Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

Identification of the Proteomes of c-kit and Sca-1 Expressing Populations of Mice Cardiac Stem Cells

Adriana Andreia Silva Pires Gomes

Mestrado em Bioquímica

Especialização em Bioquímica Médica

2010

Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

Identification of the Proteomes of c-kit and Sca-1 Expressing Populations of Mice Cardiac Stem Cells

Dissertação Orientada pelos Professores Doutores Carlos Alberto Cordeiro e Luís Rosário

Adriana Andreia Silva Pires Gomes

Mestrado em Bioquímica

Especialização em Bioquímica Médica

2010

Acknowledgements

To Prof. Carlos Cordeiro and LuísRosário, my supervisors, for the constant support of my ideas, for the enlightening conversations and for helping me grow as a scientist.

To Dr. Gonçalo Costa, for teaching me all the practical skills, and also for the constant encouragement.

To Ana Marta and Alex Matsuada, for all the lab work we shared and for all the good times at IGC.

To Marta, Ana, Filipa and all my colleagues at the Enzymology group, for the excellent working environment and for all the talks while scientific “waiting” took place.

To Prof. Ana Ponces, for being the best teacher I have ever had.

To all my friends from the house, from graduation, and from Caldas. I never forget you.

To My Mother and my Sister, who have always been there for me. To my aunts Betty and Isa, thank you for all the long talks. To Sofia, for calling all the time.To Samuel, Henrique, Ines, Daniela, who will only understand this many years from now. And for all the family that has always been so supportive.

To João Paulo, for being all the motivation I’ll ever need. For being patient with my moods during hard times.For existing.

I dedicate all my work to my grandfather, João de Deus. I hope you’re proud of me, as I am of you.

Abbreviations

2D- Two dimensional

ASC- Adult Stem Cells

BSA- Bovine Serum Albumin

CCTs-Cardiac Cell Types

CSCs- Cardiac Stem Cells

CSM- Cardiac Smooth Muscle

Da- Dalton; kDa- Kilodalton

DAB- Diaminobenzidine

DNA- Deoxyribonucleic Acid

ECs- Endothelial Cells

ESCs- Embryonic Stem Cells

ESI- Electrospray Ionization

FACS- Fluorescence Activated Cell Sorting

FBS- Fetal Bovine Serum

FSC- Forward angle Light Scatter

FTICR- Fourier Transform Ion Cyclotron

H_2O-Water

HBSS- Hank's Balanced Salt Solution

LC- Liquid Chromatography

LDH-A- Lactate Dehydrogenase-A

MALDI- Matrix-Assisted Laser Desorption Ionization

MARCKS- Myristoylated Alanine-rich C-kinase substrate

MDR1- Multi Drug Resistance

MI-Myocardial Infarction

MSC- Mesenchymal Stem Cells

MS-Mass spectrometry

MW- Molecular Weight

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PMF- Peptide Mass Fingerprint

SDS-PAGE- Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SSC- Side angle Light Scatter

TA- Transiently Amplifying Cell

TGF-□- Transforming Growth Factor

TOF - Time-of-Flight

mL- microliter; m-micro

mL- milliliter; m-mili

M- $mol.dm^{-3}$

Abstract

Heart failure and other heart diseases are the most important death causes in developed countries. When the heart fails, it heals by scar formation, which compromises its normal ability to pump sufficient blood to meet the metabolic demands of the body. As the heart was usually viewed as a post mitotic organ, the renewal of its tissue by endogenous cells was considered non-existent. However, recent studies in which small dividing cells with stem cell surface marker were found in the heart and identified as Cardiac Stem Cells (CSCs), changed this view and gave a new perspective in therapeutic approaches to heart related diseases. As CSCs are a recent discovery, it is essential to study and characterize these cells to better understand the way in which they regenerate the heart damaged tissue, the molecular factors involved in their mobilization and differentiation and the molecular factors produced by these cells.

In the present work, the membrane and nucleus of CSCs expressing the stem cell markers Sca-1 and c-Kit were isolated using differential centrifugation, and the proteome of this nuclear and membrane fractions, along with a whole cell sample for the c-kit^{+} CSCs was analyzed by Peptide Mass Fingerprint, using MALDI ionization coupled with FT-ICR detection, which allowed an increase in sensitivity and resolution. 122 proteins were identified, 48 for the c-kit^{+} CSCs and 74 for the Sca-1^{+} population. Uncharacterized proteins were found in both kinds of cells, along with proteins involved in the proliferation pathways common to both populations, as is the case of the Protein Chibby Homolog 1, or specific of c-kit^{+} population (Alpha Enolase) or to the Sca-1^{+} population (47 kDa heat shock protein). Other differences were found and discussed. This work is the first attempt at the unraveling of the proteome of Cardiac Stem Cells.

Key Words: Proteomics, FACS isolation of CSCs, Cardiac Stem Cells, Membrane proteins, Nuclei proteins

Resumo

Até ao século XX, o coração era considerado um órgão pós-mitótico, sem capacidade de auto-regeneração. Estudos recentes levaram a uma mudança de paradigma, provando a existência de células estaminais no coração, Células Estaminais Cardíacas (CEC), responsáveis por uma taxa diminuta de renovação do tecido cardíaco, e revelando novas possibilidades de terapia para doenças cardíacas. Infelizmente, pouco se sabe ainda acerca dos mecanismos subjacentes à diferenciação e migração destas células, pelo que há necessidade de estudos neste sentido, exigindo uma investigação mais detalhada. No presente projecto caracterizou-se o proteoma de duas populações de Células Estaminais Cardíacas, caracterizadas por apresentarem os marcados de superfície celular c-kit e Sca-1. Através da identificação do proteoma de membrana e núcleo destas células, pretende-se obter conhecimento acerca dos mecanismos subjacentes à diferenciação e proliferação de CSCs, utilizando o modelo animal de ratinhos Balb C, com o objectivo de melhor compreender o potencial destas células na regeneração cardíaca

No Capítulo 1 descreve-se com detalhe as propriedades das Células Estaminais Cardíacas. Estas células são multipotentes, pelo que podem diferenciar-se nas três linhagens cardíacas: Cardiomiócitos, Músculo Liso vascular e Células Endoteliais, e têm um papel importante na regeneração cardíaca.Estas célulasdemonstram capacidade de auto-renovação, apresentam elevada plasticidade, e possuem ainda a capacidade de se dividirem assimetricamente, gerando uma nova Célula Estaminal Cardíaca e uma célula progenitora, que se irá diferenciar numa das três linhagens cardíacas já referidas. Cada vez mais existem resultados que indicam que as proteínas secretadas pelas Células Estaminais Cardíacas na vizinhança de uma lesão cardíaca têm um papel importante na regeneração. É fundamental conhecer as vias de sinalização autócrinas e parácrinas destas células e perceber de que modo influenciam a diferenciação e migração das Células Estaminais Cardíacas.

Devido a serem uma descoberta recente, pouco se sabe acerca do metabolismo e da sua biologia destas células. O conhecimento do proteoma das Células Estaminais Cardíacas é fundamental para a aplicação destas células em terapia, uma vez que a

maioria das funções celulares é exercida por proteínas. O estudo do seu proteoma recorrendo à espectrometria de massa é a estratégia proposta neste trabalho. A abordagem proteómicaBottom-Up à caracterização do proteoma das CSCs permite a identificação de várias proteínas numa mistura. A amostra proteica é digerida enzimaticamente, através da incubação com trispsina, os péptidos resultantes são analisados por espectrometria de massa, e as proteínas identificadas por *PeptideMassFingerprint* usando o motor de busca MASCOT (www.matrixscience.com).

A partir do homogenato proteico, as proteínas a identificar são separadas de acordo com a massa por electroforese unidimensional (SDS-PAGE), separando as proteínas de acordo com a sua massa molecular. Após a digestão das amostras, a mistura de péptidos é analisada por MALDI-FTICR (Matrix-Assisted Laser DesorptionIonization, com detecção por Fourier Transform-IonCyclotronRessonanceMassSpectrometry). A análise por FTICR-MS permite a mais elevada exactidão de massa e resolução, dois factores cruciais para o sucesso da identificação de proteínas em misturas complexas. O conhecimento do proteoma das Células Estaminais Cardíacas permite o acesso a informação acerca dos mecanismos que levam à migração e diferenciação das Células Estaminais Cardíacas.

No Capítulo 2, é descrito o método de isolamento de Células Estaminais Cardíacas e os resultados obtidos. Ratinhos Balb C foram executados e os seus miocárdios submetidos a destruição manual e várias sequências de centrifugações e filtrações, obtendo-se uma suspensão de células cardíacas. Desta suspensão, através do uso da técnica de FluorescentActivatedCellSorting, as duas populações de Células Estaminais Cardíacas forma isoladas, utilizando sondas fluorescentes, isto é, fluorocromos conjugados com anticorpos monoclonaisanti c-kit e anti Sca-1.Citometria de fluxo é uma técnica amplamente usada para medir características de partículas biológicas. As células a serem analisadas são preparadas em suspensão celular single-cell, e o fluxo-uni-celular é bombardeado com um laser. Quando o laser atinge as células previamente marcadas com uma sonda fluorescente, a fluorescência emitida permite seleccionar e isolar estas células marcadas. A emissão de fluorescência pelas sondas utilizadas permite o isolamento das células que se ligam as mesmas. 1134807 Células Estaminais Cardíacas foram isoladas para a população c-kit^{+}, a partir de 82

ratinhos. 77656 células foram isoladas para a população Sca-1^{+}, a partir de 30 ratinhos, tenho esta população sido mais fácil de isolar, como previsto pela literatura.

A partir das amostras obtidas no Capítulo 2, foram isoladas fracções membranares e nucleares de cada população, através do método de centrifugação diferencial, no Capítulo 3. As populações foram isoladas com sucesso, facto verificado através de Western Blott, uma vez que para a fracção membranar o marcador de membrana (Sca-1 e c-kit) é observado em maior quantidade que o marcador nuclear (phospho-Histona H3), e na fracção nuclear apenas o marcador nuclear é observado. Devido a escassez de amostras de Células Estaminais Cardíacas c-kit^{+}, uma amostra de wholecell, isto é, extracto proteico não fraccionado contendo proteínas provenientes de todos os componentes celulares, foi também efectuada, através de 3 diferentes métodos de extracção de proteínas, uma optimização que permitiu perceber a necessidade de elevadas quantidades de células c-kit^{+}com vista à obtenção de quantidades de proteína suficientes para a visualização por SDS-PAGE, fundamental para a última parte do trabalho desenvolvido.

O estudo do proteoma das Células Estaminais Cardíacas foi desenvolvido no Capítulo 4. A partir das fracções membranares e nucleares de Células Estaminais Cardíacas c-kit^{+} e Sca-1^{+}procedeu-se a separação das proteínas por SDS-PAGE, e seguidamente a digestão com tripsina das bandas do gel, obtendo um conjunto de péptidos a analisar por MALDI-FTICR. Após a análise dos espectros através de ferramentas bioinformáticas, identificaram-se 134 proteínas, 58 para as Células Estaminais Cardíacas c-kit^{+}, e 74 para as Células Estaminais Cardíacas Sca-1^{+}.

Das 35 proteínas identificadas para a fracção wholecell de Células Estaminais Cardíacas c-kit^{+}, 8,3% estão envolvidas nos mecanismos de proliferação celular (Stress-Induced Phosphoprotein-1, Alpha-Enolase, ProteinChibbyHomolog 1), e 11,1 % são proteínas não caracterizadas e de função desconhecida. Na fracção membranar desta população de Células Estaminais Cardíacas identificaram-se as mesmas proteínas não caracterizadas e de função desconhecida, e ainda 10% de proteínas envolvidas nos mecanismos de proliferação. Na identificação de proteínas nucleares desta população de Células Estaminais Cardíacasforam identifcadas proteínas de função ubiquitária.

Para a população de Células Estaminais Cardíacas Sca-1^{+}, das 45 proteínas identificadas, 2% estão relacionadas com a proliferação celular e 4% com os processos de diferenciação celular. Todas as proteínas identificadas para a fracção membranar de Células Estaminais Cardíacas c-kit^{+} foram encontradas nesta fracção, demonstrando a similaridade de ambas as populações de células estaminais. No entanto, mais estudos serão necessários para determinar quais as diferenças mais relevantes entre os proteoma das duas populações de Células Estaminais Cardíacas. As proteínas identificadas na fracção nuclear de Células Estaminais Cardíacas Sca-1^{+} estão maioritariamente relacionadas com a regulação da transcrição e são comuns a maioria das células mamíferas, destacando-se 10% de proteínas relacionadas com a regulação da proliferação celular.

Proteínas não caracterizadas foram encontradas em todas as amostras. Estas proteínas, devido a serem aparentemente muito abundantes nestas populações devem ser alvo de estudos futuros, que possibilitarão determinar o seu envolvimento em processos relevantes para a biologia de células estaminais. Ainda, algumas diferenças entre as proteínas identificadas envolvidas nos processos de proliferação e diferenciação de células estaminais deverão ser futuramente exploradas através de estudos funcionais que permitirão estabelecer um fenótipo característico de cada população destas células.

Contents

Chapter 1

General Introduction

Present Work Objectives

Heart failure and other heart diseases are the most important causes of death and disability in developed countries[1]. When the heart fails, it heals by scar formation, which compromises its normal ability to pump sufficient blood to meet the metabolic demands of the body[2]. As the heart was usually viewed as a post mitotic organ, the renewal of its tissue by endogenous cells was considered non-existent, but in recent studies, small numbers of dividing cells were found in the heart,called Cardiac Stem Cells (CSCs), and changed this view[3,4,5].Thesecells have properties similar to stem cells and express transcription factors associated with cardiac development, and gave a new perspective for possible treatments of heart related diseases[4,6].

Cardiac Stem Cells divide asymmetrically, generating another CSC and a Transiently Amplifying Cell (TA cell), a cell committed to the differentiation path, and stillable to replicate several times[7]. It is essential to study and characterize these cells to better understand the way in which they regenerate the heart damaged tissue, the molecular factors involved in their mobilization and differentiation and the molecular factors produced by these cells[8].

In this work a study of the proteome of the CSCs membrane and nuclei was performed, along with the determination of the changes that may occur to this proteome during some of the early differentiation steps of these cells, when they express different surface markers[6]. This characterization is important to unravel the mechanisms of regeneration, as in general the experiences attempted until now to regenerate the damaged heart have not explained satisfactorily this mechanism and the factors involved in it[9].

This study comprises the unraveling of the c-kit^{+} whole cell, membrane and nuclei fractions and Sca-1 membrane and nuclei fractions proteomes. The major objective was to find proteome differences between these cells types that might possibly be used as markers for different stages of differentiation, and also to achieve a proteome characterization that allows reliable identification of cardiac myocytes precursors and identification of proteins involved in the regulation of these cells differentiation and proliferation processes, as these are unknown at the present.

Stem Cells

Origin of Stem cells

Adult stem cells are defined by the ability to renew themselves, under appropriate conditions, and differentiate into one or more adult cell types[8]. They exist in many organs and are responsible for organ homeostasis and tissue regeneration[8,10].

There are two main hypotheses for the origin of cardiac stem cells. These cells may be *autochthonous*, which means that they are remnants from embryonic cells that stayed in the differentiated tissue, or they might be *allochthonous*, that is, they are recruited from the bone marrow or other organs through the blood stream[11].

The results of studies characterizing cardiac cell populations expressing different stem cell surface markers suggest that the origin of each population might be different, protecting the heart homeostasis by a redundant stem cell system involving *allochtonous* and autochthonous cells, but this conclusion contrasts with the lack of effective regeneration[11]. An important step for this achievement is to correctly identify proteins expressed by the different populations of stem cells in the heart, as their similarity (or absence of it)with other stem cells may offer important clues[6] in this matter[12].

Potency and Plasticity

The ability of stem cells to differentiate into adult cell types is called Potency, and stem cells can have different levels of potency.

Totipotent cells, which can be found in the embryo of vertebrates in its early stages, have the ability to differentiate into all types of cells of the organism, eventually giving rise to the an entire living being[13].

Pluripotent stem cells are cells which may differentiate and form each of the three germ layers: endoderm, ectoderm and mesoderm. It is the case of embryonic stem cells (ESCs), which are derived from the inner cell mass of the blastocyst stage of the early embryo and are pluripotent[14].

Multipotent stem cells can differentiate into multiple organ specific cell types[10]. Cardiac stem cells are multipotent cells, and they can differentiate into the three

Cardiac Cell Types (CCT): cardiomyocytes, vascular smooth muscle cells (VSM) and endothelial cells (ECs)[15].

Stem cells have always been consideredimmortal cells, meaning that they have the ability to self-renew indefinitely. However, recent studies indicate that self-regeneration might not be their most determining characteristic, as this is not verified for all stem cells[16].

