiescence. Such results open the hypothesis that CM secreted factors in homeostasis conditions also plays a role in the regulation of CSC quiescence regulation, besides low oxygen and other stem cell niche factors (Leri *et al.*, 2014; Bellio *et al.*, 2016).

Our results suggest an hCSC response to I/R injury hiPSC-CM-derived factors focused on paracrine activation of migration, proliferation and stress response mechanisms, which is in line with the findings described in the literature. Moreover, this work adds relevant information of activation of several pathways, that, to our knowledge, have not been previously described in hCSC regenerative action mechanisms, including activation of EIF2 signaling. Future studies should include an in-depth characterization of hiPSC-CM aggregates secretory profile as well as validation of the hCSC pathways described using complementary targeted methodologies and functional assays.

5. Acknowledgements

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6. Supporting Information

Supporting information files 4.1, 4.2, 4.3, 4.4 and 4.5 available at: https://www.dropbox.com/sh/3kemuejjxov0mar/AADZG8V1Bg0tAEeoekCN05_Za?dl=0

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Chapter V

Advancing the knowledge on immunomodulatory properties of human cardiac stem cells

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Author contribution

The data presented in this chapter results from the work performed at Coretherapix SLU, Tigenix Group laboratories in Madrid, Spain, as part of the student´s working period abroad. The conception and design of the experiments were performed by Rámon Menta and Dr. Eleuterio Lombardo (Coretherapix SLU, Tigenix Group), with supervision of Dr. Itziar Palacios. The student performed immunomodulatory assay experiments.

Abstract

Transplantation of allogeneic human cardiac/stem progenitor cells (hCSC) is currently being tested in several phase I/II clinical trials as a novel and promising therapy for restauration of myocardial tissue function in acute myocardial infarction (AMI) patients. Previous findings demonstrate that these cells have an immune suppressive profile, interacting with different populations from the immune system, resulting in overall attenuation of myocardium inflammation. However, transplanted hCSCs are still recognized and cleared from the injured site impairing long retention times in the tissue that could translate into a higher clinical benefit.

In this work, through modeling allogeneic hCSC/T-lymphocyte interaction *in vitro* by direct contact, transwells inserts and hCSC conditioned medium, our results demonstrate that hCSCs exert an immune-suppressive effect on T-lymphocyte proliferation not only through the previously described cell-contact dependent PD1/PDL-1 axis but also through a paracrine mechanism associated with indoleamine 2,3-dioxygenase (IDO) enzyme mediated tryptophan metabolism. Such findings constitute a step forward in better understanding the mechanisms of action of transplanted hCSCs in allogeneic settings.

Contents

1. Introduction

Human cardiac/stem progenitor cells (hCSCs) transplantation is arising as a promising therapy for acute myocardial infarction (AMI), one of the most prevalent causes of death worldwide (Benjamin *et al.*, 2017). CSCs are considered by several authors as the preferred candidate cell source for AMI patients, mainly due to their function in the heart, well-documented paracrine regenerative properties (Chimenti *et al.*, 2010; Sharma *et al.*, 2017) and the success of transplantation studies in myocardial infarction animal models (Tang *et al.*, 2010; Crisostomo *et al.*, 2015).

Such success in preclinical stages has led to a rapid translation to the clinic, and several phase I and II clinical trials using hCSCs as an autologous therapy emerged (e.g. SCIPIO and CADUCEUS trials). Autologous transplantation, although carrying lower immunogenicity risks, holds several limitations. Not only might the quality of the cells be compromised by patients age and co-morbidities (Dimmeler and Leri, 2008; Wu *et al.*, 2016; Sharma *et al.*, 2017), but also due to logistic, economical and time-constraints issues. To overcome such limitations, in the last years the field has been moving towards allogeneic CSC sources (e.g. ALLSTAR and CAREMI trials).

Clinical trials have demonstrated physiological improvements, increase in viable tissue and in heart functional outcome (Cahill, Choudhury and Riley, 2017). However, an obstacle still preventing CSCs to meet their full clinical potential and to provide evident clinical benefit over standard-of-care is their limited retention and engraftment in the heart (Hong and Bolli, 2014; Mathur *et al.*, 2017), a problem aggravated in the allogeneic setting (Huang *et al.*, 2010; Malliaras *et al.*, 2012).

Upon AMI, an immune response is triggered, that although essential for proper tissue remodeling and stabilization, carries unwanted inflammatory mediated damage (Epelman, Liu and Mann, 2015) and, in the case of cell therapy approaches, might be also involved in the elimination of injected cells. Effective activation of T-cells, one of the main mediators of inflammatory damage upon AMI,requires simultaneous engagement of T-cell receptor (TCR) and CD28 receptor. TCR binds to antigens presented in major histocompatibility complex (MHC, human leukocyte antigens, HLA in humans) and CD28 receptor binds to B7 (CD80/CD86) costimulatory molecules. hCSCs express HLA class I (that attract killer cytotoxic T-cells), but very low levels of HLA class II molecules (that stimulate antibody producing B-cells), and do not express costimulatory molecules CD80/CD86 (Lauden *et al.*, 2013), presenting therefore a weak immunogenic profile. Besides depicting the immune phenotype of hCSCs, several studies have shed some light on how hCSCs interact with monocytes (Dam *et al.*, 2017), natural killer cells (Boukouaci *et al.*, 2014) and T-lymphocytes (Lauden *et al.*, 2013; van den Akker *et al.*, 2018). All these studies suggest that the immunologic behavior of allogeneic hCSCs might be linked to their therapeutic effects rather than eliciting deleterious immune reactions, with an immunomodulatory effect resulting in attenuation of myocardium inflammation and prevention of adverse scar tissue formation. Also, programmed death ligand 1 (PDL-1) mediated cell-cell interaction was identified as one of the main mechanisms in hCSC immunomodulatory properties, promoting stimulation of regulatory T-cells and subsequent inhibition of T-lymphocyte activation and proliferation (Lauden *et al.*, 2013).

Besides direct cell-cell interactions, paracrine immunomodulatoy effects based on extracellular vesicles have been described for hCSCs (van den

Akker *et al.*, 2018). Moreover, tryptophan (Trp) metabolism through the enzymatic activity of indoleamine 2,3-dioxygenase (IDO) has been described as a key immunosuppressive mechanism for human adipose-derived mesenchymal stem cells (hASCs) (DelaRosa *et al.*, 2009; Menta *et al.*, 2014; Mancheño-Corvo *et al.*, 2015), a cell type already used in several allogeneic cell transplantation studies (Bajek *et al.*, 2016).

Aiming at better understanding the immunomodulatory mechanisms of hCSCs in an allogeneic setting, we further investigated the capacity of hCSCs to inhibit the proliferation of T-lymphocytes *in vitro*. Taken together, our results add to the knowledge on the tolerogenic immune behaviour of hCSCs, showing that hCSC mediated immune modulation is not dependent exclusively on the PDL-1/ programmed cell death-1 (PD-1) pathway axis, but also via Trp degradation by IDO enzyme action. Such findings open new avenues in designing novel hCSC allogeneic transplantation therapies for AMI patients, including strategies to promote a higher hCSC engraftment and longer residence time in the tissue.

2. Materials and Methods

2.1. hASCs isolation and culture

Human ASCs were isolated from adipose tissue lipoaspirates obtained from healthy adult donors as described elsewhere (Mancheño-Corvo *et al.*, 2015). hASCs specific surface markers were verified by flow cytometry: hASCs were positive for HLA-I, CD90, and CD105, and negative for HLA-II, CD40, CD80, CD86, and CD34. The following primary antibody dilutions were used: CD105 (1:50), remaining (1:10). A total of ten thousand events were acquired using a FACSCalibur (BD

Biosciences). Percentage of positive cells was calculated using the FSC-express software.

Cells were cultured at 37°C in humidified incubators (5% CO_2, 3% O_2) in DMEM medium containing 10% FBS. Medium was replaced every 7 days. Cells were subcultured when the culture achieved about 90% confluency using Trypsin-EDTA 0,05% for 5 minutes at 37°C. Prior to immunomodulatory experiments, hASCs were treated with mitomycin-C (20 µg/mL) at 37°C for 30 min. All cell culture reagents were purchased from Gibco, Life Technologies unless otherwise stated. Cells were used at a population doubling level of 14.

2.2. hCSC isolation and culture

Human CSCs were obtained from human right atria appendage myocardial tissue, isolated and characterized as described elsewhere (Lauden *et al.*, 2013). Cells were cultured at 37°C in humidified incubators (5% CO_2, 3% O_2) in Expansion Medium (ExpM, composed by DMEM:F12: Neurobasal medium (1:1), supplemented with 1% penicillin streptomycin, 10% Fetal Bovine Serum embryonic stem cell-qualified, N2 Supplement (1X), B27 Supplement (1X), 0.9 mM L-Glutamine, 50 µM β-Mercaptoethanol (Sigma), Insulin Transferrin Selenium (0.5X), 10 ng/mL bFGF, 20 ng/mL EGF-I and 30 ng/mL IGF-II (Prepotech), all percentages in (v/v). Medium was replaced by 50% every 3 days. Cells were subcultured when the culture achieved about 80% confluency using Trypsin-EDTA 0,05% for 5 minutes at 37°C. Prior to immunomodulatory experiments, hCSCs were treated with mitomycin-C (20 µg/mL) at 37°C for 30 min.

hCSCs from 3 different donors were used in this study: hCPC04 (Female, 17 years), hCSC-40 (Female, 79 years) and hCSC-48 (Female, 45 years). Cells were used at passages 4–6.

2.3. Isolation of hPBMCs

Blood samples were provided by the National Transfusion Centre of the Comunidad Autónoma (Madrid, Spain). Human peripheral blood mononuclear cells (hPBMCs) were isolated from the buffy coats by density centrifugation gradient using Ficollplaque Plus (GE Healthcare Biosciences AB), in accordance with manufacturer instructions. Blood samples from 3 different donors were used in the experiments.

2.4. CFSE labelling

Proliferation of hPBMCs was accessed with carboxyfluorescein succinimidyl ester (CFSE) labeling as previously described (DelaRosa *et al.*, 2009). Briefly, cells were washed, resuspended in a 20 µM CFSE solution (10^7 hPBMCs per 200 µL of solution) and incubated under constant shaking at 37°C for 10 min. The reaction was stopped by slowly adding ice-cold RPMI medium supplemented with 10% FBS (v/v). Cells were then cultured overnight, and one aliquot was used to set up and control the FL-1 voltage for CFSE.

2.5. IDO and PDL-1 expression

hCSCs were seeded in culture well plates and either left unstimulated or stimulated with IFN-γ (3 ng/mL). After 24 h and 48 h of culture, cells were trypsinized and stained with anti-PDL-1 antibody (2.5:100, ThermoFisher Scientific) and anti-IDO antibody (4:100, ThermoFisher Scientific) and isotype control IgG (2.5:100, BD Biosciences) using the kit for

intracellular staining from eBioscience, following the manufacturer's instructions. A total of ten thousand events were acquired using a FACSCalibur (BD Biosciences). hASCs were used as a positive control IDO expression.

2.6. Generation of conditioned supernatants

hASCs ($0.2x10^6$) and hCSCs ($0.5x10^6$) were seeded per well in 6 well plates, treated with mitomycin-C (20 µg/mL, at 37°C for 30 min) and cultured in RPMI medium supplemented with 10% FBS (v/v) with or without IFN-γ stimulation (3 ng/mL) for 24, 36 and 48 hours. Supernatants were collected, centrifuged to remove dead cells and debris and stored at -20°C until further use for immunomodulatory assays.

2.7. Immunomodulatory assays

Immunomodulatory assays experimental design is illustrated in figure 5.1. Briefly, CFSE-labeled hPBMCs were stimulated with phytohemagglutinin (PHA, 1 µg/mL) (Sigma-Aldrich) and cultured in the presence of mitomycin C-treated hCSCs at three different ratios (hCSC:hPBMC ratios 1:10, 1:17 and 1:25) in RPMI + 10% FBS (v/v).

Cultures were performed in culture well plates in i) direct contact (DC); ii) using transwells (TW) inserts with a 0.4 mm pore size (Corning) to allow exchange of soluble factors but separation of both cell types; and iii) using hCSCs conditioned medium (Cond.M). Cells were cultured at 37°C in RPMI medium supplemented with 10% FBS (v/v), in humidified incubators (5% CO2, 3% O2). hASCs were used as a positive control for T cell proliferation inhibition via IDO (ratio 1:25 hASCs:hPBMCs). Parallel control experiments with hPBMCs alone were also performed. After 72

and 96 h (DC and TW experiments) or after 96 h of culture (Cond.M experiments), CFSE-labeled hPBMCs were harvested, labeled with 7-AAD and anti-CD3 antibody, and cell proliferation of the $CD3^+$/ $7\text{-}AAD^-$ population (viable CD3 T lymphocytes) was evaluated by flow cytometry (FACSCalibur, BD Biosciences) according to loss of CFSE signal. A total of ten thousand events were acquired using a FACSCalibur (BD Biosciences). Division index and percentage of inhibition of proliferation were calculated using the FSC-express software.

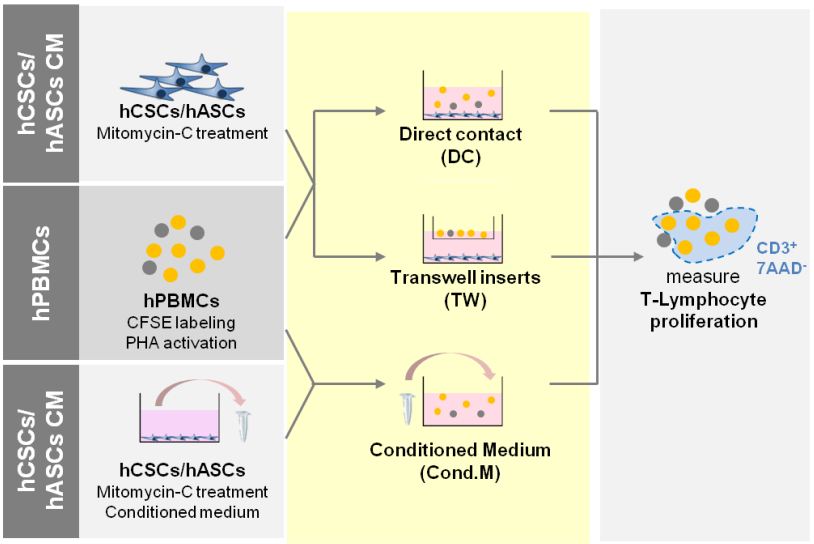

Figure 5.1. Schematic representation of immunomodulatory assay experiments. Carboxyfluorescein succinimidyl ester (CFSE) labeled and phytohemagglutinin (PHA) activated human peripheral blood mononuclear cells (hPBMCs) were cultured with mitomycin-treated hCSCs/hASCs either in: direct contact (DC); in a transwell support (TW); or in contact with mytomicin-C treated hCSCs/hASCs conditioned medium (Cond.M). Viable T-lymphocyte proliferation was accessed by CFSE labelling of the $CD3^+$ $7AAD^-$ hPBMC population.

2.8. IDO activity

IDO enzyme activity was measured by determining both Trp and kynurenine (Kyn) concentrations in conditioned medium. About 200 µL of conditioned medium was added to 50 mM phosphate buffer and 25 mL of

2 M trichloroacetic acid. After centrifugation for 10 min at 15,600 g, the supernatant was collected and analyzed by high-performance liquid chromatography (HPLC), Waters 717plus Autosampler using a Waters 1515 Isocratic Pump, and a Waters 2487 Dual Absorbance Detector. Separation was performed using a C18 4.6 50mm column,(Teknokroma Analítica S.A.) in 10 min runs with 40 mM sodium citrate (pH 5.0), 1% acetonitrile as mobile phase at a flow rate of 1 mL/min. Trp and Kyn were detected at an absorbance of 280 and 360 nm, respectively.

2.9. Statistical analysis

Statistical analyses were performed with GraphPad Prism6 (GraphPad Software Inc.). All data are shown as mean with standard deviation. Data was analyzed by One Way ANOVA Tukey test. P-values below 0.05 were considered significant. Three different hCSC biological replicates (from 3 different donors) were used.

3. Results

3.1. IDO expression is induced in hCSCs in response to IFN-γ

We first characterized the hCSC expression of the immune-relevant molecules PDL-1 and IDO without and with IFN-γ stimulation (representing an inflammatory environment).

While hASCs only express PDL-1 when activated, hCSCs express PDL-1 constitutively, and activation with IFN-γ further upregulates its expression (figure 5.2). These results are in agreement with the data previously reported by Lauden et al., 2013. Neither hCSCs nor hASCs displayed any constitutive expression of IDO but both cell types displayed significant expression upon stimulation with IFN-γ (Fig.2).

PDL-1 upregulation and IDO expression upon IFN-γ activation suggest that hCSCs enhance their immunomodulatory profile in an inflammatory environment.

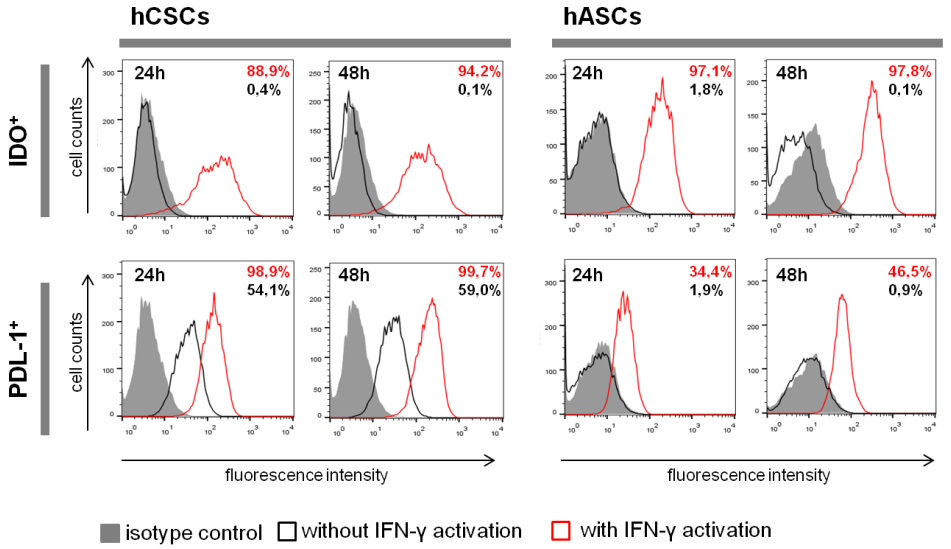

Figure 5.2. hCSCs display a favorable immune-suppressive phenotype. Representative expression of immune relevant molecules IDO and PDL-1 after 24 and 48 h in untreated (black line histograms) and IFN-γ activated (red line histograms) cells against isotype controls (gray-filled histograms). The percentages (%) of positive cells are indicated. hASCs were used as a positive control for IDO expression. hCSC results shown for donor hCSC-48. Other donors presented similar results.

3.2. hCSCs impair activated T lymphocyte proliferation

Following the characterization of a favorable immune-suppressive phenotype of hCSCs, we then examined whether these cells were capable of inhibiting T lymphocyte proliferation in an allogeneic setting. Stimulated hPBMCs were cultured in direct contact with hCSCs at different ratios.

As previously reported (Lauden *et al.*, 2013), hCSCs do exert an immune suppressive role by inhibiting T lymphocyte proliferation. Even though to a less extent when comparing with hASCs, hCSCs have a significant

suppressive effect in T lymphocyte proliferation in a dose-dependent manner (figure 5.3). Although no significant difference was registered between time points, there is a tendency for higher T lymphocyte proliferation inhibition in 96 h vs 72 h of incubation (figure 5.3) in all ratios analyzed, suggesting that this effect is also time-dependent.