Stem cells do have a highly plastic phenotype, meaning that they may quickly convert themselves into other stem cell types, and many types of stem cells and their descendants can interconvert in response to extracellular signals[14]. Studies indicate that even fully differentiated somatic cells keep the potential to reactivate genes that could reverse their fate, returning the cell to an embryonic state[13]. The study of the extracellular signals receptors and pathways is essential to understand plasticity, as these signals can propagate through intracellular signal-transduction pathways that form the genetic network which controls pluripotency[14].

Stem cell categories

There are three main categories of stem cells: Embryonic Stem Cells (ESCs)[6], Mesenchymal Stem Cells (MSCs)[17], and Adult Stem Cells (ASC)[10]. ESCs are derived from the inner mass of the blastocyst, can be grown *in vitro* and propagate indefinitely maintaining their undifferentiated state and they may differentiate into the three germ layers (pluripotent cells)[18]. Despite their enormous potential use in therapy, the use of human ESCs raises an ethic problem, because their only source is the embryo, and therefore their use is tightly controlled and they are still not used for human therapy[17,19].

MSCs are multipotent cells which are present in most tissues during development[2,6]. There is a great disagreement around the definition of Mesenchymal Stem Cells. The most accepted definition, in general, is that they are bone marrow cells that self-renew and give rise to mesenchymal tissues, although some of these cells actually differentiate into cell types that are not present in the embryonic mesoderm[17,20]. Their role in organs regeneration is not very clear, but they may play an important part in cardiac regeneration[21].

ASCs, also called progenitor cells, are multipotent stem cells which reside in adult organism organs or tissues, in extremely low numbers[9]. These cells are a recent discovery, and they populate organs like the bone marrow (hematopoietic stem cells), brain, blood vessels, liver, skin and the heart[22]. Most studies indicate that these cells have slow division rates, having small impact in tissue regeneration in case of disease in natural conditions (without further medical intervention), but have a great potential for clinical applications using cell-based therapies[23].

Stem cell niches

Stem cells usually exist in a protected microenvironment called niche, where they divide, differentiate and die[7]. The niches are located deeply inside the tissue, insuring protection from damaging stimuli[24]. The adult heart typically shows discrete structures in the interstitium where CSCs are clustered, forming cardiac niches, and are connected between themselves and with myocytes and fibroblasts by junctional and adhesion proteins (connexins and cadherins)[25].

In these structures, they are able to stay in a stem state due to the existence of supporting cells, fibroblasts and myocytes, which secrete and modulate growth signals and anchor these cells to the niche, protecting them from the outside influence of growth and differentiation factors[4]. Stem cells do not exist in the absence of supporting cells within the niche[26].

The structure of the niche is specifically tailored to suit the needs of the resident stem cells. However, the stem cells themselves seem to have an important role in the organization of their niches[24].Niche homeostasis is regulated by division of stem cells, which preserves the ideal proportion of primitive and committed cells within the organ[27].

Stem cell division

Stem cells have a small division rate, and their replication is mostly confined to niches. They are able to divide asymmetrically, yielding another stem cell and a Transiently Amplifying cell[7]. Transiently Amplifying cells are able to divide and proliferate, leading to cell differentiation and producing committed progeny, regenerating the tissue[7]. The new stem cell will maintain the stem cell pool.

Stem cells can also divide symmetrically into two committed cells, if there is great need of regeneration, decreasing the number of primitive cells, or into two stem cells, with the purpose of expanding the stem cell compartment[7,26]. This last process is usually associated with some disequilibrium of the organ homeostasis, possibly due to disease[26].

Any of these developmental choices has a direct impact in the stem cell pool size, the number of progenitors and precursors and therefore the possibilities of regeneration[24,26].

Cardiac Stem Cells and Heart Failure

Heart diseases, as the ischemic heart disease, coronary heart disease, heart failure and myocardial infarction (MI) are the most common causes of mortality in the developed countries, leading to more than 7 million deaths worldwide each year[1,28].

Cardiac diseases, especially MI, usually lead to rearrangement of the cardiac structure that includes thinning of the infarcted tissue, cardiac dilatation and tissue remodeling[29]. This remodeling, at cellular level, is associated with apoptosis and necrosis of cardiac myocytes, hypertrophy, fibrosis and infiltration of inflammatory cells[29]. The oxidative stress that follows the infiltration of inflammatory cells and the segregation of cytokines and growth factors in the inflammatory response leads to adverse cardiac remodeling, only hours after the MI, contributing to heart failure[28,30].

Stem cell transplantation appears to be a promising treatment to repair the damaged heart and restore cardiac function, as these cells are able to engraft and release factors such as proangiogenic factor Vascular Endothelial Growth Factor (VEGF) antiapoptotic factor Insulin Growth Factor (IGF-1) and antiapoptotic and proangiogenic cytokine IL-10, repairing the damaged tissue by replacing death cells and contributingagainst apoptosis and necrosis by releasing anti-apoptotic and pro-angiogenic factors, thereby diminishing myocyte loss and inhibiting adverse cardiac remodeling[8,29]. These cells also enhance the formation of vascular structures, restoring ventricular wall thickness and improving the contractile function of the heart[28,31].

Despite all the potential of the suggested cell therapy, the identification of the appropriate cell types, factors released from stem cells and their role in cardiac

regeneration and repair is still an open topic, to which the present study will contribute with the identification of receptors and other proteins expressed by Sca-1^{+} and c-kit^{+} CSCs .

Cardiac Stem Cells

Until recently, the adult heart was considered a post-mitotic organ,meaning that it exerted its function during its life-time with the same cells that were present at the end of the growth phase, which implied that it was not able to repair any form of damage. According to this paradigm, the number of myocytes would be established at end of the growth phase, and this population of terminally differentiated myocytes would be irreplaceable through the life of the organ and the organism[32,33,34].

This view was challenged by the discovery of stem cells in the heart, which are responsible for a low, but existent, tissue self-renewal[11]. These cells, Cardiac Stem Cells, are stored in niches, a microenvironment in which they are kept in a quiescent state[7]. In the niches, there are supporting cells, Fibroblasts and Myocytes, which anchor CSCs to the niche and modulate growth signals[7,6].

As most adult organ stem cells, CSCs have very strict differentiation possibilities, and they differentiate into three main cardiac cells progenies:Cardiaomyocytes, vascular Smooth Muscle cells and Endothelial cells (ECs)[7]. Their main role is to regenerate damaged tissue, although this function becomes compromised with age[2].

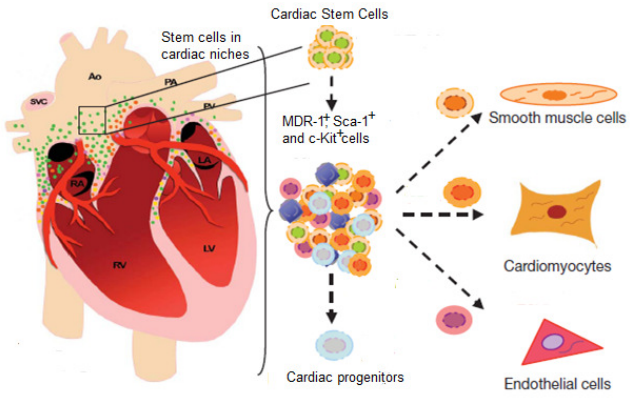

Figure 1.1- Cardiac Stem Cells exist in niches in the heart, and when exposed to differentiation and growth factors give origin to cells from the cardiac lineage: smooth muscle cells, cardiomyocytes and endothelial cells. Adapted from Bu, L. et al. Nature 2009

CSCs and progenitor cells express early cardiac genes and transcription factors essential for differentiation into cardiac lineage[6]. They also have specific surface proteins which may allow their isolation and use in cell-transplantation based therapy[10].

Stem cells surface antigens

CSCs express three main stem cell-related surface antigens: c-kit, a stem cell marker present in cells that in early fetal life colonize the yolk sack, liver and other organs[35], Sca-1 a reactive protein that is involved in self-renewal of hematopoietic stem cells[36], and MDR1 (Multi Drug Resistance), a protein extruding toxic substances and Hoechst dye[37]. All this markers have been identified in both mice and human cells[1,3,17].

Stem cell-related surface markers are still present in early committed cells, making it difficult to distinguish the actual primitive cells in the cardiac population[7,38] . CSCs do not express markers of hematopoietic, skeletal muscle,or neural cell commitment, and the ligand for the c-kit receptor is expressed in fetal and neonatal myocardium, supporting the possibility that CSCs could have been in the heart from fetal life[15].

Cardiac Stem Cells based therapy

Therapy with CSCs has been attempted, with the injection of CSCs into the heart, but so far the reconstitution of larger portions or even the hole of the damaged tissue has not been possible[17]. Moreover, the newly formed tissue has a structural organization which differs from the rest of the myocardium and which resembles more the fetal-neo-natal myocardium structure[17]. This limitation can be overcome with the application of bioengineered scaffolds, structures created from living organs that are decellularized in order to maintain the transplanted cells in a correct position[58], and also with the manipulation of the factors involved in the transplantation of the new cells[10]. The self-assembling environments can be loaded with peptides which enhance progenitor cell homing and differentiation, for a successful repair of the injured heart (Figure 1.2).

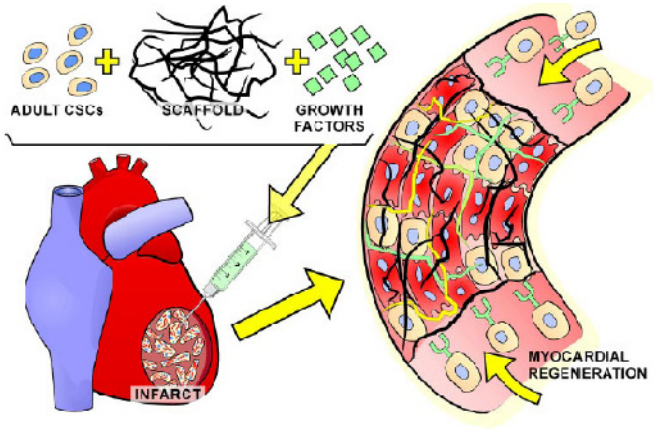

Figure 1.2-Adult Cardiac Stem Cells (CSCs) can be used in myocardial regeneration, if applied with helping factors such as growth and homing factor and a bioengineered scaffold to access a correct positioning of the newly formed tissue. Adapted from Leri, A., 2005[17]

Proteome of Cardiac Stem Cells

There is growing evidence that proteins secreted from cardiac stem cells into the vicinity of an injury in an infarcted heart can modulate the microenvironment and play an important role in functional improvement[29]. Proteins secreted by cells are very important, as their secretions are specific for each cell type, and reflect the local environment of the cells[39].Secreted proteins with regulatory effects on CSC proliferation have been identified in the medium of CSCs cultures[39].

Better knowledge about the proteome of CSC cellswill provide informationrelated to the mechanisms driving stem cells engraftment and their differentiation process[23]. This will lead to a deeper insight into the autocrine and paracrine pathways of secreted proteins, improving theunderstanding of how the cell surface receptors and the signaling proteins stimulate and influence the stem cells differentiation process[23].

Paracrine or autocrine factors are mostly proteins, secreted by cells which can induce changes in the neighboring cells or in the same cell, respectively. These factors usually belong to these protein families: Fibroblasts growth factors, Hedgehog proteins, Wnt proteins, and Transforming Growth Factor (TGF-β) superfamily. There are also neurotrophins, cytokines, interleuckins and various other factors that play an important role in these regulation processes[28].

Proteomics is a suitable approach for the study of stem cell proteins, as it allows the study of proteins and their functions in complex biological systems[23].

Proteomics

Proteomics is the term that defines the study of the set of proteins encoded by the genome of an organism cell type, and represents a major area of study, due to the fact that most cell functions are carried out by proteins[40]. This subject comes naturally after another very meaningful subject, Genomics, which is the unraveling of the genome of the organisms[41].Using the genomeinformation,the sequence of the full complement of genes in an organism, the databases predict the gene products, proteins, which are used for protein identification in proteomics area[42].The peptide sequence of a given protein may differ from the gene sequence translated product in a database due to gene mutation, gene splicing and post-translational modifications, so the databases are in constant growth to achieve better identification results[43].

Although sequencing the DNA is a relatively straightforward task, with the Polymerase Chain Reaction (PCR) and the automated sequencing techniques presently available, the study of the proteome provides a greater challenge due to the fact that a genome of an estimated 25000 genes can yield over two million proteins, in humans, due to mechanisms such as the gene splicing[44]. And this scale of research for itself is very demanding in terms of resources and analytical methods[45]. Proteome characterization includes the identification, study and quantification of the complete set of proteins expressed by the genome in a given cell, tissue or organism, and it includes isophorms, polymorphisms and modifications, and also characterization of protein-protein interactions and of complexes such as the ones involved in protein translactional[46].

Mass Spectrometry

The mass analyzer is central to the proteomics process. Mass Spectrometry (MS) is a technique that has been developed in the early days of the last century by J.J. Thompson[47] (who discovered the isotopes) and his student F.W. Aston[48], who built the first functional mass spectrometer, which he used to identify the isotopes of chlorine, bromine and krypton[45].

MS deals with ions because of the easiness to manipulate the motion and direction of ions experimentally and detect them[49]. The analysis by MS comprises three main steps: ionization of the sample, converting the species into gas-phase ionic species, separation and mass analysis of the molecular ions on the basis of their m/z (mass-to-charge) ratio, and finally the display of the measure of the ion current due to the mass-separated ions in form of a mass spectrum[49].

The introduction of the "soft ionization" techniques in the late 80's, as Matrix-Assisted Laser Desorption Ionization (MALDI) and Electrospray Ionization (ESI), allowed the analysis of large, non-volatile, polar and thermally labile biomolecules[44]. The discovery of these techniques was recognized by the awarding of the Nobel Prize in Chemistry to John Fenn for his work in the ESI[50] technique, and to Koichi Tanaka for his work in MALDI technique[44,51].

At the same time that these awards were attributed, Proteomics started to gain relevance. With the developmentof 2D-PAGE gels, advanced chromatography technologies and the several innovations in mass analyzer technology, such as the introduction of high field magnets leading to a raise in sensitivity and range mass of the technique, it was finally possible to identify proteins in a large scale, and in the present MS has been developed to an extent that supersedes the 2D gels that were the first technique used for large scale proteomics[45,52].

Bottom Up Approach

The bottom up approachto MS analysis is the most used method to study highly complex samples and for large scale analysis. Its name comes from the fact that the identification is started from a group of peptides, reaching in the end the parent protein identification[56]. In this approach, the protein sample is proteolytically digested into small peptides which are then analyzed by mass spectrometry, and the resulting peptide *m/z* ratios or sequences are used to identify the corresponding proteins through the use of bioinformatics tools[56]. This technique is called *Peptide Mass Fingerprint*, as the set of peptides fragments resultant from the digestion is unique to each protein, and their masses are used as a fingerprint to identify the parent protein[53,56]. Another possibility is tandem MS and analysis of the resulting fragments (MS/MS)[54]. The most widely used method of identification is the database search, in

which the experimental data acquired are compared with the predicted, *in silico*generated fragmentation patterns of sequence-known peptides[55]. This approach is well suited for quantification of the samples, through chemical modification of proteins (using tandem mass tags)[56].

Mass spectrometer

Mass spectrometry enables the measurement of the *m/z* ratio of gaseous ions, as described before[45]. This is achieved through measurements made by a mass spectrometer that consists of an *Inlet*, which might be a liquid chromatograph or a direct insertion probe to place the sample, an *Ion Source*, which produces ions from the sample, one or more*Mass Analyzers*, which separate the ions according to their *m/z* ratios, a *Detector*, to register the number of ions emerging from the mass analyzer, and a *Data System* which processes the data, producing a mass spectrum and which controls the instrument through feedback. The ion source, the mass analyzer and the detector are all in a vacuum system[45].

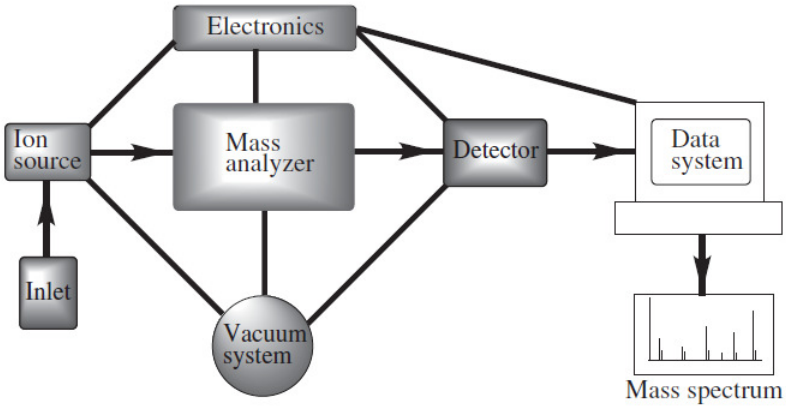

Figure 1.3- Mass spectrometer configuration. From*Fundamentals of contemporary mass spectrometry*, Wiley 2007[49]

Ion Sources

The ion sources enable the vaporization and ionization of the sample so that it can proceed through the analyzer[45]. The most common ionization sources are Electrospray Ionization and MALDI, which was used in the current work.

For the use of the MALDI techniqueproteolytically digested samples are added to a matrix, spotted onto a solid target and co-crystalized with the matrix[54]. The matrix, which typically contains a UV sensitive aromatic compound, absorbs laser energy from a laser beam, and transfers it to the acidified analyte, usingthe thermic energy of the laser to cause desorption of matrix and singly charged $[M^{+}H]^{+}$ ions of analyte into the gas phase(M is the mass of analyte molecule)[54,55]. Once the ions are vaporized they are accelerated by an electric field, and a mass analyzer can be used to measure their *m/z* (mass to charge ratio)[54].

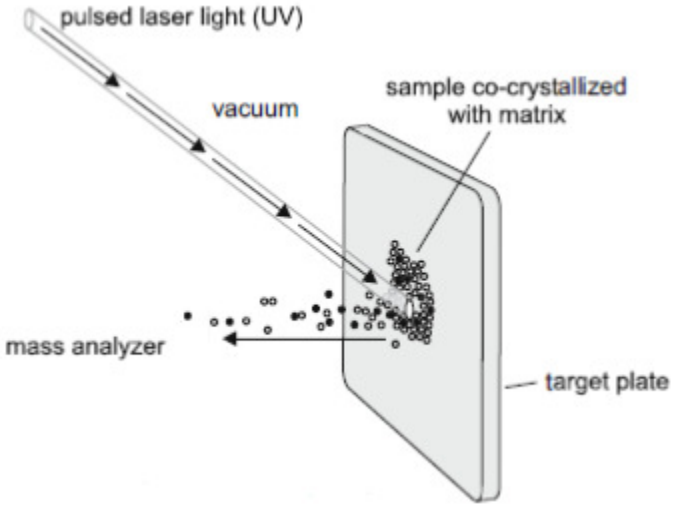

Figure 1.4- Schematic illustration of the MALDI ionization method: the sample co-crystalized with the matrix is dried in the target plate and is placed in the vacuum system of the mass spectrometer. After irradiation with laser light the sample and matrix molecules desorb from the condensed plate. Adapted from Schuchardt&Sickmann, 2007[54]

The main drawback of this technique is a strong dependence on sample preparation methods[55]. There are some variations of this method for different conditions, and also much more ionization methods that are not so common and therefore will not be specified here.

Mass Analyzers - Fourier Transform Ion Cyclotron

There are four main types of mass analyzers: Ion Trap, Time-of-Flight (TOF), Quadrupole and Fourier Transform Ion Cyclotron (FTICR-MS)[45].

The mass analyzer used in the current work was the FTICR-MS, which has the advantages of having the highest resolution over a broad range of *m/z* and also the

highest sensitivity and mass accuracy, as small amounts as tens of moles of peptide loaded onto a MALDI probe can be detected by this analyzer[55]. This detector works by trapping ions in a cell within a static magnetic field[55]. In this field, ions assume a cyclotronic motion in the cell in a plane perpendicular to the magnetic field[45]. The *m/z* ratio can be determined by measuring the frequency of motions of the ions, as cyclotron motion is periodic and characterized by its cyclotron frequency, the frequency with which an ion repeats its orbit[55]. This frequency is determined by the strength of the magnetic field (*B*), the charge of the ion (z) and the mass of the ion (*m*): $f_{c=\frac{zB}{2\pi m}}$

and so, for a constant magnetic field the m/z ratio of the ion is determined by measuring its cyclotron frequency[56]. An electronic pulse is then used to disturb the motion of the ions in the cell into coherence (which produces an electrical signal measurable between detection plates), and then an electrical signal is measured as the ion motion decays to incoherence (which produces no electrical signal)[55]. The time domain signal is converted to a frequency domain by the Fourier transform (Figure 1.5), and *m/z*values are derived from frequency of motion, yielding a mass spectrum[45].

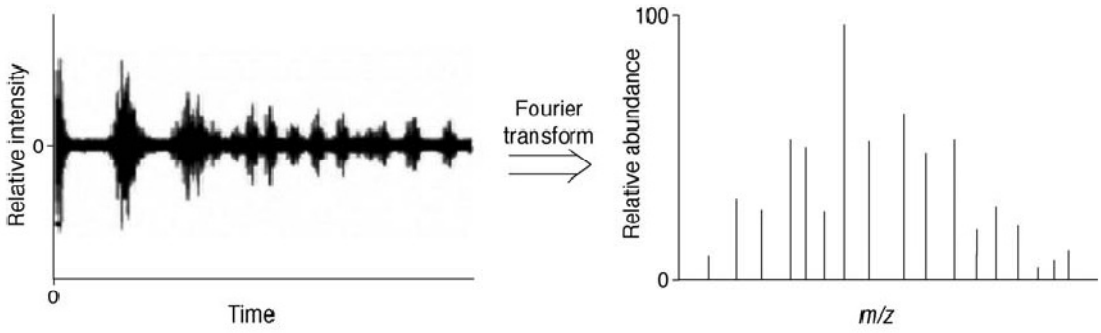

Figure 1.5- Time domain free-ion decay signal and Fourier-transformed normal mass spectrum. Adapted from *Lane, 2005*[39]

Frequency can be measured with more accuracy and precision than any other physical property[49]. Once all ions have been detected, an electric "quench" signal is applied to eject the sample before the next lot of ions is introduced into the cell[55].MALDI-FTICR analysis of samples is a robust method, that combines extremely high resolving power with mass accuracy, and allows more precise identifications and dramatically reduces false positive peptide identifications[59].

Mass Spectrum and Protein Identification

After the sample has gone through the mass analyzer, the computer generates a mass spectrum consisting of *m/z* peaks of different sizes[57]. Then, the user may acquire a list with the *m/z* values for a given sample, which can be compared with the online database artificially obtained m/z values for a theoretical digestion of a known protein[57].A score is attributed to the match based on cross relation, fragment ion, frequencies and hypergeometric probability[55]. Some of the databases also provide a statistic measure between sequence and spectra[55]. The most used match database is Mascot, although the probability model details are not published or available for public consult[57].

Conclusion

In the present work, the proteome of cardiac stem cells c-kit$^+$ and Sca-1$^+$ was accessed through mass spectrometry based techniques. These techniques are ideal for this task as they allow high sensibility and can analyze complex samples as the ones in study. The unraveling of the proteome of these cells will lead to a better comprehension of their regulation and their role in cardiac regeneration.

References

1.Rosamond W., Flegal, K. et al for the American Heart Association Statistics Committee, Stroke Statistics Subcommittee, *Heart disease and stroke statistics- 2008 update: a report from the American Heart Association Association Statistics Committee and Stroke Statistics Subcommittee*, Circulation117:e25-146

2.Jun Wu, FaquanZeng, Richard D. Weisel and Ren-Ke Li, Chapter: "*Stem Calls for Cardiac Regeneration by Cell Theraphy and Myocardial Tissue Engineering*", in "Engineering of Stem Cells", Springler Berlin/ Heidelberg, Vol 114, 2009

3.Urbanek, K. et al, *Intense myocytes formation from cardiac stem cells in human cardiac hyperthrophy*, PNAS, 100:18, 10440-10445, 2003

4.Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, and Anversa P., *Chimerism of the transplanted heart*, N Engl J Med, Vol. 346: 5–15, 2002

5.Oh, H., Schneider, M., *et al*, *Cardiac progenitor cells from adult myocardium: homing, differentiation and fusion after infarction*, PNAS, 100, 12313-12318, 2003

6.Leri, A. et al, *Cardiac stem cells and mechanisms of myocardial regeneration*, Physiol Rev, 85, 1373-1416, 2005

7.Urbanek, K., et al, *Stem cell niches in the adult mouse heart*, PNAS Medical Science, Vol.103, 9226-9231, 2006

8.Battey, J.F., et al, Stem Cells: *Current Challenges and Future Promise, Developmental Dynamics*, Vol. 236, 3193-3198, 2007.

9. Jun Wu, FaquanZeng, Richard D. Weisel and Ren-Ke Li, Chapter: "*Stem Calls for Cardiac Regeneration by Cell Theraphy and Myocardial Tissue Engineering*", in "Engineering of Stem Cells", Springler Berlin/ Heidelberg, Vol 114, 2009

10.Lyngbaek, S. et al, *Cardiac regeneration by resident stem and progenitor cells in the adult heart*, Basic Res Cardiol, 102, 101-114, 2007.

11.Di Nardo, et al, *Cardiac progenitor cells: potency and control*, Journal of Cellular Physiology, 224, 590-600, 2010

12.Bergmann, O. et al, *Evidence for cardiomyocyte renewal in humans*, Science, 324, 98-104, 2009

13.Yamanaka, S., Blau, H., *Nuclear reprogramming to a pluripotent state by three approaches*, Nature, 465, 704-712, 2010

14.Pera, M., Tam, P., *Extrinsic regulation of pluripotent stem cell,* Nature, 465, 713-719, 2010

15.Beltrami, A., et al, *Adult cardiac stem cells are multipotent and support myocardial regeneration*, Cell, 114, 763-776, 2003

16.Zipori, D., *The Stem state: Plasticity is essential, whereas self-renewal and hierarchy are optional,* Stem cells, 23, 719-726, 2005

17.Leri, A., Kajstura, J. and Anversa, P., *Cardiac Stem Cells and Mechanisms of Myocardial Regeneration*, Physiol Rev,85, 1373-1416, 2005

18.Chambers I and Smith A. *Self-renewal of teratocarcinoma and embryonic stem cells*. Oncogene 23, 7150–7160, 2004.

19.Fenno, L. E., et al, *Human Embryonic Stem Cells: Emerging Technologies and Practical Applications*, Current Opinion in Genetics & Development, 18, 324-329, 2008

20.Minguell JJ, Erices A, and Conget P. *Mesenchymal stem cells*, ExpBiol Med, 226(6), 507-520, 2001

21.Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H,Noiseux N, Zhang L, Pratt RE, Ingwall JS, and DzauVJ.Paracrine, *Action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells*, Nat Med, 11, 367–368, 2005

22.Jackson, et al,*Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells*, J Clin Invest, 107(11), 1395-1402, 2001

23.Stastra, M., et al, *Cardiac Stem /Progenitor Cells, Secreted Proteins and Proteomics*, FEBS Letters, 583, 1800-1807, 2009

24.Fuchs E, Tumbar T, and Guasch G. *Socializing with the neighbors:stem cells and their niche,* Cell, 116, 769–778, 2004.

25.Perez-Moreno et al,*Sticky bussiness: orchestratingcellular signals at adherens junctions,* Cell, 112, 535-548, 2003

26.Spradling A, Drummond-Barbosa D, and Kai T. *Stem cells find their niche*, Nature 414, 98–104, 2001

27.Zhong, W., *Diversifying neural cells through order of birth and asymmetry of divison,* Neuron, 37, 11-14, 2003

28.Bekkers, S., et al, *Myocardial Regeneration via Intracardiac Stem Cells, in Rebuilding the Infarcted Heart*, Informa Healthcare, 6, 73-86, 2007

30.Anversa, P. et al, *Myocyte death in heart failure,* Current Opinion in Cardiology, 11, 245-251, 1996

31.Kumar, D. et al, *Embryonic stem cells: differentiation into cardiomyocytes and potential for heart repair and regeneration,* Coronary Heart Disease, 16, 111-116, 2005

32.Agah R, Kirshenbaum L.A., Abdellatif M., Truong L.D.,Chakraborty S., Michael L.H., and Schneider M.D., *Adenoviral delivery of E2F-1 directs cell cycle reentry and p53-independent apoptosis in postmitotic adult myocardium in vivo*, J Clin Invest, 100, 2722–2728, 1997.

33.Chien K.R. and Olson E.N.,*Converging pathways and principles in heart development and disease,* Cell, 110, 153–162, 2002.

34.McCay C.M., Pope F., Lunsford W., Sperling G., and SambhavapholP.,*Parabiosis between young and old rats,Gerontologia*1, 7–17, 1957.

35. Jiang X., Gurel O., Mendiaz E.A., et al, *Structure of the active core of human stem cell factor and analysis of binding to its receptor kit*, EMBO J, 19, 3192-203, 2000

36. Miles C., Sanchez M-J., Sinclair A., Dzierzak E., *Expression of the Ly-6E.1 (Sca-1) transgene in adult hematopoietic stem cells and the developing mouse embryo*, Development, 124, 537-47, 1997

37. Bunting K.D., Zhou S., Lu T., Sorrentino B.P., *Enforced P-glycoprotein pump function in murine bone marrow cells results in expansion of side population stem cells in vitro and repopulating cells in vivo,* Blood, 96, 902-909, 2000

38.Reyes, M. et al, *Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells,* Blood, 98:9, 2615- 2627, 2001

29.Singla, D.K., *et al*, *Stem cells in the infarcted heart*, Int J Cardiovasc Imaging, Springerlink.com, 2010

39.Stastna, M. et al, *Identification and functionality of proteomes secreted by rat cardiac stem cells and neonatal cardiomyocytes*, Proteomics, 10, 254-253, 2010

40.Wilkins, M. et al, *From proteins to proteomes: large scale protein identification by two dimensional electrophoresis and amino acid analysis*, Nature Biotechnology, 14, 61-65, 1996

41.Tyers, M., Mann, M., *From genomics to proteomics*, Nature, 422, 193-197, 2003

42.Bhattacharya, S., *Direct Genomic and Genetic Analyses in Disease Discoveries*, Proteomics Insights, 2, 23-26, 2009

43.Pruess, M. et al, *The proteome analysis database: a tool for the* in silico*analysis of whole proteomes*, Nucleic Acid Research, 31:1, 414-417, 2003

44.Pearson, H., *Geneticists play the numbers game in vain*, Nature, 423, 576, 2003

45.Lane, C.S., *Mass Spectrometry-based Proteomics in the Life Sciences*, Cellular and Molecular Life Sciences, 62: 848-869, 2005

46.Aebersold, R., Mann, M., *Mass spectrometry based proteomics*, Nature, 422, 198-207, 2003

47.Thompson, J.J., *Rays of Positive electricity and their application to chemical analysis*, Longmans Grenn , London, 1913

48.Aston, F. W., *Mass spectra and Isotopes*, Arnold, London, 1933

49.Dass, C., *Fundamentals of contemporary mass spectrometry*, Wiley, Chapter 1, 2007

50.J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, *Electrosprayionization for mass spectrometry of large biomolecules*, Science, 246, 64–71, (1989).

51.M. Karas and F. Hillenkamp, *Laser desorption ionization of proteins with molecularmasses exceeding 10,000 Daltons*, *Anal. Chem.,*60, 2299–2301, 1988

52.K. Tanaka, H.Waki, H. Ido, S. Akita, and T. Yoshida, *Protein and polymer analyses upto m/z 100,000 by laser ionization time-of-flight mass spectrometry*, Rapid Commun Mass Spectrom,2, 151–153, 1988

53.Han, X., Aslanian, A., Yates, J., *Mass spectrometry for proteomics*, Current opinion in Chemical Biology, 12, 483-490, 2008

54.Schuchardt, S., Sickmann, A., *Protein identification using mass spectrometry: A method overview*, Plant Systems Biology, 97, 141-170, 2007

55.Yates, R. J., et al, *Proteomics by Mass Spectrometry: Approaches, Advances and Applications*, Annual Reviews in Biomedical Engineering, 11, 49-79, 2009

56.Bogdanov, B., Smith, R., *Proteomics by FTICR MS: top down and bottom up*, Mass Spectrometry Reviews, 24, 168-200, 2005

57.Yates, R. J., *Mass Spectral Analysis in Proteomics*, Annual Reviews in Biomolecular Structure, 33, 297-316, 2004

58. Ott, H., et al, *Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart,* Nature Medicine Technical Reports, 2007

59.Mann, M., Kelleher, N., *Precision Proteomics: the case for high resolution and high mass accuracy,* PNAS, 105, 18132-18138, 2008

Chapter 2

Isolation of c-kit^{+} and Sca-1^{+} CSCs

Introduction

For the study of the proteome of cardiac stem cells it was necessary to obtain a reasonable amount of protein, and therefore, a high amount of cells. For experimental convenience we chose adult Balb C mice as animal model system, as this strain of albino mice was available at the facilities and has a long reproductive life-span[1], and are also noted for displaying high levels of anxiety and for being relatively resistant to diet-induced atherosclerosis, making them a useful model for cardiovascular research[2].c-kit and Sca-1 expressing CSCs were successfully isolated from this animal model. It is described that Sca-1 expressing CSCs are the most common in the adult mice heart, and our results agree with this statement[3].