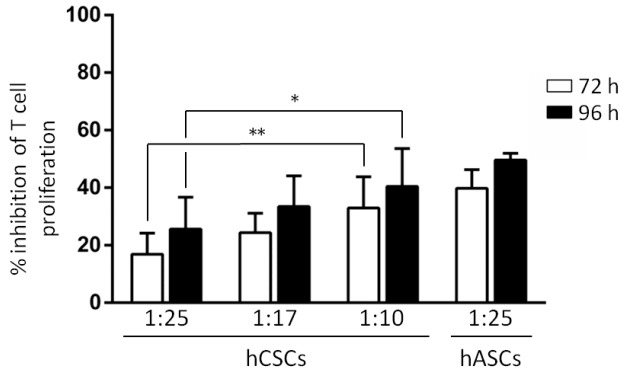

Figure 5.3. hCSCs inhibit T lymphocyte proliferation in a time and hCSC concentration dependent manner. CFSE-labeled hPBMC were stimulated with PHA and cultured alone or in direct contact with hCSCs (ratios 1:10; 1:17; 1:25 hCSC:hPBMC). After 72h (white bars) and 96h (black bars), proliferation of the viable population of CD3 viable T lymphocytes ($CD3^+/7AAD^-$) was assayed by loss of CFSE staining. Percentage of inhibition of proliferation was determined using FSC Express software against proliferation of activated hPBMCs alone. hASCs were used as a positive control for T cell proliferation inhibition (ratio 1:25 hASCs:hPBMCs).*P<0.05 **P<0.01 indicates significant differences between time points.

3.3. hCSCs immunomodulatory capacity can occur in the absence of cell-cell contact

To evaluate the importance of IDO enzyme and Trp metabolism in the immunosuppressive capacity of hCSCs, we carried out T lymphocyte proliferation assays in which hCSCs are not in direct contact (DC) with hPBMCs, and therefore cannot exert their immunomodulatory activity through the PDL-1/PD1 axis.

We carried out a hCSC:hPBMC co-culture in transwell (TW) setting, allowing paracrine interaction between the two cell types. At 72 h of incubation, although slightly lower when comparing to DC, hCSCs do

exert a significant suppressive effect on T lymphocyte proliferation in TW conditions. Moreover, such difference between TW and DC conditions was lost after 96 h of incubation (figure 5.4A).

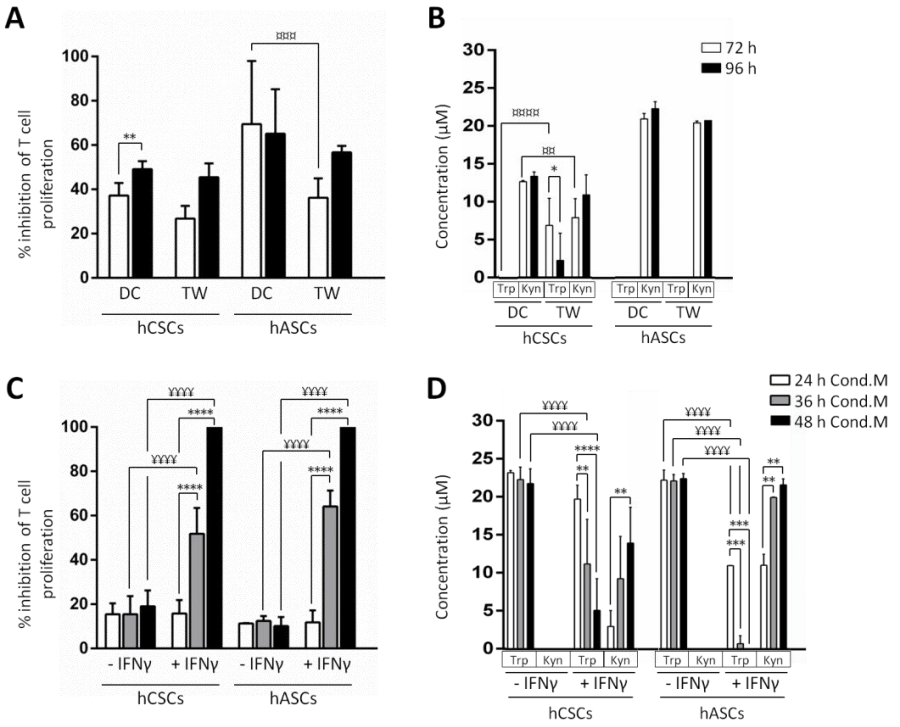

Figure 5.4. hCSCs inhibit T lymphocyte proliferation via a paracrine mechanism. (A) CFSE-labeled hPBMC were stimulated with PHA and cultured alone, in direct contact (DC) or in a transwell setting (TW) with hCSCs (ratios 1:10 hCSC:hPBMC). (B) Concentrations of Trp and Kyn were determined by HPLC in the supernatants. (C) CFSE-labeled hPBMC were stimulated with PHA and cultured alone or in conditioned medium (Cond.M) from hCSCs cultures activated or not with IFN-γ. Conditioned mediums were generated for 24 h (white bars), 36 h (grey bars) and 48h (black bars). (D) Concentrations of Trp and Kyn were determined by HPLC in the conditioned media. Proliferation of the viable population of CD3 T lymphocytes ($CD3^+/7AAD^-$) was assayed by loss of CFSE staining after 72 h (white bars) and 96 h (black bars) for TW and DC experiments (A) and after 96 h for Cond.M experiments (C). Percentage of cells per generation and % of inhibition of proliferation was determined using FSC Express software against proliferation of activated hPBMCs alone. hASCs were used as a positive control for T cell proliferation inhibition (ratio 1:25 hASCs:hPBMCs).**P<0.01, ***P<0.001, ****P<0.0001 indicates significant differences between time points; ¤¤¤P<0.001, indicates significant differences between culture type (CT vs TW); and ¥¥¥¥P<0.0001 indicates significant differences between -IFN-γ vs + IFNγ.

Trp metabolism was also accessed by measuring Trp and Kyn (a Trp metabolite described as cytotoxic for T lymphocytes, Terness et al., 2002a) concentrations in the conditioned mediums. As shown in figure 5.4.B, Trp is fully depleted already at 72 h in the DC condition, whereas in the transwells setting it is also significantly diminished when comparing to stimulated hPBMCs alone. Also, the accumulation of Kyn occurred in both experimental setups (figure 5.4B).

Besides TW experiments, hCSC Cond.M was generated for 24 h, 36 h and 48 h using control and IFN-γ stimulated hCSCs. Similarly with hASC control, hCSCs-derived Cond.M significantly inhibited T lymphocyte proliferation, with a significant increase in IFN-γ stimulated cells (51.79±11.67 % vs 15.49±8.10 % with 36 h Cond.M; 100±0.00 % vs 19.01±7.22 % with 48 h Cond.M) (figure 5.4.C). Moreover, Cond.M from longer IFN-γ stimulated hCSCs cultures prompted higher inhibition of T lymphocyte proliferation (figure 5.4.C). Such findings are also in accordance with the Trp and Kyn measurements, in which Trp is gradually depleted and Kyn gradually accumulates in the supernatant of hCSC cultures (figure 5.3.D).

4. Discussion

Allogeneic hCSC based therapies continue to be explored as an alternative for AMI patients. However, hCSC regenerative medicine approaches have yet to prove evident and robust clinical benefit over standard-of-care. Although described as having a positive immunomodulatory role rather than eliciting further inflammation (Lauden *et al.*, 2013; Boukouaci *et al.*, 2014; Dam *et al.*, 2017), one of the main challenges to be addressed in hCSC transplantation-based therapies is the rapid elimination of the injected cells by the host immune system. A

better knowledge of the immunological properties of hCSCs is therefore paramount in developing strategies to increase the transplanted cells retention time in the myocardium that would consequently increase their regenerative benefits.

hCSCs have been described as having an immune suppressive profile in allogeneic settings by directing natural killer cell cytokine secretion towards an anti-inflammatory profile and lower cytotoxicity (Boukouaci *et al.*, 2014), by modulation of monocytes, macrophages and dendritic cells towards an anti-inflammatory phenotype (Dam *et al.*, 2017) and by activation of T regulatory lymphocytes with subsequent inhibition of T lymphocyte proliferation via PDL-1/PD1 direct cell communication (Lauden *et al.*, 2013) as well as via extracellular vesicle-mediated paracrine communication (van den Akker *et al.*, 2018).

In this work, we assayed the immunomodulatory capacity of hCSCs in an inflammatory setting by IFN-γ activation and explored the hypothesis of paracrine IDO-mediated T lymphocyte proliferation inhibition.

IFN-γ is highly expressed in inflammation settings (such as after an AMI). This pro-inflammatory pleiotropic cytokine is produced primarily by the host T lymphocytes in response to antigen recognition and has also shown to induce the expression of immune-relevant molecules in several stem cell populations, including neural stem cells (Kulkarni, Ganesan and O'Donnell, 2016) and mesenchymal stem cells (Polchert *et al.*, 2008; DelaRosa *et al.*, 2009). IFN-γ was shown to cause hCSCs to up-regulate expression of both class I and II HLA molecules (Lauden *et al.*, 2013), which indicates that their administration into an inflammatory myocardium environment might probably increase their recognition and clearance by host T lymphocytes. On the other hand, IFN-γ supplementation has also

previously shown to up-regulate hCSC expression of PDL-1, resulting in a hCSC stronger immune-suppressive profile (Lauden *et al.*, 2013).

IFN-γ activation has also been correlated with increased IDO expression in hMSCs, including hASCs, which in turn has shown to be a key enzyme involved in the immunomodulatory capacity of these cells (Li *et al.*, 2014; Mancheño-Corvo *et al.*, 2015). IDO exerts its immune effects by suppressing T lymphocyte proliferation and promoting T lymphocyte death through degradation of Trp, an essential amino acid required for cell proliferation and subsequent accumulation of cytotoxic Trp metabolites (including Kyn) (Terness *et al.*, 2002; Fallarino *et al.*, 2003). IFN-γ also causes the activation of tryptophanyl-transfer RNA synthetase (WRS, a aminoacyl synthetase that incorporates Trp into proteins) in IDO-expressing cells, which has been postulated to be a compensatory mechanisms, allowing IDO expressing cells to better cope with Trp depletion (Mellor *et al.*, 2003).

In our results, we show that IFN-γ activation is correlated with an increase in hCSC PDL-1 and IDO expression. Although with an overall weaker immune-suppressive profile when comparing to hASCs, we also showed that hCSCs are able to inhibit T lymphocyte proliferation in a time and hCSC cell concentration dependent manner when in direct co-culture.

Moreover, we showed Trp depletion and Kyn accumulation in activated hCSC Cond.M. Concordantly, stimulated hCSC Cond.M. showed a superior anti-proliferative effect when compared to unstimulated hCSCs Cond.M, suggesting a relevant role of IDO-mediated Trp metabolism in the immunomodulatory paracrine effect of hCSCs.