Experimental methods to isolate these cells are still in development, as it a great challenge to obtain viable CSCs[4]. To isolate the desired cells with an acceptable purity it was necessary to sort them by Fluorescence Activated Cell Sorting (FACS), a laser-based technology used to measure characteristics of biological particles, a particularlyimportant techniquefor biological studies, as it allows qualitative and quantitative analysis of whole cells that have been labeled with an antibody conjugated with a fluorochrome (fluorescent probe)[5]. The sample used is a single cell suspension that flows past a laser beam in a single file flow[5]. Two different phenomena occur as the laser beam strikes the cell: light is scattered by cells, which is directly related to the cell structural and morphological properties, and fluorescence is emitted by the fluorochrome attached to the marker antibody[5]. Both of these phenomena are possible to measure, allowing the recollection of data and the sorting (isolation) of specific kinds of cells[5].

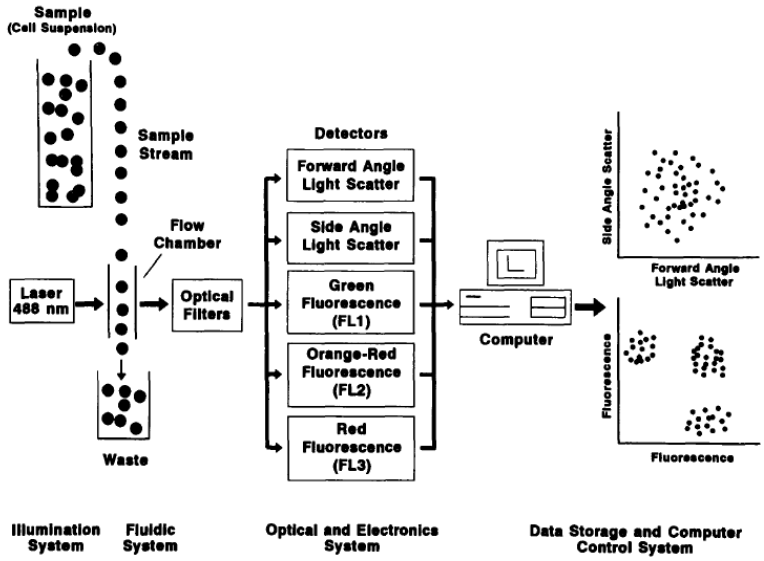

Figure 2.1- A scheme representing the main components of a flow cytometer. A single-cell suspension passes by a focused laser beam that is generated by the illumination system. The resulting scattered light and fluorescence are gathered by an optical and electronic system that translates the light signals into information which is saved in the data storage and computer control system. After this, information can be processed in the form of charts for analysis. Adapted from *Jaroszeki, Molecular Biotechnology, 1999*

Using FACS it is possible to isolate Sca-1 and c-kit expressing cells after marking the samples with a monoclonal antibody for c-kit or Sca-1 conjugated with a fluorochrome[6].The amount of Sca-1 expressing cells obtained for each mouse was much greater than of c-kit expressing cells, making these cells a good experimental model for further experiences.

Materials and Methods

Heart explant for isolation of c-kit$^+$ and Sca-1$^+$ Cardiac Stem Cells

Balb C mice were sacrificed by CO_2 asphyxiation, and an open thoracotomy was performed. The obtained hearts were injected with 1mL of Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) without Ca^{2+} or Mg^{2+}. Hearts were then removed and placed in a plate of HBSS for cleaning, on ice, to remove residual blood. Then, they were cut into approximately $1mm^3$ pieces, to which Collagenase A 1,1% (Roche) in HBSS and F12$^+$SCF medium (Kaighn's media (Sigma-Aldrich), supplemented by 10% Fetal Bovine Serum (FBS), 3% Penicillin/Streptomicin (Gibco), 1% Insulin Tranferrin Sodium Selenite(Sigma-Aldrich) and Stem Cell Factor) were added. The samples were then incubated at 37 ºC, for 25 minutes with agitation at 140 rpm, and filtered with a 40 μm nylon mesh into a tube, thus yielding a cell suspension. The pellet from the filtration was rinsed with HBSS and further filtered to the tube with the cell suspension. This was centrifuged at 1200 rpm for 10 minutes at 4 ºC, and the pellet was suspended with 1mL of FACS buffer (HBSS, 10% FBS)and kept on ice. Protocol adapted from *Smits,2009*[7].

Isolation of c-kit$^+$ and Sca-1$^+$ cells by Flow Cytometry with FACS

Cell suspension from the previous step was used in this procedure. Cells were counted using a Haemacytometer. Unstained sample was prepared with approximately $1x10^6$ cells. Samples of c-kit$^+$ cells were stained using c-kit PE (δ Mouse CD 117, clone 2B8 Rat IgG2b, BD #553355) with a dilution of 3/100, and Sca-1$^+$ cells were stained using Sca-1 FITC (δ Mouse LY-6A/E, clone E13-161.7 Rat IgG2a, BD #553335) with a dilution of 1/100 . Cells were then washed with FACS Buffer, centrifuged for 10 minutes at 1200 rpm, 4ºC, and suspended the pellet with FACS Buffer (cells are typically prepared as a suspension in buffered saline solution or in a liquid growth media. As the samples were prepared for further proteomics analyses, the buffer used could not have any proteins that would contaminate the proteomic results, and thereby the FACS Buffer was an appropriate choice).

Flow cytometry sorting of c-kit$^+$ and Sca-1$^+$ cells

Cardiac Stem Cells expressing c-kit and Sca-1 were isolated using a FACSAriabenchtop High Speed Cell Sorter produced by Becton Dickinson (BD). The analysis and sort gates were restricted to the stem cell gate excluding larger cells and dead cells, as determined by their characteristic forward and side scatter. The cells were sorted directly into Eppendorf tubes containing Phosphate Buffered Saline (PBS). The results were analyzed using Cell Quest software (BD Biosciences, san Diego, CA) aiming at obtaining 200 000 cells, and the obtained cells were stored at -80ºC.

Quantification of the amount of protein obtained

To quantify the amount of protein in each sorted sample, 10 µL of cell suspension were mixed with 500µL of Dye Reagent Concentrate (Coomassie® Brilliant Blue G-250, phosphoric acid and methanol, Bio Rad), and the absorbance of the mixture was read at 595nm, against a negative control containing PBS instead of cell suspension. The resulting absorbance was used to calculate the protein concentration of the solution using a calibration curve made with measurements of Bovine Serum Albumin (BSA) with the following equation: $y = 10{,}13x - 0{,}36$ in mg/mL.

Results

Quantification of protein per concentration of c-kit+ cells

Protein quantification was made using previously obtained samples of c-kit$^+$ CSCs, with approximately 50 000 cells and 500 µL. Calculations were made using a calibration curve for BSA, with the equation $y = 10{,}13x - 0{,}36$ (Table 2.1).

Table 2.1- Protein concentration of c-kit$^+$ cell samples were calculated using the equation $\underline{y = 10{,}13x - 0{,}36}$

c-kit$^+$ cells (units)	Absorbance 595nm	Concentration (mg/mL)	Volume (µL)	Protein (mg)
50000	0,012	0,0367	500	0,0184
545000	-	-	-	0,2001

The minimum of protein required to successfully analyze the proteome of these cells by mass spectrometry is 0,200mg for each sample. Assuming a linear relation between number of cells and protein amount, to achieve the total of 0,200 mg of protein it is necessary to have approximately 545 000 cells (Table 2.1). This amount of cells was used as target for the FACS experiences.

FACS profiles of c-kit+ cells

The typical fluorescence emission profile obtained in these experiences of mice c-kit$^+$ CSCs marked with an antibody anti-c-kit PE, a fluorochrome that emits fluorescence in the orange-red wavelength, is shown in Figure 2.1.The arrows indicate the electronic gates used to determine the stem cell fraction. The cells that emit fluorescence in the left side of the plot are mainly erythrocytes. Unfortunately, it is easily observed that the c-kit$^+$ population represents only a small fraction of the total cell sample.

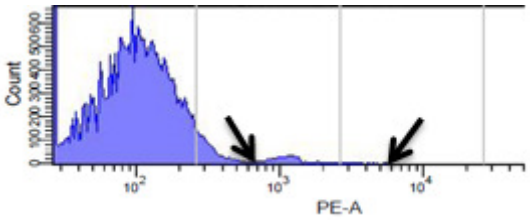

Figure 2. 2-- Count of mice c-kit$^+$ CSCs versus PE emission profile. Arrows indicate the gates used to sort these cells

To select viable cells it was necessary to obtain the graphics of Forward and Side Scattered Light. These properties come from the fact that when cells cross the laser beam inside the Optical System of the FACS (Figure 2.2), they scatter light.

Light can be scattered in the same direction as the laser, Forward angle Light Scatter (FSC), and this parameter provides basic morphological information such as cell size[5,8]. Light scattered at 90º relatively to the incident beam is collected as the Side angle Light Scatter (SSC), and is an indicator of cytoplasm granularity and surface/membrane irregularities or topographies[5,8]. The relation between these two properties allows the selection of cellular populations in heterogeneous populations, and also allows the selection of viable cells and exclusion of death cells and cell debris[8].

In Figure 2.2 a typical SSC-A (amplitude) versus FSC-A (amplitude) for c-kit$^+$ cells is represented. The selected gate was chosen by the operator, and selects viable cells leaving out cell debris and dead cells.

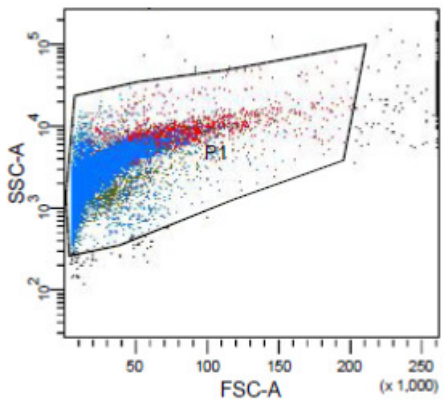

Figure 2.3 – SSC-A versus FSC-A plot. Selected gate represents viable cells. Spots outside the gate are considered cell debris or dead cells. Each dot represents a cell, blue dots are the ones chosen by the drawn gate, and green dots are cells that emit fluorescence in the PE wavelenght.

After the selection of the first gate (represented as P1 in Figure 2.3), a second one is drawn using the properties of amplitude and width of FSC, in order to correctly select the small cells that include the stem cell population (Figure 2.4).

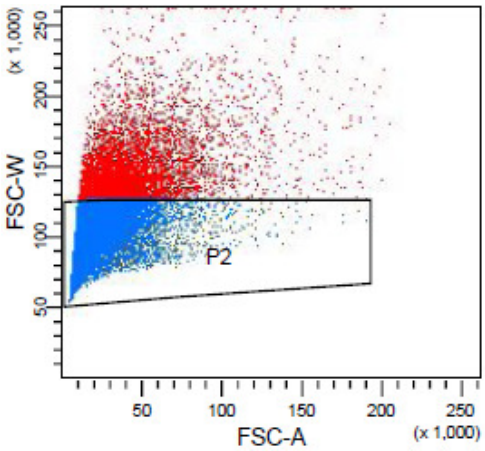

Figure 2.4-FSC-Width versus FSC-Amplitude. Selected gate represents viable small cells. Each dot represents a cell, blue dots are the ones chosen by the drawn gate, and green dots are cells that emit fluorescence in the PE wavelenght.

Gate P2 is a smaller gate that limits the total of events selected and allows a better sorting of viable cells. The cells represented in Figure 2.4 are already a selected population chosen in the P1 gate of Figure2.3. From P2, cells that emit in the characteristic wavelength of the fluorochrome PE, orange-red, are selected (Figure 2.5).

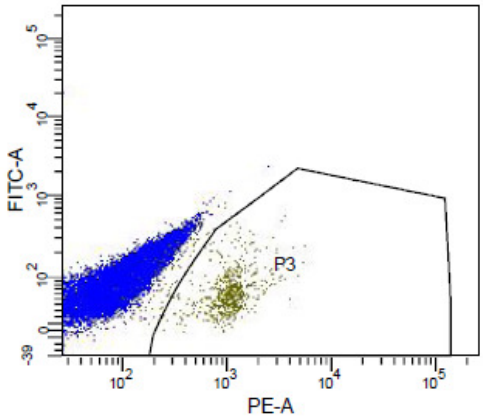

Figure 2.5- Fluorescence emission in the PE area (Orange-red wavelength) and FITC area (green wavelength). Each dot represents a cell, dots in blue have no fluorescence associated with PE, and dots in green are cells bound with an anti-c-kit$^+$ PE antibody.

P3 represents the gate that contains c-kit$^+$ cells, chosen from the selected population of P2. This guarantees that the chosen cells are viable cells. The plot depicts the emission of fluorescence in the PE area and FTIC (another fluorochrome,which emits in the green wavelength area). As the samples were not marked with any FITC-bound antibody, this allows the exclusion of cells with no fluorescence (the ones in the bottom area on the left side with a FITC emission distributed around zero) and cells with autofluorescence, which spread along a imaginary diagonal axis (x=y) of the chart[9].

The distribution of the non-fluorescent population around zero is due to the device calibration[9]. When there are no cells present the FACS instrument obtains the background noise and subtracts it with statistical variations to the signal recorded from each cell, which may cause some cells that have close to zero fluorescence to be a little above or a little bellow the zero value[9].

After several experiments in order to obtain three replicates of c-kit$^+$ cells, these were successfully isolated and used for the proteomic studies. Results of the isolation experiments are summarized in Table 2.2.

Table 2.1 –c-kit$^+$ cells isolated by FACS using an antibody anti-c-kit$^+$ PE. Each event is a sorted cell.

Replicate	BalbCMice	Gender	Total Events (Date)	Total Events (Replicate)
1st replicate	8	Male	695000	695000
	8	Female		
2nd replicate	8	Male	67906	197967
	8	Female		
	10	Female	130061	
3rd replicate	10	Female	16720	241840
	10	Female	51120	
	10	Female	115000	
	10	Female	59000	
	82		1134807	1134807

For the first replicate isolation, the results were very promising. For the second replicate, however the number of sorted cells was much smaller than the expected and this trend was maintained along the isolation of cells for the third replicate. The number of cells isolated for each replicate was smaller than the expected, due to limitations in the number of mice available for the isolations. This affected further experiences, as will be described in Chapters 2 and 3.

FACS profiles of Sca-1$^+$ cells

The typical fluorescence emission profile of mice Sca-1$^+$ CSCs marked with an antibody anti-Sca-1 conjugated to FITC, a fluorochrome that emits fluorescence in the green wavelength, is shown in Figure 5. The Sca-1$^+$ population is not as well defined as the c-kit$^+$ population (Figure 2.2). However, it is possible to identify it by comparing the emission of fluorescence of the FTIC fluorochrome (Figure 2.6a) with the auto fluorescence (Figure 2.5b), measured at another wavelength (orange-red).

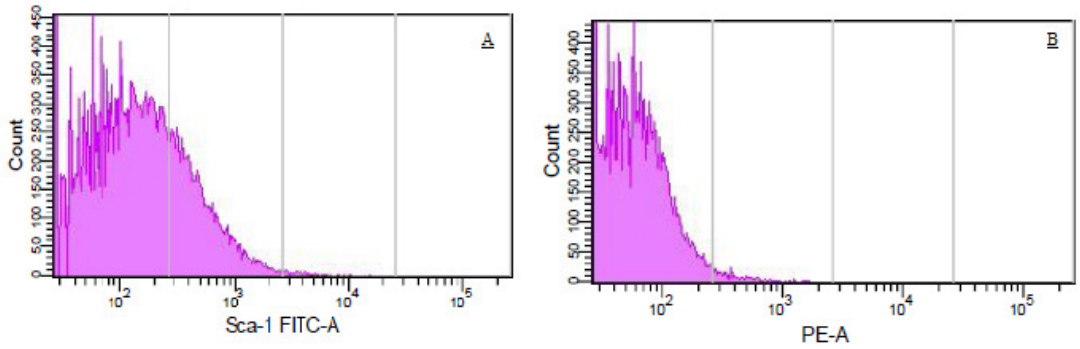

Figure 2.6-A)Count of mice Sca-1^{+} cells versus FITC emission profile. B- Auto-fluorescence emission from Sca-1^{-} cells. Sca-1 expressing population can be located by subtracting the auto-fluorescence represented in B to the total fluorescence of A.

To select viable cells it was necessary to obtain the graphics of Forward and Side Scattered Light, as previously explained. In Figure 2.7 a typical SSC-A (amplitude) versus FSC-A (amplitude) for Sca-1^{+} cells is represented. The drawn gate was chosen to select viable cells leaving out cell debris and dead cells.