By co-culturing hPBMCs and hCSCs we also show no significant differences between DC and TW setups in terms of T lymphocyte

proliferation, suggesting that paracrine–mediated are in fact the central mechanism of action of hCSCs.

These findings provide evidence, that although playing a role in the process, PDL-1 mediated T regulatory cell modulation is not the exclusive neither the central mechanism involved in T lymphocyte proliferation inhibition. This finding further supports the prominent paracrine-based beneficial CSC activities in the host tissue.

In this work, we give evidence of Trp metabolism as a novel mechanism involved in the hCSC mediated T lymphocyte proliferation suppression properties. We also hypothesize that similarly to what is already described for hASCs (DelaRosa *et al.*, 2009; Menta *et al.*, 2014; Mancheño-Corvo *et al.*, 2015), IDO is the main player in hCSC Trp metabolism in inflammation settings. Complementary studies to further test this hypothesis should include analysis of WRS expression, Trp supplementation and IDO inhibition experiments to further validate IDO Trp metabolism as a main player in hCSC immunomodulatory properties in the host tissue.

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Chapter VI

Unveiling human cardiac fibroblast membrane proteome

This Chapter was adapted from:

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Author Contribution

Maria João Sebastião participated in the experimental setup and design, performed part of the experiments, performed data analysis and wrote the chapter.

Abstract

Cardiac fibroblasts (CFs) are one of the main cell populations in the heart and play important roles in tissue homeostasis and myocardial fibrosis. The study of these cells has been hampered by the lack of reliable membrane markers: none of the antigens currently used for characterization and isolation of CFs is unique for this cell type. This issue has also raised doubts regarding a distinct identity of cardiac fibroblasts when compared to other myocardium cell populations with similar morphologies. In this work, we report a comprehensive description and functional analysis of human CFs (hCFs) membrane enriched fraction proteome by advanced mass spectrometry-based proteomic tools. A total number of 1478 proteins were identified, including 774 membrane proteins (52%). We also report the identification of a subset of 30 membrane proteins that in this workflow were only identified in hCFs by comparison with the membrane-enriched proteome lists of human cardiac stem cells, human mesenchymal stem cells, and human dermal fibroblasts. The data reported in this work are a valuable source of information for further studies aiming at defining a membrane molecular signature of human cardiac fibroblasts (hCFs), and a step forward in research regarding membrane proteins with key roles in hCF function in homeostasis and disease.

Contents

1. Introduction

Cardiac Fibroblasts (CFs) play key roles in myocardium homeostasis and in response to injury. CFs are the main cell type involved in synthesis and turnover of extracellular matrix (ECM), function as local immune modulators, and are part of the cardiac electrophysiology network (Furtado, Costa and Rosenthal, 2016). Upon myocardial injury and other cardiac pathologies, CFs undergo activation (differentiation to myofibroblasts), proliferate and produce an excess of ECM, a process referred as fibrosis. Fibrotic tissue is stiffer and less conductive, which leads to impaired normal heart function, with increased tissue workload and arrhythmias (Furtado, Costa and Rosenthal, 2016). Fibrosis is a pathological feature present in numerous forms of heart diseases: it is estimated that about 45% of all deaths in the developed world involve cardiac fibrosis (Wynn, 2007).

Due to their central role in cardiac fibrosis, a lot of attention has been drawn to the study of CFs. Although some progress was made recently (Furtado, Costa, *et al.*, 2014; Furtado, Nim, *et al.*, 2014; Furtado, Costa and Rosenthal, 2016; Ivey and Tallquist, 2016), the lack of a proper molecular definition enabling an accurate identification of these cells has been one of the major impediments to CF research. Some of the most commonly used markers for CFs characterization are collagens, vimentin, discoidin domain receptor (DDR2), Thy1 antigen (CD90) and smooth muscle actinin (SMA). None of these molecules is neither expressed by all CFs neither unique for this cell type (Furtado, Costa and Rosenthal, 2016). Recently, a novel antibody, mEF-SK4 (unknown antigen) has been identified as a robust fibroblast marker of the adult mice heart, but unfortunately, this antibody is not suitable for human cells (Pinto *et al.*, 2016).

Taking into account the lack of reliable membrane CFs markers, and the key role that membrane proteins such as integrins (Civitarese *et al.*, 2017), receptors (Pellman, Zhang and Sheikh, 2016), ion channels (Li *et al.*, 2009) and cell junction constituents (Ongstad and Kohl, 2016) have in the context of cardiac fibrosis and in CF coupling with cardiomyocytes (CMs), a deeper characterization of CF membrane proteins is of outmost importance. In the present study, we examined the proteome profile of human CFs (hCFs), with a focus on membrane proteins, using an optimized membrane protein enrichment protocol previously reported by our group (Gomes-Alves *et al.*, 2015). In order to define a protein signature distinctive of hCFs, we further compared the membrane-enriched proteome of these cells with similar membrane-enriched fractions from another fibroblast population [human dermal fibroblasts (hDFs)] and from two adult stem cells populations [human cardiac stem cells (hCSCs) and human mesenchymal stem cells (hMSCs)], shown to have high resemblance to hCFs in terms of morphology, cell surface and transcriptional markers which has been hampering the establishment of a distinct identity (Furtado, Costa and Rosenthal, 2016).

From this specific comparison of membrane-enriched fractions (hCF vs hCSC+ hMSC+ hDF), a subset of 72 proteins were identified only on hCFs, including 30 membrane proteins. The data herein reported constitutes a valuable source of information to further decipher hCFs functions and will certainly serve as a building block for a complete definition of hCFs membrane proteome.

2. Materials and Methods

2.1. Cell culture

hCFs (adult ventricular, cat. 306v-05a) were purchased from Cell Applications Inc. (San Diego, USA). The cells were cultured as monolayers in humidified incubators at 37 °C at 5% CO2 in complete DMEM containing 10% (v/v) fetal bovine serum (FBS), supplemented with 1% (v/v) penicillin streptomycin (Pen/Strep). hCFs were used between passages 5 and 6.

hCSCs were isolated as previously described by Lauden et al(Lauden *et al.*, 2013). hDFs were obtained from Inbiobank Stem Cell Bank. Both hCSCs and hDFs were cultured in DMEM:F12 Neurobasal medium (1:1), supplemented with 1% (v/v) Pen/Strep, 10% (v/v) FBS, N2 Supplement (1X), B27 Supplement (1X), 0.9 mM L-Glutamine, 50 µM β-Mercaptoethanol (Sigma), Insulin Transferrin Selenium (0.5X), 10 ng/mL bFGF, 20 ng/mL EGF-I and 30 ng/mL IGF-II (Prepotech). hMSCs were obtained from Inbiobank Stem Cell Bank (cat. BM#19) and cultured with DMEM low glucose medium supplemented with 10% (v/v) FBS, 1% (v/v) Pen/Strep and 1% (v/v) L-Glutamine. hCSCs, hDFs and hMSCs were cultured as monolayers in humidified incubators at 37°C, 5% CO_2 and 3% O_2. Unless otherwise noted, all reagents and chemicals are from Thermo Fisher Scientific.

2.2. Immunofluorescence microscopy

Cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) and fixed in 4% (w/v) paraformaldehyde (PFA) and 4% (w/v) sucrose in DPBS for 20 min. Afterwards, cells were permeabilized for 10 min in 0.1% (v/v) Triton X-100 in DPBS and blocked with 0.2% (v/v) Fish Skin Gelatin (FSG) in DPBS for 30 min, at room

temperature (RT, 18-20°C). Cells were then incubated with primary antibodies diluted in 0.13% (v/v) FSG, 0.1% (v/v) Triton X-100 for 2 h at RT. Cells were washed with DPBS and incubated with secondary antibodies diluted in 0.13% (v/v) FSG, 0.1% (v/v) Triton X-100 for 1 h at RT in the dark. The following primary antibodies were used: anti-vimentin (1:100, Abcam, ref ab16700), anti-DDR2 (1:200, Antibodies Online ref ABIN2158508) and anti-SMA (1:100, Agilent ref M085129-2). Unless otherwise noted, all reagents and chemicals are from Sigma.

2.3. Western Blot

Cell pellets were lysed in Lysis Buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% (w/v) Triton X-100, and 1X (w/v) complete protease inhibitor cocktail (Roche)) for 30 min at 4 ˚C. Total protein was quantified using the Micro-BCA Protein Assay Kit (Thermo Scientific). Proteins were precipitated with 10% (v/v) Trichloroacetic acid during 10 min at RT. Samples were centrifuged again at 15,000 x*g* for 10 min at 4 °C, incubated in cold EtOH 80% for 10 min at -20 ºC. Extracts were clarified by centrifugation at 15,000 x*g* for 10 min at 4 °C and air-dried.

Proteins were denatured with sample buffer according to manufacturer instructions, loaded in an electrophoresis gel (NuPAGE 4-12% Bis-Tris Gel) (Thermo Fisher Scientific) under reducing conditions for 40 min (200 V, 400 mA) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using iBlot system (Thermo Fisher Scientific), according to the manufacturer's instructions.

Membranes were blocked by incubation for 1 h with blocking solution (0.1% (v/v) Tween 20 and 5% (v/v) dry milk in DPBS), and incubated overnight with primary antibody diluted in blocking solution. Blots were developed using the enhanced chemiluminescence (ECL) detection

system after incubation for 1 h at room temperature with horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit antibodies (GE Healthcare) at 1:5,000 dilution. Chemiluminescence detection was performed by incubating the membranes with Amersham ECL Prime western blotting detection reagent (GE Healthcare) and analyzed under ChemiDoc XRS System (Bio-Rad). The following primary antibodies were used: anti-vimentin (1:100, Abcam ref ab16700), anti-DDR2 (1:1000, Antibodies Online ref 2158508), anti-SMA (1:1000, Agilent ref M085129-2), and anti-α Tubulin (1:5000, Sigma ref T6199). Unless otherwise noted, all reagents and chemicals are from Sigma.

2.4. Proteomic Analysis

hCFs membrane enriched fractions (available at ProteomeXchange with the dataset identifier PXD007839) were analyzed and filtered using lists of membrane enriched fractions of hCSCs, hMSCs and hDFs previously generated by our group and partners (Gomes-Alves *et al.*, 2015) (available in ProteomeXchange with the dataset identifiers PXD007839 and PXD001117 and Peptide Atlas with the dataset identifier PASS00827). For all experimental conditions, at least two biological replicates with 2/3 technical replicates were run.