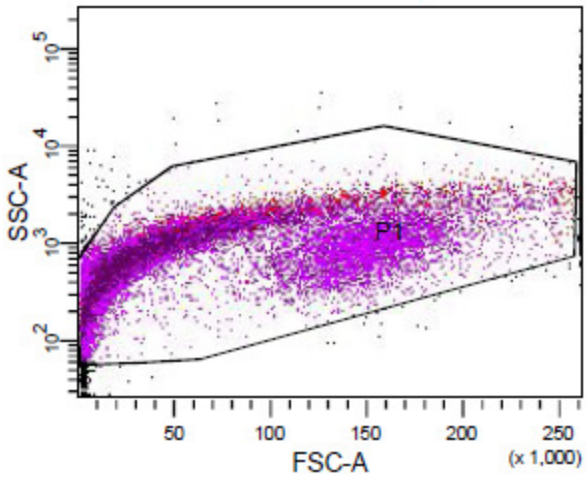

Figure 2.7-SSC-A versus FSC-A plot. Selected gate represents viable cells. Spots outside the gate are considered cell debris or dead cells. Each dot represents a cell, purple and red dots are the ones chosen by the gate, and dark purple dots are cells that emit fluorescence in the FITCwavelength.

After the selection of the first gate, a second one is drawn using the properties of amplitude and width of FSC, in order to correctly select the small cells that include the stem cell population (Figure 2.8)

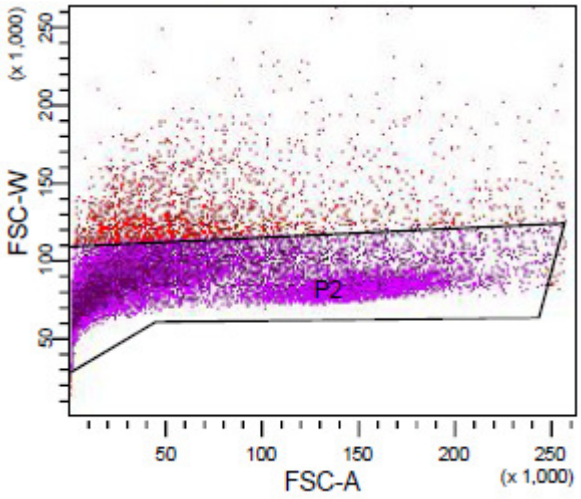

Figure 2.8-FSC-Width versus FSC-Amplitude. Selected gate represents viable small cells. Each dot represents a cell, purple and dark purple dots are the ones chosen by the drawn gate, P2.

Gate P2 is the smaller gate that limits the total of events selected and allows a better sorting of viable cells, as explained before. From P2, cells that emit in the characteristic wavelength of the fluorochromeFITC, green, are selected (Figure 2.9).

P3 represents the gate that contains viable Sca-1^{+} cells, chosen from the selected population of P2. The plot depicts the emission of fluorescence in the PE area and FTIC. As the samples were not marked with any PE-bound antibody, this allows the exclusion of cells with no fluorescence and cells with autofluorescence.

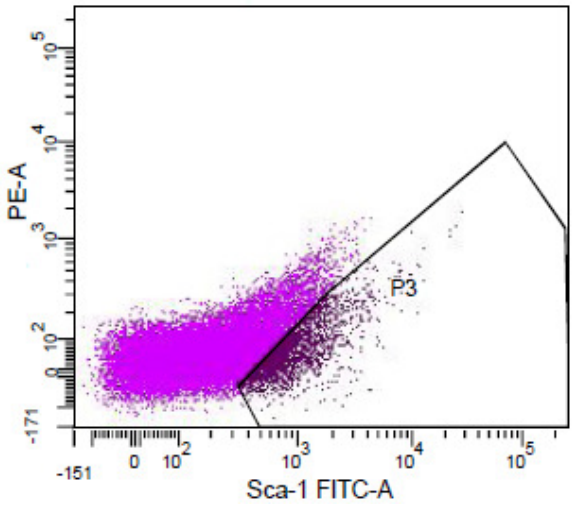

Figure 2.9- Fluorescence emission in the FITC area (Green wavelength) and PE area (Orange-red wavelength). Each dot represents a cell, dots in purple have no fluorescence associated with FTIC, and dots in dark purple are cells bound with an anti-Sca-1 FITC antibody.

After several experiments in order to obtain three replicates of Sca-1$^+$ cells, these cells were successfully isolated and used for the proteomic studies.The results of the isolation experiments are summarized in Table 2.3.

Table 2.2- Sca-1$^+$ cells isolated by FACS using an antibody anti-Sca-1$^+$ FITC. Each event is a sorted cell.

Replicate	BalbC Mice	Gender	Total Events (Date)	Total Events (Replicate)
1st replicate	5	Female	241059	241059
2nd replicate	5	Female	277493	277493
3rd replicate	10	Female	159104	259104
	10	Female	100000	
	30			777656

Sca-1$^+$ CSCs were available in greater amounts than c-kit$^+$ CSCs[1]. The number of sacrificed mice was greatly reduced to obtain a larger amount of cells for the Sca-1$^+$samples. This was already verified by other authors, since Sca-1$^+$ CSCs are the predominant stem cell population in the mice heart. According to these observations, Sca-1$^+$ cells are 100 to 700-fold more frequent than c-kit$^+$ cells. The amount of sorted cells contributed to the success of the further studies made on this cell type[3].

Discussion

The use of antibodies against the stem-cell surface antigens Sca-1 and c-kit made it possible to isolate CSCs expressing these antigens from the cardiac original population. Sca-1 expressing CSCs were easily isolated and sorted in high amounts. However, c-kit expressing CSCs were much more difficult to isolate from much greater numbers of subjects (82 mice) than the ones used to isolate Sca-1 expressing CSCs (30 mice).

Although the actual final number of c-kit$^+$ CSCs was superior to number of obtained Sca-1$^+$ CSCs, this was due only to a very successful first isolation of c-kit$^+$ CSCs. The reason for this better result was that this experience was made by 3 operators, a condition that we were never able to repeat due to lack of availability of the operators. It remains a challenge to isolate and study these cells, and in the future optimization of the presently used methods is required.

References

1. *http://www.informatics.jax.org/external/festing/mouse/docs/BALB.shtml*, consulted at October 2010

2.*http://jaxmice.jax.org/strain/000651.html*, consulted at October 2010

3. Beltrami, A., Anversa P., *et al*, *Adult cardiac stem cells are multipotent and support myocardial regeneration*, Cell,114, 763-776, 2003

4. Messina, E., Giacomello, A., *et al*, *Isolation and expansion of adult cardiac stem cells from human and murine heart*, Circulation Research, 95, 911-921, 2004

5.Jaroszeski, M., Radcliff, G., *et al*, *Fundamentals of Flow Cytometry, Molecular Biotechnology*, 11, 37- 53, 1999

6. Oh, H., Schneider, M., *et al*, *Cardiac progenitor cells from adult myocardium: homing, differentiation and fusion after infarction*, PNAS, 100, 12313-12318, 2003

7. Smits, A. M., *et al*, *Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physolagy and pathophysiology*, Nature Protocols, Vol.4 No.2, 232-243, 2009.

8. Shapiro, H., *Practical Flow Cytometry*, 4th edition, by Jonh Wiley &Sons, 2003

9. Herzenberg, L., Parks, D., et al, Interpreting Flow Cytometry Data: a Guide to the Perplexed, Nature Immunology, 7, 681-686, 2006

Chapter 3

Cell fractioning of Sca-1^{+}and c-kit^{+} CSCs

Introduction

The present work aims at identifying the proteome of Cardiac Stem Cells. In order to achieve this goal, two different approaches were used: cell fractioning of the Sca-1^{+} and c-kit^{+} CSCs for the proteome analysis and whole cell samples of c-kit^{+}.

The identification of membrane proteins can provide valuable information into the biological principles of pluripotency and other biological processes of CSCs. Membranes have a fundamental role in the protection from harmful environment fluctuations, regulate and mediate the transport of ions, propagate signaling cascades and mediate the communication between cells and compartments [1]. The comparison of these proteomes will contribute to the comprehension of their differences and roles in the heart physiology, and also allows the identification of biomarkers to exploit these cells for therapeutic purposes [2].

The study of the nuclei proteome is also a very relevant area, as the transcription factors involved in the processes of differentiation are located in this compartment, and the protein families such as transcription factors and chromatin remodeling enzymes are crucial to the maintenance of a stem state or to ignite the differentiation routes[3]. It is essential to know which transcription factors are present in CSCs in order to optimize conditions for their culture *in vitro* and for potential therapeutic purposes.

Finally, the study of the whole cell proteome is a very challenging task, since this consists in a few thousands of different proteins at each instant. However, the information obtained by this method is valuable as it allows a general overview of the protein expression, and also indicates which proteins are more abundant in the cell[4]. This approach was used in order to study c-kit^{+} cells, which are most difficult to isolate and still represent a challenge in obtaining fractions from the original samples.

Methods

Protein extraction from c-kit⁺ CSCs, whole cell approach

Three different methods were accessed in order to choose the most successful one, based on *Baharvand, BBRC, 2206*[5].

Method A- Samples were treated with 0.07% DTT in acetone for 1 hour at -20ºC. The samples were centrifuged at 14000 x g for 10 minutes at 4ºC (Eppendorf Centrifuge 5804R). The supernatant was collected, and after the acetone evaporated, the proteins were separated by SDS-PAGE.

Method B- Samples were centrifuged at 14000 x g for 90 minutes at 4ºC (Eppendorf Centrifuge 5804R). The pellet and the supernatant were separated, incubated overnight with 0.07% DTT in acetone and centrifuged again at 14000 x g for 10 minutes at 4 ºC. The resulting supernatants were collected in eppendorfs, and after the acetone evaporated, the proteins were separated by SDS-PAGE.

Method C- 8 eppendorfs of sample were incubated with 0.07% DTT in acetone for 2 hours at 4 ºC, and centrifuged at 14000 x g for 25 minutes at 4ºC (Eppendorf Centrifuge 5804R). The pellet from the first eppendorf was then diluted in Sample Buffer (water, 0.5M Tris-HCl, Glicerol, 10% SDS, β-Mercaptoetanol, 0.5% v/v Bromofenol Blue in water), agitated for 15 minutes and added to the second pellet, and successively until all the pellets were in the same volume of Sample Buffer.The resulting pellets were separated by SDS-PAGE.

Preparation of the membrane enriched fraction

Cell fractioning was made according to *Dormeyer, 2008*[6]. Briefly, cells were harvested by centrifugation at 14000 x g for 30 minutes at 4ºC, and ressuspended in Lysis Buffer (50 mMTris-HCl pH 7,8, 250 mM sucrose and 2mM EDTA), supplemented with 1:100 protease inhibitor cocktail (Roche Diagnostics, Switzerland), and incubated on ice for 10 minutes. Cells were then lysed by 30 passes through a $30^1/_2$ gauge needle. Cell debris, unbroken nuclei, ER membranes and mitochondrial membranes were removed by centrifugation at 1000 x g for 10 minutes at 4ºC. The resulting pellet is stored for nuclei isolation (Pellet A).

The supernatant is layered onto a 60% sucrose cushion and centrifuged at 20817 x g for 20 hours at 4ºC. The membrane-enriched fraction on top of the sucrose cushion is collected and diluted 1:2 with cold 50mM Tris-HCl pH 7.8, and pelleted at 20000 x g for 2 hours at 4ºC. The supernatant is discarded and the membrane pellet is washed for 1 hour at 4ºC with 100µL of 100mM Na_2CO_3 pH 11.5, and centrifuged at 20000 x g for 30 minutes at 4ºC. The obtained pellet is the membrane enriched fraction.

Preparation of the nuclei enriched fraction

Pellet A obtained at the previous method is suspended in 200 µL of B buffer [(20% Glycerol, 1,5mM $MgCl_2$, 10mM KCl, 20mM Tris-HCl pH 7.9, in water) supplemented by 4µL of 100mM PMSF and 4,15µL DTT 0,72M], and 2/3 of C buffer [(20% Glycerol, 1.5mM $MgCl_2$, 1.2M KCl, 20mM Tris-HCl pH 7.9, in water) supplemented by 10 µL PIC 100x, 4 µL PMSF 100mM and 4,15 µL DTT 0.72M]. The sample is then rolled at 4ºC for 45 minutes and pelleted at 20000 x g for 45 minutes at 4ºC. The supernatant is collected and contains nuclear proteins.

Separation of proteins by SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Samples were diluted in 5 times in Sample Buffer (water, 0.5M Tris-HCl, Glicerol, 10% SDS, β-Mercaptoetanol, 0.5% v/v Bromofenol Blue in water), heated at 100ºC for 5 minutes, and applied in a Acrylamide vertical slab 1,5 mm (12% Acrylamide for the resolution gel, and 4% acrylamide in the concentration gel) in a Mini Protean (Bio Rad) filled with Running Buffer (25mM Tris-HCl, 192mM Glycine, 0.1% SDS, pH 8.3). Electrophoresis was carried out at 100V, 200mA and 100w, until the Bromofenol blue from the sample buffer exited the gel. Slabs were then stained either with Coomassie Brilliant Blue R-250 or by Silver Staining.

Silver Staining

Slabs were stained using the *Kit PlusOne Silver Staining, Protein* (GE Health Care). Briefly, slabs are incubated in a fixation solution overnight (30% ethanol, 10% glacial acetic acid), and the incubated with a sensitizing solution (30% ethanol, 4% sodium thiosulphate, 6,8%sodium acetate, 0,5% glutardialdehyd) for 1 hour and 30 minutes.

The slab is washed 2x15minutes in water, incubated with silver solution (2.5% silver nitrate solution) for 1 hour and 30 minutes, and then washed 2x 1minutes in water. The slab is then revealed with developing solution (0.025g.mL^{-1} sodium carbonate, 0.08% formaldehyde) until the dark stains appear in the protein locations, and the reaction is stopped by incubating with stopping solution (5% acetic acid) overnight. The slab can be stored after the staining in water at 4 ºC.

Western Blotting

Membrane Transference

After the protein separation is achieved by SDS-PAGE and without staining, the slab is layered onto a PUDF membrane previously activated in methanol and 15 minutes in Transfer Buffer (5.8mg.mL^{-1}Tris-HCl, 2.9 mg.mL^{-1} Glycine, 37.5 mg.mL^{-1} SDS, 20% methanol), standing in 3MM paper. The slab is then covered with 3MM paper, and pressed with a roll to undo any bubbles. The sandwich is applied in the membrane transference support with ice and the transference is run at 100V for 65 minutes. The membrane is then stained with Ponceau S to monitor sample transference, and stored overnight in blocking solution (5% skimmed milk in TBS (10mM Tris-HCl, 150mM NaCl, pH7.5)) at 4ºC.

Incubation with the antibodies

The membrane is incubated with a solution of 1:1000 primary antibody (in 1% skimmed milk in TBS-T (TBS with 0,1% Tween 20) overnight, after discarding the blocking solution. The membrane is washed 3 x 10 minutes with TBS, incubated with the secondary antibody solution (1:20000 in 5% skimmed milk in TBS-T) for 3 hours and washed with TBS 4x15 minutes.

Revealing the Slab

Revelation of the Western Blot carried out using the Roche revelation kit in a dark chamber. Briefly, the Detection solution is made according to Roche instructions (100 Solution A: 1 Solution B). The membrane is placed in this solution for 1 minute. The film is placed on top of the membrane inside the revealing cassette, and stays in the closed cassette enough time for the bands to print on it. The film pellicle is then placed

in the revealing solution (Roche) for 3-5minutes and in the fixing solution (Roche) for 5 minutes. Immune-reactivity was detected with Diaminobenzidine (DAB) as a chromomeric substrate, following the manufacturer instructions.

Antibodies

The following antibodies were used for the Western Blot: anti Sca-1 (δ Mouse LY-6A/E, clone E13-161.7 Rat IgG2a, BD #553335), rabbit anti-phospho-histone H3 (Upstate Biotechnology). Secondary antibodies used were anti-mouse and anti-rabbit IgG (at 1:5000 dilution)

Results

Fractioning of Sca-1^{+}and c-kit^{+}CSCs

Sca-1^{+} CSCs isolated in the previous steps were fractioned according to the procedure described in methods, *Preparation of the membrane enriched fraction*. The collected membrane fractions were analyzed by Western Blot using a primary antibody anti-Sca-1 as the membrane fraction marker (1:5000) and another primary antibody anti-phosfohistone3 (1:5000) as a marker for nuclei fraction. Secondary antibodies are described in the Methods *Antibodies* section. As it is visible in Figure 3.1, there is a clear enrichment of Sca-1 in the Membrane fraction.