Membrane enriched fractions:

For all samples analyzed in this work (hCFs, hDFs, hMSCs and hCSCs), proteins were extracted, enriched for membrane-associated fractions and quantified as described elsewhere (Gomes-Alves *et al.*, 2015). Briefly, cell pellets were resuspended in lysis buffer [50 mM Tris (pH 7.8); 250 mM Sucrose; 2 mM EDTA] with protease inhibitors and incubated on ice for 10 min. Cells were lysed with 30 passes through the 301/2 Gauge needle at 4 °C. The cell debris, unbroken nuclei, and other membrane

proteins were removed by centrifugation at 1,000 x*g* for 10 min at 4 °C. The supernatant was layered onto sucrose buffer 1:1 (60% sucrose) and centrifuged at 160,000 x*g* for 70 min at 4 °C. The membrane fraction (interface) on top of the sucrose cushion was collected and diluted 1:2 with 50 mM Tris (pH 7.8) and centrifuged at 100,000 g for 1 h at 4 °C. The obtained protein pellet was washed with 100 mM Na_2CO_3 (pH 11.5) for 2 h at 4°C followed by ultracentrifugation at 100,000 x*g* for 90 min at 4 °C. Finally, the resultant membrane enriched fraction was rinsed twice with cold water. Protein content was measured using BCA™ Protein Assay Kit (Pierce).

Samples solubilized in 0,32% (w/v) Rapigest SF Surfactant (Waters) (were subjected to reduction (DTT, 10 mM, 40 min, 56 °C) and alkylation (IAA, 20 mM, 30 min in the dark, room temperature 18-20°C), a further 10 min reduction with DTT (room temperature) was performed to quench the excess of IAA). Proteins were digested with trypsin overnightat 37 ºC (trypsin:sample = 1:50, in solution). To guarantee a complete digestion a second step of in-solution digestion (3h, 37 ºC) was performed using a ratio of 1:100 (in 80% acetonitrile). Samples were then dried in the speedvac. Resultant tryptic peptides were resolubilized in 5% formic acid and desalted/concentrated in C18 zip-tip microcolumns prior to nanoLC-MS analysis.

NanoLC-MS Analysis:

hCFs

hCFs tryptic peptides were analyzed by NanoLC–MS/MS using a hybrid Quadrupole Time-of-Flight (ABSciex TripleTOF 6600). Separation was performed at 300nl/min using an Ekspert 400 nanoLC (Eksigent) with CHiPLC (cHiPLC ChromXP C18-CL analytical column, 75 µm x 15 cm, 3 µm, 120 Å, Sciex; cHiPLC ChromXP C18-CL trap column, 200 µm x 0.5

mm, 3 µm, 120 Å, Sciex). The gradient was as follows: 0-1 min, 5% B (0.1% formic acid in acetonitrile, Fisher Chemicals, Geel, Belgium); 1-91 min, 5-30% B; 91-93 min, 30-80% B; 93-108 min, 80%; 108-110 min, 80-5% B; 110-127 min, 5% B. The electrospray ionization (Sciex NanoSpray III ion source, New Objective PicoTip emitter SilicaTip FS360-20-10-N-20-C12) was achieved with source gas 1 of 12, gas 2 of 0, curtain gas of 30, interface heater temperature of 80 and an ion source voltage floating of 2300. A top 50 acquisition method was used with a cycle made of one full MS1 scan in the TOF, followed by a precursor selection of 50 most intense ions applying an exclusion window of 12 seconds and followed by the acquisition of 50 product ion scans generated in the Quadrupole analyzer, and detected in the TOF. Collision induced dissociation took place in the quadrupole, using nitrogen. Spectra were acquired in positive mode. External calibration was performed using beta-galactosidase digest (ABSciex). The 40 most intense precursor ions from the MS spectra were selected for MS/MS analysis. Data were acquired with the Analyst software TF 1.7 (ABSciex). The raw MS and MS/MS data were analyzed using Protein Pilot Software v.5.0 (ABSciex), with Paragon™ and ProGroup™ algorithms for protein identification. The search was performed against Swissprot protein database with taxonomic restriction to *Homo Sapiens*. Trypsin was selected as digestion type, Iodoacetamide was selected as source of Cys alkylation and the TripleTOF 6600 as the Instrument. The ID focus was on biological modifications and amino acid substitutions. The search effort was set as thorough and a FDR analysis was performed. Protein identification was considered when unused scores were greater than 1.3 (corresponding to 95% confidence and global FDR < 1%).

The complete list of identified proteins and peptides identified in hCFs is presented in Supporting Information file 2. Analysis of the protein lists

was performed using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/), and Ingenuity Pathway Analysis v01.7 (IPA, Quiagen). Hits were classified as membrane proteins if containing at least 1 predicted transmembrane domain by the Hidden Markov Model (HMM)-based method THMM online tool (http://www.cbs.dtu.dk/services/TMHMM/).

Statistically significant representation of biological functions and canonical pathways was identified based on IPA p-value. This probability score is calculated taking into account the total number of proteins known to be associated with a given function or pathway, and their representation in the experimental dataset. IPA's calculated p-value is displayed as -log (p-value).

Proteomic data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD007839.

hCSCs

Sample tryptic peptides were analyzed by NanoLC–MS/MS using a hybrid Quadrupole Time of Flight (ABSciex TripleTOF 6600) similarly to hCFs samples. This dataset has also been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD007839.

Protein lists from hCSCs enriched-membrane fractions resulting from a previous publication (Gomes-Alves *et al.*, 2015) were also added in order to enlarge hCSC membrane-associated proteome coverage [analysis details and proteomic data available in ProteomeXchange Consortium

(http://proteomecentral.proteomexchange.org) with the dataset identifier PXD001117.

The complete list of identified proteins in hCSCs is presented in Supporting Information file 5.

hMSCs and hDFs

MS analysis details and protein lists from the hMSCs and hDFs enriched-membrane fractions were previously deposited in Peptide atlas (http://www.peptideatlas.org/) with the dataset identifier PASS00827.

The complete list of identified proteins in hMSCs and hDFs is presented in Supporting Information file 6 and 7, respectively.

3. Results & Discussion

As reported in the literature (Furtado, Costa and Rosenthal, 2016; Pinto *et al.*, 2016), Vimentin, DDR2 and SMA showed not to be uniquely and specifically expressed by hCFs. In our assessment these proteins were identified in hCFs as well as in adult stem cells (hMSCs and hCSCs) and in other fibroblast population (hDFs) by proteomics, immunofluorescence and western blot (figure 6.1 A-C). Moreover, platelet-derived growth factor receptor α (PDGFRα), Filaminin A, and collagens Col1α and Col2α (proteins which are routinely used as CFs markers) were also identified by proteomics in all cell types used as reference (figure.6.1 A).

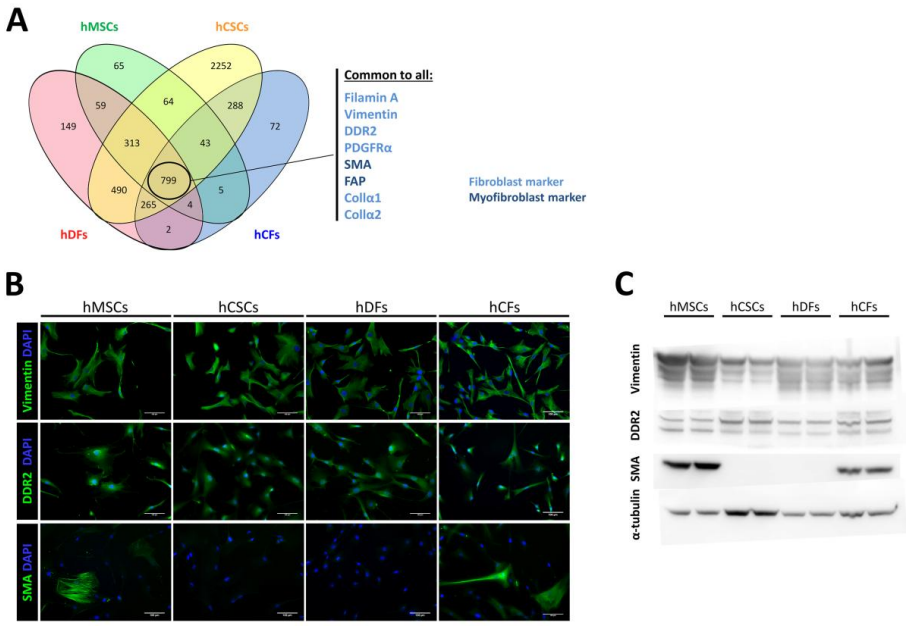

Figure 6.1. hCF lack a distinctive molecular marker signature. Vimentin, DDR2 and SMA, some of the most commonly used markers for hCFs, were identified in hMSCs, hCSCs, hDFs and hCFs through: (A) proteomic analysis- Venn diagram; (B) immunofluorescence labelling; and (C) western blot analysis. Scale bars 100µm.

hCF proteome profiling was performed in samples enriched for membrane fraction. The analysis rendered the identification of 1478 proteins, of which 774 (52%) were classified as membrane proteins (figure 6.2 A, Full list of identified proteins in Supporting Information file 6.1; Full List of Identified Membrane Proteins in Supporting Information file 6.2). Biological functions and canonical pathways enrichment analysis was performed using IPA software (Full list in Supporting Information file 6.3). We focused the analysis on terms associated with relevant features of fibrosis and in CF-CM coupling.