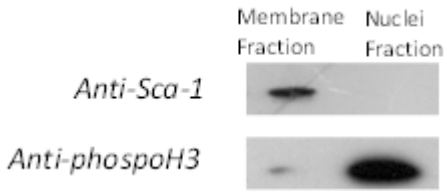

Figure 3. 1-Western Blot analysis of the membrane and nuclei fractions obtained for the Sca-1^{+} CSCs samples. The membrane fraction is enriched in Sca-1, and has only traces of the nuclei marker, phosphohistone-3

The same method was applied to the c-kit^{+} CSCs isolated in the previous step. The collected membrane fractions were analyzed by Western Blot using a primary antibody anti-c-kit (1:5000) as the membrane fraction marker and another primary antibody anti-phosfohistone-3 (1:5000) as a marker for nuclei fraction. Secondary antibodies are described in the Methods *Antibodies* section. The results seem to indicate an even greater enrichment of the membrane fraction

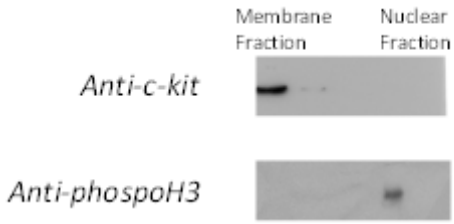

Figure 3. 2-Western Blot analysis of the membrane and nuclei fractions obtained for the c-kit^{+} CSCs samples. The membrane fraction is the only one to present c-kit, and apparently has no traces of the nuclei marker, phosphohistone-3.

c-kit+ CSC whole cell samples

Samples of c-kit+ CSCs isolated before the start of this work by Alex Matsuada (IGC) using the methods described in Chapter 2 (Table 3.1) were submitted to the protein extraction methods previously referred(*Protein extraction from c-kit+ CSCs, whole cell approach*).

Table 3.1- Cell sorting of c-kit+ CSCs for whole cell proteomic analysis. Methods A, B and C are described in the methods section Protein extraction from c-kit+ CSCs, whole cell approach

Replicate	BalbCMice	Gender	Total Events (Date)	Total Events (Replicate)
Method A	8	Male	50000	50000
Method B	8	Male	50000	117906
	2	Male	67906	
	18	Female		
Method C	5	Female	25000	85727
	4	Female	60727	
	45			253633

Extracts were the separated by SDS-PAGE, silver stained for more sensitivity and the efficacy of each method was evaluated.

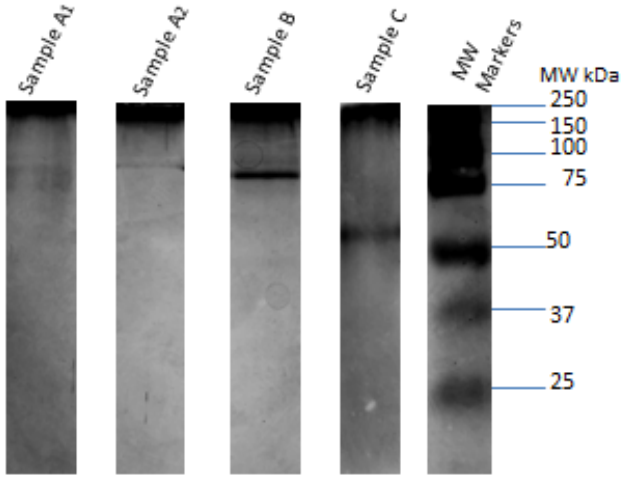

Figure 3.3-SDS-PAGE of samples A, B and C silver stained. Sample A1- half of sample A; Sample A2 - half of sample A; MW Markers- Bio Rad Precision Plus Proteins Standards all Blue

Method B seems to be best for protein visualization by SDS-PAGE. This is also due to the fact that the sample used has more cells, leading to a higher concentration of protein. This indicates that a high amount of cells is necessary to have good separation by SDS-PAGE and further proteomics analysis, as suggested in Chapter 2.

Cell fractioning was successful, as there is clear enrichment of the membrane and nuclei fractions in their respective markers (c-kit and Sca-1, phosphohistone H3, respectively). Better results could be achieved with higher rates of centrifugation during the sucrose cushion centrifugation, using an ultracentrifuge. However, due to lack of availability, the use of lower centrifugation speeds is supported by *Intoh, A. et al, Proteomics, 2009*[7], with very acceptable results.

The whole cell approach for c-kit$^+$ CSCs samples was successful. In the future, greater amounts of cells are necessary to achieve a good SDS-PAGE separation of proteins in the mixture.

References

1.Pettey, H., *Molecular biology of membranes: structure and function*, Springer Verlag, Plenum Press, New York, 2007

2.Rabilloud, T., *Membrane proteins ride shotgun*, Nature Biotechnology, 21, 508-510, 2003

3. Williamson, A., *et al*, *Quantitative proteomics analysis demonstrates post-transccriptional regulation of embryonic stem cell differentiation to hematopoiesis*, Molecular & Cellular Proteomics, 7.3, 459-473, 2008

4. Cox, J., Mann, M., *Is proteomics the new genomics?* Cell, 130, 395-399, 2007

5.Baharvand, H., Salekdeh, G., *et al*, *Comparative proteomic analysis of mouse embryonic stem cells and neonatal- derived cardiomyocytes*, BBRC, 349, 1041-1049, 2006

6.Dormeyer, W., Heck, A., *et al*, *A practical guide for the identification of membrane and plasma membrane proteins in human embryonic stem cells and human embryonal carcinoma cells*, Proteomics, 8, 4036-4053, 2008

7.Intoh, A., Asashima, M., *et al*, *Proteomic analysis of membrane proteins expressed specifically in pluripotent murine embryonic stem cells, Proteomics*, 9, 126-137, 2009

Chapter 4

Mass Spectrometry characterization of the c-kit^{+} and Sca-1^{+} CSCs proteome

Introduction

The protein expression of CSCs is largely unknown, due to the very recent discovery of these cells and consequent study of their properties[1]. The potential therapeutic application of these cells depends on the understanding of their metabolic conditions and gene expression[2]. There is also growing evidence that proteins secreted from cardiac stem cells into the vicinity of an injury in an infracted heart can modulate the microenvironment and play an important role in functional improvement[3]. Knowing the proteome of these cells will allow the access to information about the mechanism driving stem cells engraftment and their differentiation process[2,3]. Using the MALDI – FTICR and a precision proteomics approach, it is possible to obtain a good amount of accurate identifications and assemble the proteome of CSCs[21]. In the present study I used this technique to further characterize the proteome of mouse c-kit$^+$ and Sca-1$^+$ CSCs.

Proteins secreted by cells are very important, as their secretions are specific for each cell type, and reflect the local environment of the cells[3]. Paracrine and autocrine factors, mediated by either endogenous or transplanted CSCs, contribute to regeneration and repair processes[4]. These paracrine or autocrine factors are proteins secreted by cells which can induce changes in the neighboring cells or in the same cell. These factors usually belong to these protein families: Fibroblasts growth factors, Hedgehog proteins, Wnt proteins, and Transforming Growth Factor (TGF-β) superfamily[3,5]. There are also neurotrophins, cytokines, interleukins and various other factors that play an important role in these regulation processes.

The unraveling of the proteome of cardiac stem cells will lead to a better understanding of the pathologic alterations of heart function, and contribute to the future use of CSCs in therapy.

Methods

Samples

Protein samples for analysis were obtained as described in Chapter 2 and 3.

c-kit$^+$whole cell samples from the slab represented in Figure 3.3 were sliced from the gel with a scalpel in 1 mm^3 pieces, obtaining approximately 25 bands from each column (sample A1, sample A2, sample B and Sample C). c-kit$^+$ CSCs membrane fraction samples obtained as described in Chapter 2, Methods, *Preparation of the membrane enriched fraction.* The first replicate (see Table 2.2) was submitted to protein separation by SDS-PAGE and sliced from the gel with a scalpel inaproximately 1 mm^3 pieces, obtaining 54 bands for in-gel digestion.

Sca-1$^+$ membrane and nuclei fractions were obtained as described in Chapter 2, Methods, *Preparation of the membrane enriched fraction* and *Preparation of the nuclei enriched fraction*. Membrane fraction samples were separated by SDS-PAGE and bands were sliced from the gel, obtaining 34 bands for the first replicate, 36 for the second replicate and 37 for the third replicate. Nuclei fraction samples submitted to the same procedure, obtaining 55 bands for the first replicate, 46 bands for the second replicate and 45 samples for the third replicate.

In gel digestion

In gel digestion was performed as in *Mann, 2001*[6]. Briefly, gel bands were placed in eppendorfs and washed with water MilliQ and Acetonitrile (ACN for LC-MS, JMGS) to remove previous stain. Cys residues were reduced with 10mM Dithiothreitol in 100mM NH_4HCO_3, and alkylated (to prevent re-formation of disulfite bridges) with 55mM of Iodoacetamide (Sigma) in 100mM NH_4HCO_3. Gel pieces were then dried by centrifugation under vacuum (speedvac). Finally, proteins were digested with 6.7ng/μL for 30 minutes, incubated overnight (16-18 hours) with NH_4HCO_3 buffer at 37º. The digest was recovered to new tubes and stored at -20ºC.

Desalting and Application of Samples

Samples were desalted and concentrated as described in *Larsen, 2004*[7], using Zipt-Tip C18 microcolumns (Millipore), and were eluted directly into the MALDI plate (AnchorChip, BruckerDaltoniks) with 10µg/µL matrix solution CHCA in 50% ACN in 0.1% Trifluoracetic acid (TFA), according to the manufacturer's instructions.

Peptide Mass Fingerprint by MALDI-FTICR

Digests were analyzed by MALDI-FTICR in a Bruker Apex Ultra, Apollo II combisource, with a 7 Tesla magnet. Mono-isotopic peptide masses were determined using the SNAP algorithm in Data Analysis software version 4.0 (Brukerdaltonics). External calibration was performed by using BSA and trypsin digest spectra, processed and analyzed with Biotools software (BrukerDaltonics).

Peakerazor software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant m/z peaks.

Database analysis of the obtained spectra

The obtained mono-isotopic peptide masses were used to search for protein identification by using the Mascot software (Matrix Science, London, UK; http://www.matrixscience.com). Data was submitted and analyzed with Biotools 3.0 (BrukerDaltonics). Database searches were performed against MSDB (a non-identical protein sequence database maintained by the Proteomics Department at the Hammersmith Campus of Imperial College, London; http://csc-fserve. hh.med.ic.ac.uk/msdb.html) and SwissProt databases.

The following criteria were used to perform the search: (1) mass accuracy of 50 ppm; (2) one missed cleavage in peptide masses; and (3) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. Criteria used for protein identification in the Mascot software were (1) significant homology scores achieved in Mascot; (2) significant sequence coverage values; (3) at least 4 peptides identified and (4) similarity between the protein molecular mass calculated from the gel and for the identified protein.

Results

c-kit+ CSCs whole cell proteome characterization by MALDI-FTICR

For the analysis of the c-kit$^+$ CSCs whole cell proteome, the Peptide Mass Fingerprint (PMF) approach was used to identify the expressed proteins, previously separated by SDS-PAGE (Chapter 3, Figure 3.3). MALDI ionization was used, after the digestion of samples with trypsin and micro-purification and concentration of protein samples made with home-made columns. PMF spectra were obtained by FT-ICR, achieving a high mass accuracy. An example of a typical mass spectrum for this analysis is described in Figure 4.1.

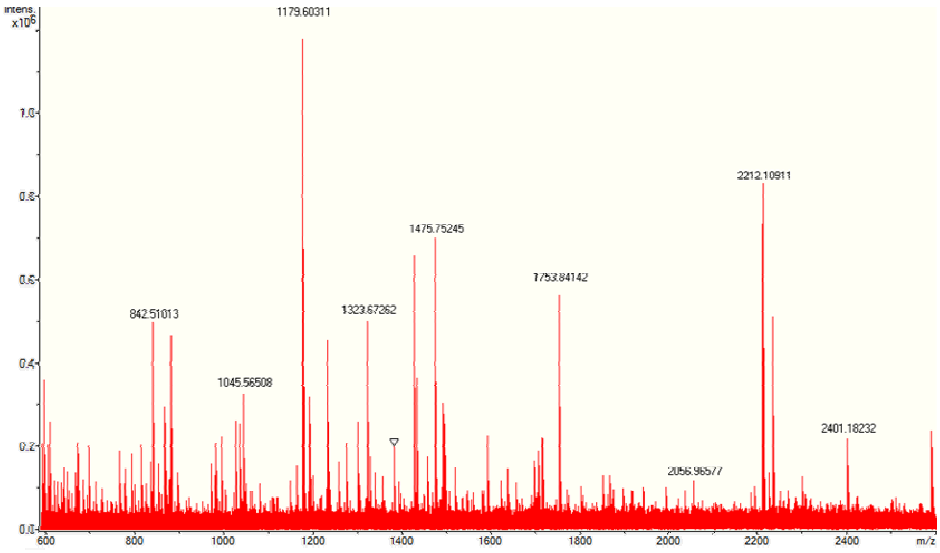

Figure 4.1 Example of a mass spectrumacquisition, the Alpha-Enolase mass spectra. Values of m/z are used for database identification of the protein (see Methods)

Samples were used in triplicate to assure reproducibility of the results (see Methods, *Samples*). Identifications are described in Table 4.1 .

Table 4.1- Proteins identified from the c-kit$^+$ CSCs whole cell sample by MALDI-FTICR. % cov is the percentage of protein sequence covered by the peptides used to identify the protein. Proteins characteristics such as cellular location and function were retrieved from their file at http://www.uniprot.org/.

Accession Number	Identified protein	Function	Compartment	Score	% cov
P27005	Calgranulin A/ Protein S100-A8	Cellular Immune Reactions, Chemotaxi	Cytoplasm	125	36
37194891	Cofilin 1	Actin polimerization	Nucleus	98	32
P26638	Seryl-tRNASynthetase, cytoplasmic	Anabolism: Aminoacyl-tRNA biosynthesis	Mitochondrion	74	24
Q8K0I5	3-Hydroxy-3-Methylglutaryl-Coenzyme A synthase 1	Anabolism: Isoprenoid biosynthetic process	Cytoplasm	89	16
Q9CQL9	AdenylosuccinateSynthetaseisozyme 2	Anabolism: Purine nucleotide biosynthesis.	Cytoplasm	67	17
Q544K9	Uridine Monophosphate Synthetase	Anabolism: Pyrimidine biosynthetic pathway	Cytoplasm	98	23
Q8CI65	ATP synthase β subunit, mitochondrial	Anabolism: ATP synthesis coupled proton transfer	Mitochondrion	87	24
13277819	Stress-induced phosphoprotein 1	Chaperone Binding Proliferation	Nucleus	89	29
Q99LL6	Col1a1 protein	Extracellular matrix structural constituent	Cytoplasm	85	18
P29966	Myristoylated Alanine-rich C-kinase substrate	Chemotaxis, Cell Signaling	Membrane	54	27
535924	LactateDehydrogenase-A (LDH-A)	Glycolitic pathway	Cytoplasm	65	29
80477474	Phosphoglycerate Kinase 1	Glycolitic pathway	Cytoplasm	78	31
P17182	Alpha-enolase	Glycolitic pathway, Proliferation	Membrane	69	12
Q9CPV4	Glyoxalase domain-containing protein 4	Cell Growth	Mitochondrion	78	11
Q9Z1D1	Eukaryotic Translation Initiation Factor 3 subunit G	Protein Translation	Cytoplasm	87	23
O75955	Flotillin-1	Structural component	Membrane	84	14
P18242	Cathepsin D	Lysosome; involved in apoptosis	Lysosome	69	18

P08732	Cytochrome b561	Membrane transport	Membrane	74	15
Q60715	Prolyl 4-Hydroxylase α-1	Metabolism: Arginine and proline metabolism	Endoplasmic Reticulum Lumen	79	36
Q99J99	3-Mercaptopyruvate Sulfurtransferase	Metabolism: Cysteine and methionine metabolism	Cytoplasm	94	12
Q9D0J2	ATP synthase, mitochondrial F1 complex, Δ subunit precursor	Anabolism: ATP synthasis	Mitochondrion	124	35
Q6P8P4	Myosin Regulatory Light Chain 2, ventricular/cardiac muscle isoform	Motor activity: Cardiac muscle contraction	Cytoplasm	113	34
Q9CZ13	Cytochrome b-c1 complex subunit 1, mitochondrial	Motor activity: Cardiac muscle contraction	Mitochondrion	74	11
Q9D6J6	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	Oxidative phosphorylation	Mitochondrion	98	24
Q9D819	Inorganic Pyrophosphatase	Oxidative phosphorylation	Cytoplasm	87	23
Q9Y3M2	Protein Chibby Homolog 1	Proliferation, inhibition of the Wnt/Wingless system pathway	Cytoplasm	78	21
Q9R1P3	Proteasome subunit beta type-2	Proteasome	Cytoplasm	95	14
13938629	Chaperonin containing Tcp1, subunit 2β	Protein folding	Cytoplasm	71	15
P30412	Peptidyl-Prolylcis-trans isomerase C	Protein folding	Cytoplasm	82	23
82919177	Heterogeneous Nuclear Ribonucleoprotein A3, isoform 1	Transcription	Nucleus	97	21
Q99LC2	Cleavage stimulation factor 50 kDa subunit	Transcription	Nucleus	93	22
Q6PAC1	Gsn protein	Uncharacterized	Unknown	82	28
Q8N357	Transmembrane protein C2orf18	Uncharacterized	Membrane	71	17
Q9H9K5	Uncharacterized protein LP9056	Uncharacterized	Membrane	123	31
Xp_425032	Hypothetical Protein	Uncharacterized	Unknown	119	29

The identified proteins represent the most abundant ones in the cell population, as the separation technique used (SDS-PAGE) has a limited resolution compared with separation methods as 2D electrophoresis or liquid chromatography (LC). 2D electrophoresis could not be used in this work due to the small amounts of

protein available, and the LC-MS was not operational during the experiments. However, it is highly recommendable that these techniques are implemented for future experiences.