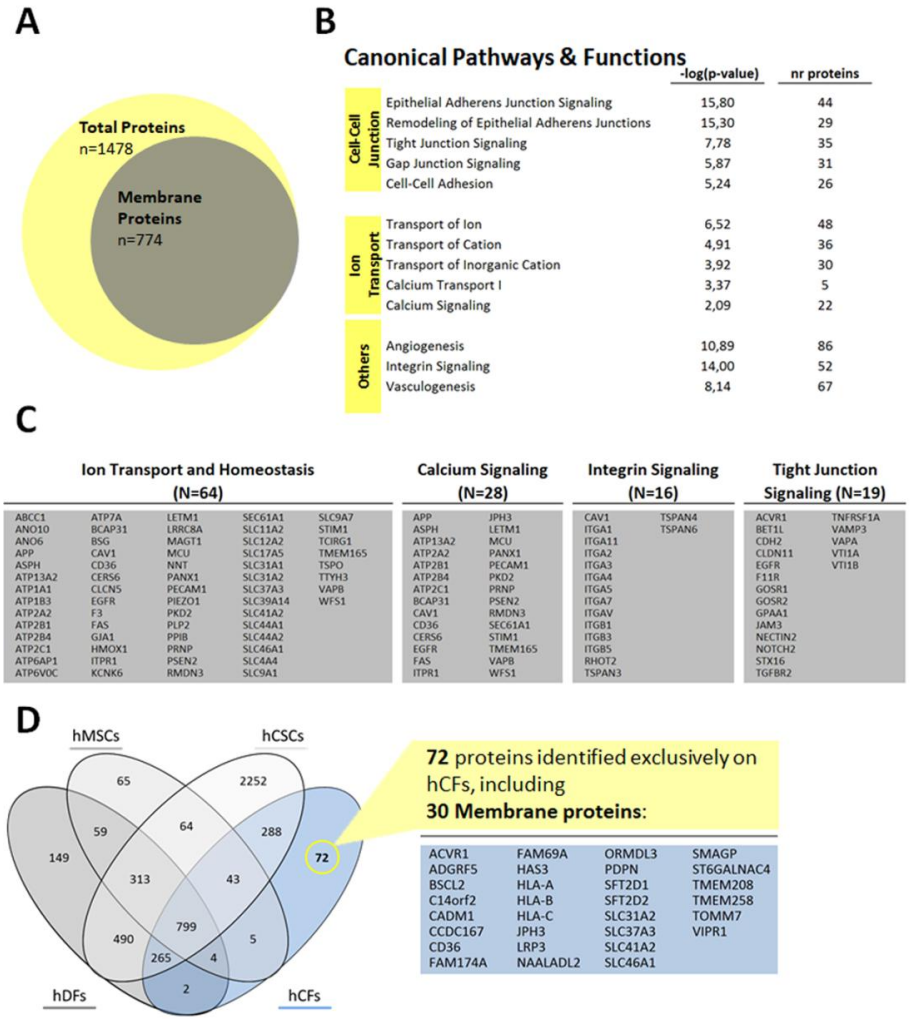

Figure 6.1. Functional analysis of proteins identified in hCFs. (A) A total of 1478 proteins were identified in hCFs, including a subset of 774 membrane proteins (52,36%); (B) Canonical Pathways and Biological Functions enrichement analysis of the 1478 proteins identified in hCFs. Cell-Cell junction, ion transport , angiogenesis, vasculogenesis and integrin signalling are highlighted; (C) Functional analysis of the identified subset of membrane proteins in hCFs: Ion transport and homeostasis, calcium , integrin and tigh junction signalling are highlinhted as highly represented cathegories; (D) Venn diagram illustrating the overlaps between membrane-associated proteome profiles of hCSCs, hMSCs, hDFs and hCFs. A total of 72 proteins were exclusively identified in hCFs, including 30 membrane proteins, highighted in the blue box.

Terms associated with cell-cell junctions, ion transport, angiogenesis, vasculogenesis and integrin signaling were found as enriched in hCFs (figure 6.2 B,C). Also, 113 proteins were identified as associated with

cardiovascular system and development (Supporting Information file 6.3). Such results reinforce not only the important role of hCFs in electrical coupling and cell-to-cell adhesion but also the strong cardiac molecular signature of these cells, as reported previously (Furtado, Costa, *et al.*, 2014; Furtado, Nim, *et al.*, 2014). To our knowledge, this work reports for the first time a deeper hCFs membrane-associated proteome characterization.

By comparing the proteins identified in hCF, hCSCs, hMSCs and hDFs membrane enriched fractions, we were able to report a subset of 72 proteins exclusively identified in hCFs. From these, 30 were identified as membrane proteins, including ACVR1, a protein involved in tight junction signaling and five proteins associated with ion transport and homeostasis (CD36, SLC31A2, SLC37A3, SLC41A2, SLC46A1) (figure 6.2 C,D). Other interesting proteins include HAS3, involved in ECM production, vasculogenesis and angiogenesis and CADM1, which is described as being involved in cardiac fibrosis remodeling and fibroblast proliferation (Cao, Shi and Ge, 2017). From our list of 30 membrane proteins exclusively identified in hCFs, 4 proteins (CD14, FAM69A, CADM1 and AVCR1) were also identified as specifically enriched in hCFs in a recent quantitative whole proteome study of human heart (Doll *et al.*, 2017).

4. Conclusion

In this work, by comparison of membrane-enriched fractions of hCF vs hCSC, hMSC and hDF, we were able to identify a panel of 30 protein marker candidates for hCFs. We are aware that these results need further validation, as the analysis of enriched-membrane fractions may provide potential false positive targets (as only partial proteomes are under analysis). Nevertheless, the list of membrane proteins identified

herein for hCFs, constitutes a valuable basis for more exhaustive studies, including knockout experiments in order to further unveil proteins with key molecular function in hCF physiology. Comparison of hCFs membrane proteome with other myocardium populations, such as human CMs and human endothelial cells may also be considered to further validate these results.

5. Acknowledgements

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The authors declare no conflicts of interest.

6. Supporting information

Supporting Information File 6.1, 6.2 and 6.3 available at: https://onlinelibrary.wiley.com/doi/abs/10.1002/pmic.201700446.

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Chapter VII

Discussion

Contents

1. Discussion

The frequency of cardiovascular diseases is expected to rise in developed countries as a result of an increase in life expectancy (WHO). Within heart disorders, Acute Myocardial Infarction (AMI) is responsible for millions of deaths worldwide, still no therapies enable the full recovery of the damaged tissue upon Ischemia/Reperfusion (I/R) injury in AMI survivors, leading to chronic heart dysfunctions and a significant loss in life quality (Benjamin *et al.*, 2017).

The work developed in this thesis aimed at characterizing human cardiac populations, including hCFs, hCSCs and hiPSC-CMs, as well as to develop relevant I/R *in vitro* human cell models. The overview of the main aims and achievements is summarized in figure 7.1.

Advanced mass spectrometry (MS) tools were used to provide a comprehensive description of human cardiac fibroblast (hCF) membrane molecular landscape as well as to unveil human cardiac stem/progenitor cells (hCSCs) mechanisms of action in a myocardial I/R injury context. Moreover, the study of the immune-suppressive capacities of hCSCs in an allogeneic transplantation context was investigated.

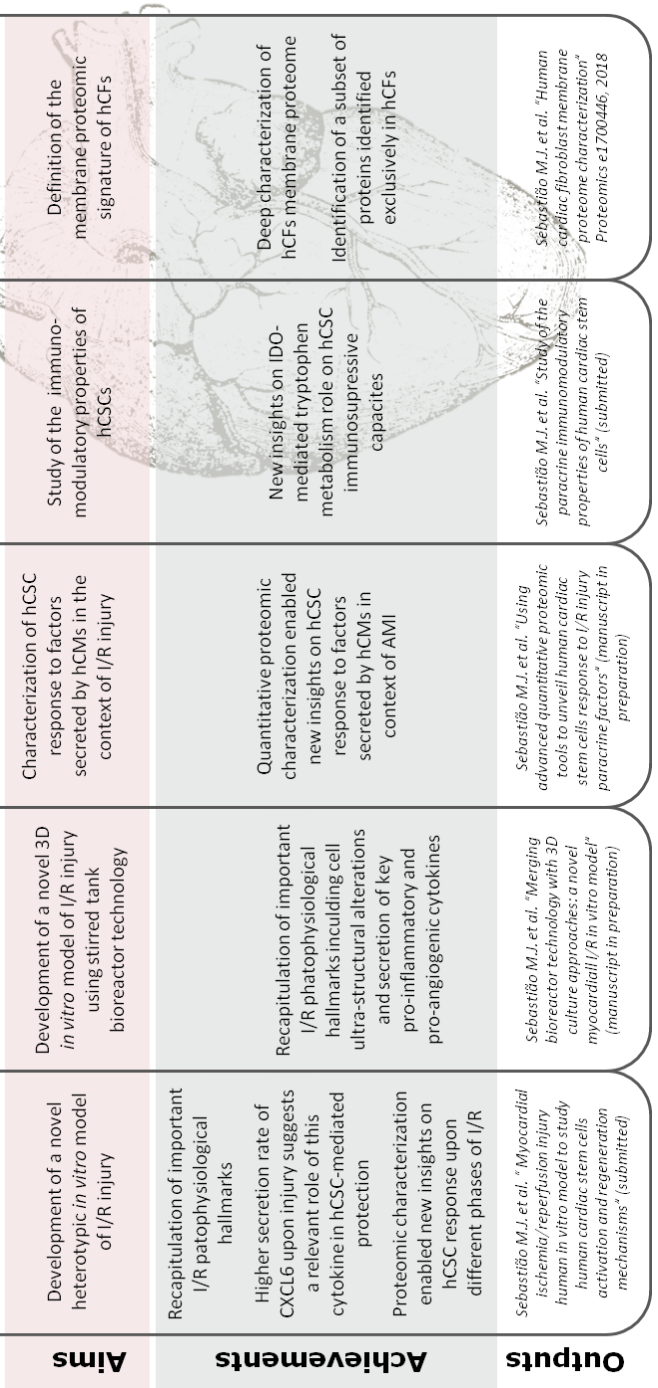

Figure 7.1. Schematic representation of the aims of this thesis and the main achievements of each chapter. I/R: Ischemia/Reperfusion; AMI: acute myocardial infarction; hCSCs: human cardiac stem cells; hCMs: human cardiomyocytes; hCFs: human cardiac fibroblasts.

In **Chapter II**, an human I/R *in vitro* model using a heterotypic co-culture system with hCSCs and human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) was implemented, including a whole proteome study of hCSC response to injury. Following this work, an alternative I/R *in* vitro model was developed in **Chapter III**, taking advantage of stirred-tank bioreactor technology and aggregate 3D culture of hiPSC-CMs. hCSCs were incubated with the conditioned medium resulting from the I/R bioreactor experiments, allowing to study CSC response to an AMI-based secretory environment by quantitative whole proteomic analysis (**Chapter IV**). The immunomodulatory capacity of hCSCs in an allogeneic transplantation context was also studied in **Chapter V**. Lastly, **Chapter VI** focused on a different myocardial population: hCFs, and in the characterization of their membrane proteome landscape.