From the total of 45 proteins identified from the c-kit$^+$ CSCs whole cell extracts, remarkably, there are 11,1% still uncharacterized proteins. This highly relevant information, since they may play important roles in the differentiation processes of CSCs. The functions of the identified protein are resumed in Table 4.2. The high level of turnover and folding related proteins (11,1%) is a very interesting phenomenon, since it might be related to stressful conditions during the acquisition of the cells, or it can be due to the specific biological properties of stem cells and their regenerative role in the heart.

Functions	% Ids
Biosynthesis	22,2%
CellMobility	5,6%
ImmuneSystemRecognitionm	5,6%
GlycolisisPathway	8,3%
CellMetabolism	5,6%
Proliferation	8,3%
CellStructure	5,6%
Motor Activity	5,6%
OxidativePhosphorylation	5,6%
Turnover	8,3%
Transcription	5,6%
MembraneTransport	2,8%
Unknown	11,1%

Table 4.2- Functions of identified proteins in c-kit$^+$ Cardiac Stem Cells. Functions are the resume from the more detailed functions indicated in Table 4.1

Uncharacterized proteins are excellent subjects for further studies, as their identification in this fraction means that they belong to the group of most expressed proteins, and they might have an important role in the biology of the stem cell. Other interesting proteins for further studies are the common proteins with the Sca-1$^+$ CSCs membrane fraction (see Results*, Sca-1$^+$ membrane enriched fraction cell proteome characterization by MALDI-FTICR*).

c-kit+ CSCs membrane enriched fraction cell proteome characterization by MALDI-FTICR

c-kit$^+$ CSCs membrane fraction proteome was analyzed by MALDI-FTICR, as described previously. For this experience, only one replicate was used, as it was impossible to obtain identifications for the other replicates, due either to the small protein amount available or to the failure of fractioning methods. A small amount of proteins was identified, and all of them were also found in the Sca-1$^+$ CSCs membrane enriched fraction (see next results).

Table 4.3- Proteins identified from the c-kit$^+$ CSCs membrane enriched fraction by MALDI-FTICR. % cov is the percentage of protein sequence covered by the peptides used to identify the protein.Proteins characteristics such as cellular location and function were retrieved from their file at http://www.uniprot.org/.

Accession Code	Protein Identification	Function	Compartment	Score	% cov
O75955	Flotillin-1	Cell Structure	Membrane	123	32
P05023	Sodium/potassium-transporting ATPase subunit alpha-1 precursor	ATP binding, Membrane Transport	Membrane	85	19
P13987	CD59 glycoprotein precursor	Cell surface signal receptor	Membrane	84	22
P15529	Membrane cofactor protein precursor	Cell surface signal receptor	Membrane	79	23
P29966	Myristoylated alanine-rich C-kinase substrate (MARCKS)	Chemotaxis, Cell signaling	Membrane	99	32
P50993	Sodium/potassium-transporting ATPase subunit alpha-2 precursor	Membrane Transport	Membrane	106	30
P51153	RAB13	Cell communication	Membrane	103	36
Q8N357	Transmembrane protein C2orf18	Uncharacterized	Membrane	98	29
Q9H9K5	Uncharacterized protein LP9056	Uncharacterized	Unknown	74	19
Q9Y3M2	Protein chibby homolog 1	Proliferation: Inhibition of the Wnt/Wingless system pathway	Nucleus	76	17

In this experience, the purification of the membrane fraction was well succeeded, as confirmed by western blot (Chapter 3, Fractioning of Sca-1^+ and c-kit^+ CSCs), as 80% of the identified proteins are membrane located. However, the total number of identified proteins was low (10 identifications). In this small amount of identifications, there are still 20% of uncharacterized proteins (see Table 4.4), which are the same as the ones identified in the whole cell extracts, supporting the validity of the results.

Table 4.4- Functions of identified proteins in the membrane extracts of c-kit^+ Cardiac Stem Cells. Functions are the resume from the more detailed functions indicated in Table 4.3

Function	% Ids
Signaling	40%
Metabolism	30%
Proliferation	10%
Uncharacterized	20%

The proliferation related Protein Chibby Homolog 1 is also common to the whole cell extract, indicating that this is probably an important protein for the maintenance of stem properties, as it is known that this protein part of the Wnt system and Wnt proteins have been identified as major physiological regulators of multiple aspects of stem cell biology, from self-renewal and pluripotency to precursor cell competence and terminal differentiation[8,9]. Also, the proteins Flotilin-1, related to cell structure, as it is a part of the lipid rafts on the cell membrane, and the MARKS, a factor involved in cell signaling and chemotaxis, are common with the whole cell extract, and seem to have an important role in this cell processes.

Sca-1^{+} membrane enriched fraction cell proteome characterization by MALDI-FTICR

Proteome analysis of Sca-1^{+} CSCs membrane fraction was made in 3 biological replicates, described in Methods *Samples*. After the isolation of the enriched membrane fraction, proteins were separated by SDS-PAGE (see *Methods*), Figure 4.2.

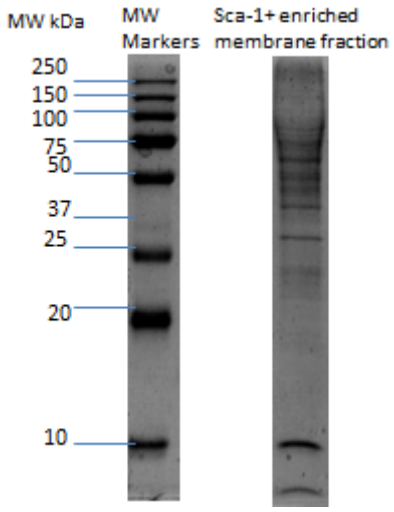

Figure 4.2- SDS-PAGE of the membrane enriched fraction of Sca-1. The land with the sample was then cut in 1% mm bands that were then proteotically digested (see methods) and analysed by MALDI-FTICR.

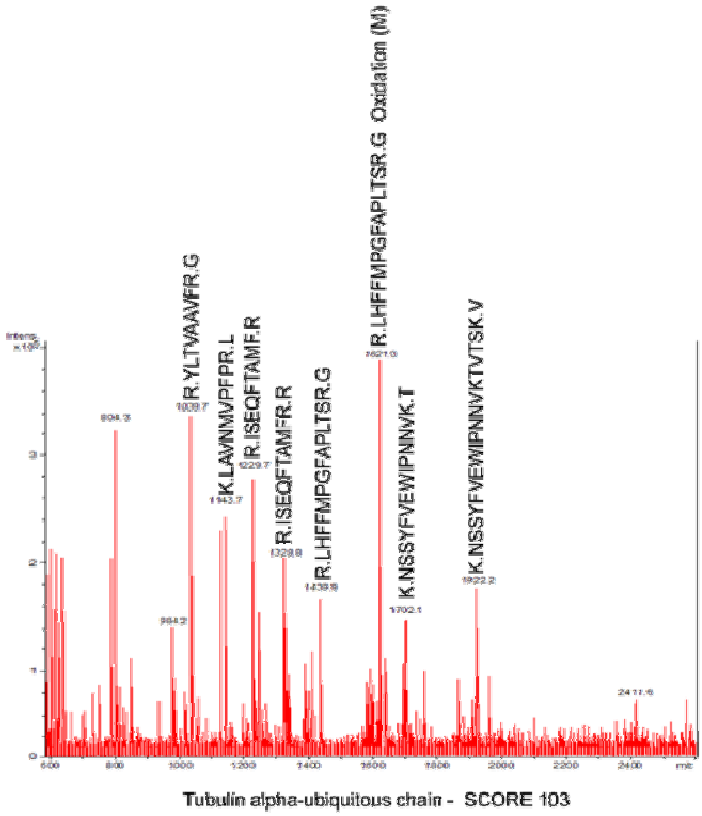

Figure 4.3-Example of a mass spectrum acquisition, the Tubulin α-1B chain mass spectra. Values of m/z were used for database identification of the protein (see Methods). The peptide sequence is represented over the peak.

45 proteins were successfully identified by PMF, and identifications are described in Table 4.5. An example spectrum with peptides sequences is described in Figure 4.3.

Table 4.5- Proteins identified from the Sca-1^{+} CSCs membrane enriched fraction by MALDI-FTICR. % cov is the percentage of protein sequence covered by the peptides used to identify the protein.Proteins characteristics such as cellular location and function were retrieved from their file at http://www.uniprot.org/.

Accession Code	Protein Identification	Function	Compartment	Score	% cov
NP_003613	Isoform 1 of Liprin-beta-1	Cell Adhesion	Membrane	123	36
P51153	RAB13	Cell adhesion	Membrane	98	24
Q15063	Isoform 1 of Periostin precursor	Cell adhesion	Secreted	87	19
P34022	Ran-specific GTPase-activating protein	Cell cycle	Cytoplasm	88	17
LOC679208	Adhesion-regulating molecule 1 precursor /Sortilin	Cell Differentiation	Cytoplasm	95	16
Q9Y3M2	Protein chibby homolog 1	Cell Differentiation	Nucleus	114	29
BAG64033	Low-density lipoprotein receptor-related protein 2 precursor	Cell Signaling	Membrane	74	12
gi|121574	Heat shock 70 kDa protein 5	Folding	Endoplasmic Reticulum Lumen	86	17
Q8WVQ1	Isoform 1 of Soluble calcium-activated nucleotidase 1	Cell signalling	Membrane	94	23
Q9NQ34	Transmembrane protein 9B precursor	Cell signalling	Membrane	78	21
P13987	CD59 glycoprotein precursor	Immunitary response	Membrane	89	20
P15529	Membrane cofactor protein precursor	Immunitary response	Membrane	85	23
P27005	Calgranulin A	Immunitary response	Cytoplasm	81	24
Q6P0Q8	Isoform 1 of Microtubule-associated serine/threonine-protein kinase 2	Immunitary response	Membrane	91	19
gi|39930503	ATP synthase gamma subunit	Metabolism	Mitochondrion	72	17

P05023	Sodium/potassium-transporting ATPase subunit alpha-1 precursor	Metabolism	Membrane	93	16
P50993	Sodium/potassium-transporting ATPase subunit alpha-2 precursor	Metabolism	Membrane	117	36
Q15035	Translocation-associated membrane protein 2	Metabolism	Membrane	98	30
gi|109082950	Myosin heavy chain 6	Motor Activity	Cytoplasm	87	29
gi|127167	Myosin regulatory light chain 2	Motor Activity	Cytoplasm	86	27
gi|54036667	Actin alpha cardiac muscle 1	Motor Activity	Cytoskeleton	95	25
P02563	Myosin heavy chain 6	Motor Activity	Cytoplasm	74	16
P29966	Myristoylated alanine-rich C-kinase substrate	Motor Activity	Membrane	86	19
P60710	Actin, cytoplasmic 1	Motor Activity	Cytoplasm	92	22
P63260	Actin, cytoplasmic 2	Motor Activity	Cytoplasm	73	13
AAA60243	GTP-binding protein	Folding	Membrane	98	29
gi|116242506	Heat shock 70 kDa protein 9	Folding	Mitochondrion	79	24
IPI00387144	Tubulin alpha-1B chain	Structural	Membrane	85	22
gi|232283	47kDa heat shock protein	Proliferation	Endoplasmic reticulum lumen	76	16
O75955	Flotillin-1	Structural	Membrane	99	28
P35052	Glypican-1 precursor	Structural	Membrane	113	37
NP_666204	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6L	Transcription	Eucleus	127	38
Q6ZMG9	LAG1 longevity assurance homolog 6 (LASS6)	Transcription	Membrane	132	31
A8K769	Secretory carrier-associated membrane protein 2	Transport	Membrane	127	29
A8K813	Prolactin regulatory element-binding protein	Transport	Cytoskeleton, cytoplasm	116	33
B4E1V1	Transmembrane protein 16F	Transport	Membrane	78	16
PS50929	ATP-binding cassette, sub-family C, member 1 isoform 6	Transport	Membrane	69	14
Q5T094	RER1 protein	Transport	Membrane	67	16
Q6P1M0	Long-chain fatty acid transport protein 4	Transport	Membrane	74	18

Q8N4V1	Transmembrane protein 32	Transport	Membrane	98	26
Q9Y289	Sodium-dependent multivitamin transporter	Transport	Membrane	69	22
Q96K37	Isoform 1 of Solute carrier family 35 member E1	Transport	Membrane	81	26
Q8N357	Transmembrane protein C2orf18 homolog precursor	Uncharacterized	Membrane	93	29
Q8WYZ0	Putative Uncharacterized Protein	Uncharacterized	Not characterized	76	17
Q9H9K5	Uncharacterized protein LP9056	Uncharacterized	Not characterized	74	18

From the total of 45 proteins identified, 62% of the proteins identified belong to the membrane (Figure 4.4). Thus, the method used for the isolation of this fraction can be considered an acceptable method for membrane protein isolation.

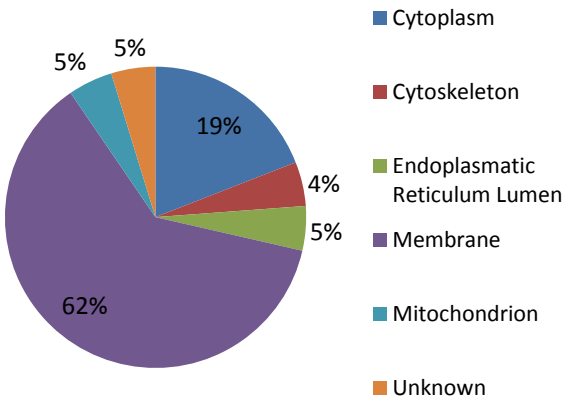

Figure 4. 4- Round chart representing the locations of the proteins identified in the membrane enriched fraction. Membrane- 62%, Cytoplasm- 19%, Cytoskeleton- 4%, Mitochondrion- 5%, Endoplasmatic Reticulum Lumen- 5%, Unknown location- 5%

Some of the identified proteins are related to proliferation and cell cycle progression regulation, as is the case of Ran Specific GTPase Activating Protein, which has a role in the control of the progression through the cell cycle[10], or the 47kDa Heat Shock Protein, which may be involved in the control of cell proliferation and cellular aging[11]. Most proteins are related to transport of metabolites across the membrane and structural and motion functions. The great amount of proteins related with motor

activity is characteristic of cardiac cells, since they belong to a muscle. Frequencies of the different functions for the identified proteins are detailed in Table 4.6.

Table 4. 6-Functions of identified proteins in the membrane extracts of Sca-1$^+$ Cardiac Stem Cells. Functions are the resume from the more detailed functions indicated in Table 4.5

Function	Id %
CellAdhesion	7%
Cellcycle	2%
CellDifferentiation	4%
CellSignaling	7%
Folding	7%
Immunitary response	9%
Metabolism	9%
Motor Activity	16%
Proliferation	2%
Structural	7%
Transcription	4%
Transport	20%
Uncharacterized	7%

From the identified proteins, there are 10 in common with the c-kit$^+$ CSCs membrane fraction and 6 in common with the c-kit$^+$ CSCS whole cell extracts. It is difficult to draw conclusions from these numbers due to the lack of replicates and small number of identifications for c-kit$^+$ CSCs membrane fraction: further studies are still necessary to successfully compare the two populations. Common proteins for c-kit$^+$ and Sca-1$^+$ populations are listed in Table 4.7.

Table 4.7- Common identified proteins in the c-kit$^+$ and Sca-1$^+$ CSCs membrane enriched fraction, and c-kit$^+$ CSCs whole cell extracts. Protein S100-A8 was only commun with c-kit$^+$ CSCs whole cell extracts, all the others are common for the three populations.