1.1. Development of human I/R injury *in vitro* models

Different culture strategies were employed in order to recapitulate distinct aspects of the human myocardium physiology, having as major aim the development of robust human *in vitro* I/R injury models. Approaches applied included the use of human adult/mature cell populations, recapitulation of several physico-chemical aspects of myocardium environment during ischemia and reperfusion (**Chapter II and III**) and the use of three-dimensional (3D) culture and bioreactor technology systems (**Chapter III**).

State of the art references have mainly relied on animal models and murine cardiac cell cultures to study myocardial I/R molecular mechanisms. Murine systems, despite central to cardiovascular research, do not fully recapitulate human cellular physiology (Davis *et al.*, 2011; Roux, Gonzàlez-Porta and Robinson-Rechavi, 2012). In that

context, human cell-based models represent an alternative to study I/R injury. Taking into account the inherent ethical constraints and lack of availability of human biopsies for the isolation of primary adult CMs, as well as the technical difficulties in maintaining these cells in culture, CMs derived from human pluripotent stem cells (hPSCs) have been increasingly used in the last years, accelerating human cardiovascular cell biology research *in vitro.* In fact, CMs derived from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) have been used in toxicology screening in the pharmaceutical industry (Denning *et al.*, 2016), and to modulate a wide range of disorders including arrhythmias, metabolic disorders, and cardiomyopathies (reviewed in Giacomelli, Mummery, & Bellin, 2017) as well as ischemic damage (Ebert *et al.*, 2014; Hidalgo *et al.*, 2018). However, hPSC-CM differentiation protocols often yield immature cells, that more closely resemble fetal and neonatal rather than adult CM phenotype (Synnergren *et al.*, 2012; Birket *et al.*, 2015; Correia *et al.*, 2018). Such issue is especially relevant in the context of I/R injury, since immature cardiac phenotypes are related with higher metabolic plasticity and higher resistance to hypoxia injury (Robertson, Tran and George, 2013; Hidalgo *et al.*, 2018), as previously mentioned in the introduction.

In fact, during the establishment of the monolayer I/R injury *in vitro* setup (**Chapter II**), hiPSC-CM after 15 days of differentiation showed resistance to I/R, with CM viability only being affected after the addition of an extra maturation step (Ribeiro *et al.*, 2015) to the differentiation protocol. Such findings are in accordance with a recent study from Hidalgo et al., in which immature hESC- and hiPSC-CMs displayed minimal cell death upon *in vitro* I/R injury. After an extra 8-day metabolic-based maturation phase, both CM cell types showed higher cell death post injury (Hidalgo *et al.*, 2018).

Indeed, upon a hormone-based maturation protocol (Ribeiro *et al.*, 2015; Correia *et al.*, 2017), both hiPSC-CM monolayer (**Chapter II**) and hiPSC-CM aggregate (**Chapter III**) cultures showed higher concentration of apoptotic cells at 1 h post reperfusion, which is consistent with the described *in vivo* pathophysiology of CM death during AMI, where reactive oxygen species (ROS) accumulation, calcium overload and mitochondrial permeability pore opening cause a burst of CM death in the first minutes after reperfusion (Hausenloy and Yellon, 2013). Moreover, a deeper cell ultrastructure analysis in hiPSC-CMs aggregates by transmission electron microscopy (TEM) (**Chapter III**) revealed morphological changes characteristic of I/R injury, including sarcomere disorganization as well as rupture of structure of mitochondria. hiPSC-CMs subjected to bioreactor I/R injury setup also showed secretion of key angiogenic factors, previously shown to be upregulated upon AMI (Vandervelde *et al.*, 2005; Torella *et al.*, 2007; Li *et al.*, 2014; Ong *et al.*, 2015).

In **Chapter II**, an heterotypic human I/R injury *in vitro* model was developed, using hCSCs and hiPSC-CMs separated by transwells. Heterotypic co-culture models using more than one cell type are more representative of *in vivo* tissue when compared to more traditional mono-culture, allowing to elucidate how the cross-talk between different cell populations influence each cell type phenotype. This is especially relevant in the context of CSC mechanisms of action, since several studies demonstrate that CSCs are activated, responding to I/R molecular cues mainly through recognition and secretion of several growth factors and cytokines. CSCs have been shown to secrete paracrine factors involved in the modulation of cell proliferation, angiogenesis, vasculogenesis and pro-survival of CMs (Torella *et al.*, 2007; Miyamoto *et al.*, 2010; Li *et al.*, 2012; Park *et al.*, 2016; Valiente-

Alandi *et al.*, 2016; Sharma *et al.*, 2017) and to be activated by factors highly released in the myocardium upon injury (Aghila Rani and Kartha, 2010; Stastna and Van Eyk, 2012; Koudstaal *et al.*, 2014; Waring *et al.*, 2014). Indeed, when comparing with mono-culture controls, our results showed that co-culture condition better recapitulated important hallmarks of CSC response to injury, including proliferation activation, secretion of relevant factors and the enrichment of proteins associated with cytoskeleton organization, oxidative stress, stress response and several pathways related with cardiac regeneration. In **Chapter III**, a different setup of I/R *in vitro* model was developed, using bioreactors and 3D aggregates of hiPSCs-CMs. Although in this setup the two cell populations were not in direct co-culture, the paracrine effect of hiPSC-CM secretome on hCSCs was also studied, by incubating hCSCs with conditioned medium from the hiPSC-CM I/R injury bioreactor experiments (**Chapter IV**). Here, paracrine factors/ extracellular vesicles released by hiPSC-CMs upon injury also affected hCSC phenotype, including upregulation of proteins associated with migration, proliferation and endocytosis.

Besides using adult/mature cell populations, several physico-chemical parameters of ischemia were recapitulated in the developed *in vitro* models (**Chapter II and III**), including not only oxygen and nutrient deprivation, but also acidosis, high lactate accumulation and hyperosmosis. In a study comparing all these factors, Lu et al. identified lactate and acidosis as critical contributors to adult rat CM death (Lu *et al.*, 2005). In our models, reperfusion phase of injury was mimicked by the re-establishment of culture conditions, including nutrient rich medium and 3% of oxygen, representing myocardial physiological normoxia (Khan et al., 2010). Most I/R *in vitro* injury setups re-establish normoxia

at atmospheric oxygen (21%), representing hyperoxic conditions that might not reflect the *in vivo* conditions.

Aiming at further improving our I/R injury *in vitro* setup, stirred tank bioreactor technology and culture of hiPSC-CMs as 3D aggregates was combined in **Chapter III**. 3D culture is increasingly being employed as a strategy to modulate cardiac tissue *in vitro*, since cultivating CMs in 3D has shown to better recapitulate contractile function and metabolic maturation of cells when comparing to 2D cultures (Zhang *et al.*, 2013; Daily *et al.*, 2015; Correia *et al.*, 2018). Although many authors employ 3D culture strategies in the development of heart tissue constructs (reviewed in Ryan et al. 2016), still few studies take advantage of these 3D systems to model myocardial I/R injury (Katare *et al.*, 2010; Mosadegh *et al.*, 2014).

Stirred tank bioreactors are attractive to cultivate 3D culture systems. Bioreactors are commonly employed to generate relevant number of high quality cells for cell therapy applications (Hosseinkhani *et al.*, 2010; Correia *et al.*, 2014; Kryukov, Ruvinov and Cohen, 2014; Gomes-Alves *et al.*, 2016; Kropp *et al.*, 2016; Cunha *et al.*, 2017), for production of biomolecules (Sousa *et al.*, 2015; Sequeira *et al.*, 2018), as well as for generation of relevant 3D tissue constructs (Tostões *et al.*, 2012; Rebelo *et al.*, 2015; Simão *et al.*, 2016; Sgodda *et al.*, 2017). Noteworthy, these bioreactors also provide unique advantages when considering *in vitro* disease modeling, especially in the context of I/R injury, in which the tight control and monitoring of oxygen and pH levels is important. Adding these advantages to the accumulated knowledge in our lab on bioreactor culture with neural ischemic setups (Amaral *et al.*, 2010), iPSCs aggregates (Abecasis *et al.*, 2017) and cardiac populations (Correia *et al.*, 2014; Gomes-Alves *et al.*, 2016), we decided to establish a myocardial I/R injury *in vitro* model in stirred-tank bioreactors

(**Chapter III**). Using this setup, we were able to successfully mimic both phases of I/R injury by readily decreasing the dissolved oxygen in culture (pO_2 <0.4%) at the start of ischemic phase and re-establishing normoxia (pO_2=3%) during reperfusion. Moreover, the bioreactor setup enabled nondestructive sampling along the experiments without disturbing ischemia and reperfusion environmental parameters.

Overall, in this thesis, multiple strategies were used in order to develop robust I/R injury *in vitro* models, recapitulating several features of *in vivo* human adult myocardial environment. Such strategies included the use of adult (hCSCs, hCFs) and mature (hiPSC-CMs) human cell populations, the use of heterotypic cultures, the establishment of different physico-chemical parameters of I/R, as well as 3D culture configuration and stirred tank bioreactor technology. Altogether, the *in vitro* I/R injury models established in this thesis constituted an important step forward in I/R injury research, allowing to recapitulate several cellular hallmarks of I/R as well as to study the mechanisms of action of hCSCs upon AMI.

In the future, efforts to further increase the relevance of such models should be pursued, including: i) addition of different human cell populations with relevant roles in I/R physiology, such as human endothelial cells, human cardiac fibroblasts and cells from the immune system; and ii) challenging the models using drugs and molecules with known effects on cardiac cellular physiology during and upon AMI.

1.2. Advancing hCSC clinical potential: unveiling hCSC mechanisms of action in an AMI context

hCSCs transplantation emerged as a clinical option for AMI patients. However, so far, clinical trials were not able to show a significant physiological improvement and benefit over pharmacological standard-of-care. Several challenges still need to be addressed in order to meet the

full clinical potential of hCSCs, including the use of advanced human based experimental models to further decipher the mechanism of action of these cells, as well as novel strategies to improve their retention in the target tissue, as recommended by the task force of the European Society of Cardiology (Mathur *et al.*, 2017). A better understanding of the mechanism of action of hCSCs is relevant not only for the advancement of cardiovascular basic biology research by itself, but also to generate knowledge on how to direct novel strategies involved in endogenous hCSCs activation and enhancement of transplanted hCSCs clinical benefits.

Analytical techniques and software development have substantially increased the power of non-targeted global proteomic MS-based strategies in the last decade, allowing the identification and/or quantification of large sets of proteins in a given sample. Such features make proteomics a unique tool for revealing novel molecular mechanisms, both in homeostasis and disease settings. Regarding CSCs, non targeted global proteomics has been applied for characterization of murine (Stastna *et al.*, 2010) and hCSC secretome (Sharma *et al.*, 2017; Torán *et al.*, 2017), hCSC receptome (Gomes-Alves *et al.*, 2015) and whole proteome studies of murine CSCs in homeostasis settings (Samal *et al.*, 2012; Torán *et al.*, submitted).

In this thesis, several strategies were combined in order to further unveil the biology of hCSCs in an AMI context, including the use of un-labelled global proteomic approaches (**Chapter II** and **IV**), as well as the development of relevant human *in vitro* models of injury (**Chapter II** and **IV**) and inflammation (**Chapter V**).

CSCs become activated after injury and transplantation, playing key roles in immunomodulation, proliferating, differentiating into cardiomyogenic

lineages, migrating to the site of injury, and secreting important paracrine factors involved in the modulation of cell proliferation, angiogenesis, vasculogenesis and cytoprotection of CMs. Although CSCs were already identified 15 years ago (Beltrami *et al.*, 2003), still several doubts remain in the scientific community regarding their ability to efficiently differentiate towards cardiac lineages and functionally integrate the tissue upon injury, specially concerning c-kit^{+} CSCs (Ellison *et al.*, 2013; Nadal-Ginard, Ellison and Torella, 2014; van Berlo *et al.*, 2014), the cell population used throughout this thesis. Although proteomic data analysis revealed an enrichment in proteins related with cell differentiation in hCSCs upon ischemia (**Chapter II**), no further evidence for hCSC differentiation was found, including change in cell morphology.

Nevertheless, our results reinforce the idea that CSCs activate proliferation and migration in context of I/R, indicated by an enrichment of proteins associated with pathways with roles on cell movement and proliferation, as well as an increased proliferation upon reperfusion phase of injury (**Chapter II**), and upregulation of proteins associated with remodeling of cell-cell junctions and several pro-migratory and proliferation pathways (**Chapter IV**).

There is a consolidated consensus regarding the importance of paracrine-mediated mechanisms in the regenerative response of CSCs, supported not only by several studies depicting CSC secretome (Stastna *et al.*, 2010; Sharma *et al.*, 2015; Park *et al.*, 2016; Torán *et al.*, 2017), but also by the benefic effect of transplanted CSCs even without tissue retention and engraftment, reported in several preclinical and clinical studies (Madonna *et al.*, 2016). In **Chapter II**, we show for the first time increased CXCL6 secretion by hCSCs upon an I/R injury, suggesting a relevant role of this angiogenic chemokine in hCSC mediated myocardial regeneration. CXCL6 was also recently identified in the secretome of

hCSCs, where addition of an anti-CXCL6 antibody inhibited the migration and angiogenic properties of CSC conditioned medium, proving the importance of this chemokine in key paracrine regenerative potential features of these cells (Torán *et al.*, 2017). Moreover, we also show an enrichment in several paracrine signaling pathways and angiogenic related proteins, as well as an increased angiogenic activity of hCSC:hiPSC-CM co-culture conditioned medium upon injury. Cytoprotection was also observed in the co-culture model (**Chapter II**), where hCSCs exerted a paracrine protective effect in hiPSC-CMs. Interestingly, we can find some parallelisms between activation of hCSC paracrine mechanisms in the co-culture system (**Chapter II**) upon I/R and the cytokines detected as secreted in the different phases of injury (**Chapter III**). Concordant with the increased IGF-1, GM-CSF, IL-8, PDGF and Oncostatin M secretion during the ischemic phase (**Chapter III**), hCSCs also demonstrated enrichment of proteins associated with pathways related with these same molecules upon the ischemic phase of injury (**Chapter II**). Moreover, HGF signaling, detected as one of the enriched pathways in hCSCs on late reperfusion (**Chapter II**) was also secreted by hiPSC-CMs upon reperfusion (ELISA results) (**Chapter III**), further strengthening the importance of the paracrine cross-talk between the two cell populations.

As previously mentioned, one of the main challenges to be addressed in hCSC transplantation is the rapid elimination of the injected cells by the host immune system (Huang *et al.*, 2010; Malliaras *et al.*, 2012), aggravated by the highly inflammatory microenvironment of an AMI patient heart. A better knowledge on the immunomodulatory properties of hCSCs is therefore paramount in developing strategies to increase the transplanted cells retention time in the myocardium, that would probably amplify their regenerative potential. Several studies point to a positive

immunomodulatory capacity of hCSCs, being able to modulate and suppress different responses of the immune system, namely inhibiting cell populations, such as T-lymphocytes. Programmed Death Ligand 1 (PDL-1) mediated direct cell-cell interaction was identified as the main mechanisms in hCSC mediated T-lymphocyte proliferation inhibition (Lauden *et al.*, 2013). In **Chapter V**, through modeling hCSC/T-lymphocyte interaction in an allogeneic context, we were able to demonstrate for the first time that hCSCs exert an immune-suppressive effect on T lymphocyte proliferation not only through the previously described PDL-1 axis but also through a paracrine mechanism associated with indoleamine 2,3-dioxygenase (IDO) tryptophan metabolism. Such findings not only contribute to a better knowledge of hCSCs immunomodulatory mechanisms, but also open new avenues in the development of new hCSC transplantation strategies. For instance, one can envision IDO high expression as a novel quality attribute for the selection of highly immunosuppressive hCSCs donors or genetic engineering of hCSCs to express higher levels of this enzyme.

All findings in this thesis support hCSCs as a cell population with significant regenerative properties in AMI context, including paracrine signaling promoting hCM protection, angiogenesis and immune-suppressive properties. Currently, clinical trials and studies in animal models including transplantation of CSCs or activation of endogenous CSCs show improvements in heart performance. For instance, in a recent report of the phase I/II clinical trial CARE-MI (NCT02439398) with transplantation of allogeneic c-kit^{+} hCSCs, safety, low immunogenicity and estimated treatment effect of 2.3% of decrease in infarct size area were registered (Fernández-Avilés *et al.*, 2018). However, such results are still far from the complete regeneration of the infarcted heart to pre-infarct myocardium functionality level, which constitutes the holy grail of

modern cardiac regenerative medicine. Improved *in vitro* studies and omics approaches, such as the ones developed in this thesis, contribute to further understand the inherent human cellular mechanisms, crucial to guide novel biological therapeutic strategies focused on the activation of endogenous hCSC populations and on hCSC transplantation.

1.3. Characterizing hCF membrane proteome

As introduced in **Chapter I**, not only CSCs and CMs play important roles in AMI physiology. CFs are the main cell type involved in extracellular matrix (ECM) kinetics, including collagen deposition upon AMI and other heart pathologies, leading to tissue fibrosis. Such central role in cardiac fibrosis makes CFs the research focus of several laboratories worldwide. However, one of the main challenges pointed out when studying this cell population is the lack of a proper molecular identity. Molecular markers currently used to isolate and characterize hCFs are not unique nor specific. Besides allowing for the identification of a high number of proteins *per* sample, untargeted MS-based approaches allow for an unbiased, discovery-oriented and high-throughput profiling of the proteome panorama of a given cell population.

With the aim of filling this gap in hCF research, and by applying an optimized membrane protein enrichment protocol previously developed by our group (Gomes-Alves *et al.*, 2015), in **Chapter VI** we provide a deep analysis of hCF membrane proteome. By direct comparison with other cardiac and stem cell populations, we identified a subset of 72 hCF specific proteins including 30 membrane proteins. The characterization of the hCF membrane proteome contributes not only to the identification of potential novel cell specific molecular markers but also to an increased understanding of hCF biology. In order to further filter and strengthen the list of proteins identified herein, additional comparison with other human

myocardial cell populations, such as smooth muscle cells, endothelial cells, CMs, and other hCFs isolated using different methodologies should be considered in future experiments. Moreover, the specificity of the proteins identified may also be validated with other methodologies, such as flow cytometry, western blot and/or immunocytochemistry.

2. Conclusions

In conclusion, overall the work developed herein contributes: i) with novel and robust human cell-based I/R injury *in vitro* models ii) by unveiling hCSCs mechanisms of action in response to AMI in endogenous and transplantation contexts and iii) by providing a detailed characterization of membrane proteins of hCFs.

The knowledge generated in this thesis has the potential to be used in the development of novel strategies targeting the enhancement of hCSCs regenerative benefits as well as the definition of novel hCF-specific molecular markers.

3. Future work

The increasing incidence of ischemic heart diseases has been directing cardiovascular research field towards a better definition and understanding of human cardiac cell populations biology in homeostasis and under pathological conditions. By using novel human cell-based I/R injury *in vitro* models and advanced proteomic tools, this thesis contributed to the field, by providing added knowledge to the mechanisms of action of hCSCs upon AMI and with an extensive characterization of hCFs membrane proteome. Still, the models and

findings obtained from this thesis raised new questions that require further investigation:

- I/R injury *in vitro* models:
  - Challenge I/R models developed with drugs with known effect on I/R injury;
  - Increase complexity and representation of *in vivo* heart tissue by adding myocardium human cell populations such as endothelial cells, hCFs, smooth muscle cells and cells from the immune system;
  - Further characterize conditioned medium content in proteins and secretory vesicles by high-throughput untargeted proteomic approaches.

- Characterization of hCSC mechanisms of action upon AMI:
  - Validate pathways identified as important for hCSC regenerative benefits with knock-out experiments and functional assays;
  - Validate IDO-mediated Triptophan metabolism importance on hCSC immunomodulation, including Trp supplementation and IDO inhibition experiments;

- Identification of hCF specific molecular markers:
  - Extend membrane fraction proteome comparison to other myocardial human cell populations, including hCMs, endothelial cells, smooth muscle cells and hCFs isolated using different methodologies.
  - Further filter the list of proteins identified as specific for hCFs by complementary validation assays.

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