Accession Number	Identified protein	Function
P27005	Protein S100-A8	Participates in the cell immune reactions, has chemotactic functions
P29966	Myristoylated Alanine-rich C-kinase substrate	Participates in the directed chemotaxis mechanism and in cell signaling pathways
O75955	Flotillin-1	Structural component, Lipid Rafts
Q9Y3M2	Protein Chibby Homolog 1	Proliferation, inhibition of Wnt/Wingless system pathway
Q8N357	Transmembrane protein	Uncharacterized

	C2orf18		
Q9H9K5	Uncharacterized protein LP9056		Uncharacterized

Common proteins include proteins related with proliferation, as Protein Chibby Homolog 1, which inhibits the Wnt signaling pathways, responsible for cell proliferation and development[8,9], and also with chemotaxis, as the Myristoylated Alanine-rich C-kinase substrate, reported as an important part of the migration mechanisms in mesenchymal stem cells[12].

The identification of protein S100-A8 in the three samples is surprising, since this protein is usually a marker for macrophages and has important immune functions[13]. It could be evidence in favor of the *allochthonous* theory for CSCs origin (See Chapter 1, *Stem cells origin*), but further studies must be performed in order to confirm their role in CSCs biology[4]. Flotilin-1 seem to be an ubiquitary protein, as it is an integrant part of the lipid rafts of the cell membrane, and is involved in several transport processes[14].

The presence of both Transmembrane protein C2orf18 and Uncharacterized protein LP9056 is very interesting, as these uncharacterized proteins are obviously abundant in CSCs, and further studies into their functions may bring important data in cell signaling, homing and differentiation of CSCs.

The other common proteins between c-kit$^+$ CSCs and Sca-1$^+$ CSCs seem to have rather ubiquitous expression, as they are involved in ion transport (Na^+/K^+-transporting ATPase subunit α-1 and α-2 precursors[15]) and inhibition of the auto-immune response (CD59 glycoprotein precursor, membrane cofactor protein[16]). Rab13, also known as *Cell growth-inhibiting gene 4 protein*, however, is not as common. Rab 13 is part of the Rab/Ras group of transporters. Rab proteins are small GTPases involved in determining organelle identity and participate in membrane fusion reactions. They are well-known effector molecules for the regulation of receptor tyrosine kinase activities and also act as molecular switches related to assorted networks of tethering factors and GTPase activating proteins. Rab proteins have also been interlinked via effector molecules, suggesting coordination of protein transport within the endomembrane system and thereby have an important role in cell to cell communication and cell signaling[17].

c-kit+ nuclei enriched fraction cell proteome characterization by MALDI-FTICR

c-kit$^+$ CSCs nuclei fraction proteome was analyzed by MALDI-FTICR, as described previously. For this experience, only one replicate was also used, for the reasons detailed previously (see results, *c-kit$^+$ CSCs membrane enriched fraction cell proteome characterization by MALDI-FTICR*), as it was the same sample that was used for the identification of the membrane fraction of these cells. A small amount of proteins was identified (Table 4.8).

Table 4.8- Proteins identified from the c-kit$^+$ CSCs nuclei enriched fraction by MALDI-FTICR. % cov is the percentage of protein sequence covered by the peptides used to identify the protein.Proteins characteristics such as cellular location and function were retrieved from their file at http://www.uniprot.org/.

Accession Code	Identified Protein	Function	Compartment
P62805	Histone H4	Transcription Regulation	Nucleus
P68431	Histone H3	Transcription Regulation	Nucleus
B4DWF8	FLJ53414 (similar to Nuclear pore complex protein Nup98-Nup96precursor)	Transport	Nucleus

The 3 identified proteins are characteristic of nuclei proteome, as histones are fundamental proteins for regulation of transcriptions, and the nucleus is protected by a membrane with pore structures, which allows the trade of metabolites with the cytoplasm. This fraction is very difficult to isolate for c-kit$^+$ CSCs, as these cells are not as numerous as the Sca-1$^+$ CSCs, therefore adding a great difficulty to the collection of protein samples for MS analysis.

Sca-1$^+$ nuclei enriched fraction cell proteome characterization by MALDI-FTICR

Sca-1$^+$ CSCs nuclei fraction proteome was analyzed by MALDI-FTICR, as described previously. Samples were obtained as described in Methods, *Samples.*29 proteins were successfully identified by PMF, as described in Table 4.9.

Table 4.9- Protein identification for Sca1$^+$ CSCs nuclei enriched fraction. Proteins were identified by MALDI-FTICR. % cov is the percentage of protein sequence covered by the peptides used to identify the protein.Proteins characteristics such as cellular location and function were retrieved from their file at http://www.uniprot.org/.

Accession Code	Protein Identification	Function	Compartment
CAA31455	Actin, cytoplasmic 2	Cell mobility	Cytoplasm
Q91XQ0_MOUSE	Dynein heavy chain 8, axonemal	Cell mobility	Cytoskeleton
BAA01862	Stress-70 protein, mitochondrial	Cell proliferation and cellular aging	Mitochondrion
Q58E49_MOUSE	Hdac1 protein	Chromatin binding protein	Nucleus
Q8CDZ0_MOUSE	Protein Shroom2	Developmental protein	Cytoplasm
ENOA_MOUSE	Alpha-enolase	Glycolisis, growth control	Cytoplasm
I52858	Nucleophosmin	Proliferation control	Nucleus
Q5F264_MOUSE	Coronin-6	Regulation of actin dependent processes	Cytoplasm
I49366	Histone-binding protein RBBP4	Regulation of chromatin metabolism	Nucleus
Q5DTQ8_MOUSE	Protein 4.1	Regulation of membrane physical properties of mechanical stability	Nucleus
JC4159	40S ribosomal protein S12	Ribosomal constituint	Cytoplasm
BAC36976	Heterogeneous nuclear ribonucleoprotein H2	RNA and nucleotide binding	Nucleus
Q499X2_MOUSE	Heterogeneous nuclear ribonucleoprotein L	RNA and nucleotide binding	Nucleus
ATR_MOUSE	Serine/threonine-protein kinase ATR	Signaling cascade	Nucleus
AAN73044	Rootletin	Structural activity	Nucleus
Q80ZV2_MOUSE	Tubb5 protein	Structural, constituent of cytoskeleton	Cytoskeleton
AAH52729	Lamin-B1	Structural, interacts with chromation	Nucleus

NUCL_MOUSE	Nucleolin	Transcription activation	Nucleus
AAF73151	Histone-lysine N-methyltransferase SUV39H1	Transcription regulation	Nucleus
AAH06955	Core histone macro-H2A.1	Transcription regulation	Nucleus
H2A1H_MOUSE	Histone H2A type 1-H	Transcription regulation	Nucleus
H2AW_MOUSE	Core histone macro-H2A.2	Transcription regulation	Nucleus
Q640M9_MOUSE	Histone acetyltransferase KAT2B	Transcription regulation	Nucleus
S06754	Histone H2A type 1-D	Transcription regulation	Nucleus
S45110	Histone H2A	Transcription regulation	Nucleus
HSBO3	Histone H3.3	Transcription regulation, predominant form of histone H3 in non-dividing cells	Nucleus
BAB25326	Suppressor of G2 allele of SKP1 homolog	Ubiquitination of proteins	Cytoplasm
Q3TWX1_MOUSE	Putative uncharacterized protein	Uncharacterized	Unknown
BAC34601	Unnamed protein product	Uncharacterized	Unknown

From the total of 29 proteins identified, it is visible that not all of them belong to the nucleus fraction. However, as 62% of the proteins identified belong to the nuclei (Figure 4.5), the method used for the isolation of this fraction can be considered a good method for nuclei protein isolation.

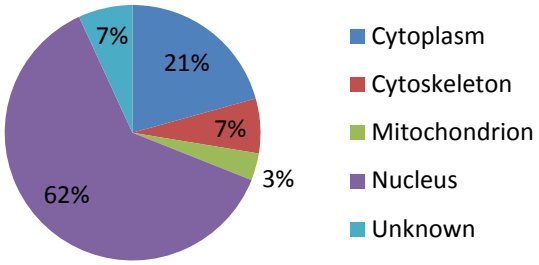

Figure 4. 5- Round chart representing the locations of the proteins identified in the nuclei enriched fraction. Nuclei- 62%, Cytoplasm- 21%, Cytoskeleton- 7%, Mitochondrion- 7%, Unknown location- 3%

There are no common proteins with the c-kit^{+} CSCs nuclei enriched fraction. This is probably due to the general lack of identification for that fraction, and more studies are needed to successfully compare both populations.

Identified proteins are mostly involved in regulation of transcription and other cell processes, as visible in Table 4.10.

Table 4.10- Functions of identified proteins in the nuclei extracts of Sca-1^{+} Cardiac Stem Cells. Functions are the resume from the more detailed functions indicated in Table 4.8

Function	Occurrences (%)
Mobility	7%
Signaling	10%
Proliferation	10%
Regulation	48%
Structural	10%
Turnover	7%
Unknown	7%

Some proteins reveal special interest for further studies, as they might have important roles in stem cell differentiation and homing processes. Is the case of Stress-70 protein, which extracts protein from the nucleus, and is implicated in the control of proliferation and cellular aging[18], and is phosphorylated upon DNA damage by ATR, another protein identified: Serine/Threonine- protein kinase ATR is an important metabolite, which function is related with control of apoptosis and might have an anti-tumor participation[19].

Nucleophosmin, a multifunctional protein frequently overexpressed in actively proliferating cells, and that has been reported to enhance proliferation in HSCs. It might also play a relevant part in differentiation processes[20].

Both Putative Uncharacterized Protein and Unnamed Protein Product are uncharacterized proteins, and are good subjects for further studies, as they might be involved in important and yet unknown cell mechanisms.

Discussion

Undifferentiated CSCs are sensitive to extracellular stimuli, such as growth factors and extracellular matrix components that can induce differentiation towards cardiomyocytes, cardiac smooth muscle cells and endothelial cells. The identification of the factors, receptors and other proteins involved in this process is crucial for the future application of CSCs in therapy.

In the present study we prepared membrane and nuclei fractions for the Sca-1^{+} and c-kit^{+} CSCs. The whole cell extract of c-kit^{+} CSCs was also prepared due to the fact that the small amount of isolated c-kit bearing cells could compromise the fractioning and thereby the achievement of identifications.

58 proteins were identified from c-kit^{+} CSCs: 45 for the whole cell extracts, 10 for the membrane fraction and 3 for the nuclei fraction. Only the most abundant proteins can be identified using the method proposed, as the SDS-PAGE separation of proteins is a method with limited sensibility. The identified membrane and whole cell protein included receptors, adhesion molecules, signaling molecules, and other proteins, some of them related with differentiation, proliferation and homing of this cells, as it is the case of Protein Chibby Homolog 1[8,9], and Myristoylated Alanine Rich C-Kinase Substract[12], proteins involved in differentiation and mobility processes, respectively. A very interesting data was the finding of four uncharacterized proteins, GSN protein, Transmembrane Protein C2orf18, Uncharacterized protein LP9056 and Hypothetical Protein. This opens way for future studies, as the abundance of these proteins might indicate that they have an essential role in the biology of CSCs. Unfortunately, very few proteins were identified for the nuclei fraction, and thereby no conclusions can be drawn from these identifications, since they were all ubiquitary proteins.

For the Sca-1^{+} CSCs, 74 proteins were identified: 45 for the membrane fraction and 29 for the nuclei fraction. The identified proteins in the membrane fraction also include receptors, adhesion molecules and signaling molecules, with some very interesting results. Proteins like Ran-Specific GTPase activating protein[10] and 47kDa Heat Shock Protein[11] seem to have a very relevant role in progression through the cell cycle and control of cell proliferation and cell aging, respectively. Another interesting

data is the common proteins with c-kit^{+} CSCs. Some of the common proteins, as Protein Chibby Homolog 1, and Myristoylated Alanine Rich C-Kinase Substract, proteins involved in differentiation and mobility processes, indicate that these cells have common pathways in these processes. The finding of uncharacterized proteins common to both populations is another evidence for the importance of their role in CSCs biology. In the nuclei fraction, protein identifications included transcription factors, signaling molecules, structural proteins, and other proteins, including important factors such as Nucleophosmin[20], involved in cell proliferation and differentiation, Serine/Threonine- protein kinase ATR[19] and Stress-70 protein[18], both involved in the regulation of cell proliferation and cellular aging. In this fraction there are also 2 uncharacterized proteins, which should be part of further characterization studies.

In conclusion, the receptors, cell adhesion molecules and proliferation and differentiation factors found in the proteomes of these two populations of CSCs are involved in the maintenance of the stem state of these cells, and should be target of further studies, including bio-informatics studies in order to better understand their role in the CSCs biology, and enzymatic and functional studies is order to study the uncharacterized proteins so that their contribution to the CSCs differentiation and proliferation processes can be fully understood.

References

1. Stastna, M. et al, *Cardiac Stem /Progenitor Cells, Secreted Proteins and Proteomics,* FEBS Letters, 583: 1800-1807, 2009
2. Stastna, M. et al, *Identification and functionality of proteomes secreted by rat cardiac stem cells and neonatal cardiomyocytes*, Proteomics, 10, 254-253, 2010
3. Bekkers, S, et al, *Myocardial Regeneration via Intracardiac Stem Cells*, in *Rebuilding the Infarcted Heart*, Informa Healthcare, Chapter 6: 73-86, 2007
4. Di Nardo, et al, *Cardiac progenitor cells: potency and control*, Journal of Cellular Physiology, 224, 590-600, 2010
5. Urbich, C. et al, *Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac progenitor cells*, Journal of Molecular and Cellular Cardiology, 39, 733-742, 2005
6. Mann M. et al, *Analysis of proteins and proteomes by mass spectrometry*, Annu Rev Biochem, 70, 437-473, 2001
7. Larsen, M.R. et al, *Improved detection of hydrophilic phosphopeptides using graphite powder microcolumns and mass spectrometry: evidence for in vivo doubly phosphorylated dynamin I and dynamin III,* Mol Cell Proteomics, 3, 456-465, 2004
8. Logan, C. and Nusse, R., *The Wnt signaling pathway in development and disease,*Annu Rev Cell DevBiol, 20, 781-810, 2004
9. Michaelis, T., Lie, D.C., *Wnt signaling and neural stem cells: caught in the Wnt web,* Cell Tissues Res, 332, 193-210, 2008
10. Coutavas, E. et al, *Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4,* Nature, 366, 585-587, 1993
11. Takechi, H. et al, *Molecular cloning of a mouse 47-kDa heat shock protein (HSP47), a collagen binding stress protein, and its expression during the differentiation of F9 teratocarcinoma cells*, Euro J Biochem, 206, 323-329, 1992
12. Stumpo, D. et al, *Molecular cloning, characterization and expression of a cDNA encoding the 80-87 kDamyristoylated alanine-rich C kinase substrate: A major cellular substrate for protein kinase C*, ProcNatlAcadSci, 86, 4012-4016, 1989

13. Zhang, H. et al, *Injection of bone marrow mesenchymal stem cells in the borderline area of infarcted myocardium: heart status and cell distribution*, 134, 1234-1240, 2007
14. Ynagisawa, M. et al, *Roles of Lipid rafts in integrin-dependent adhesion and gp130 signalling pathway in mouse embryonic neural precursor cells*, Genes to Cells, 9, 801-809, 2004
15. Quintas, L.E., et al, *Na^+/K^+-ATPase alpha isophorms expression in stroke-prone spontaneously hypertensive rat heart ventricles: Effect of salt loading and lacidipine treatment*, European Journal of Pharmacology, 565, 151-157, 2007
16. Papanastasiou, A. et al, *Cloning of a CD59-like in rainbow trout: Expression of phylogenetic analysis of two isoforms*, Molecular Immunology, 44, 1300-1306, 2007
17. Harkness, L. et al, *Identification of a membrane proteomic signature for human embryonic stem cells independent of culture conditions,* Stem Cell Research, 1, 219-277, 2008
18. Warrick, J. et al, *Suppression of polyglutamine-mediated neurodegeneration in* Drosophila *by the molecular chaperone HSP70*, Nature Genetics, 23, 425-429, 1999
19. Sears, R., Nevins, J., *Signaling networks that link cell proliferation and cell fate*, The Journal of Biological Chemistry, 277:14, 11617-11620, 2002
20. Li, J. et al, *Nucleophosmin regulates cell cycle progression and stress response in hematopoietic stem/progenitor cells*, The Journal of Biological Chemistry, 281:24, 16536-16545, 2006
21. Mann, M., Kelleher, L., *Precision Proteomics: The case for high resolution and high mass accuracy*, PNAS, 105, 18132-18138, 2008

Chapter 5

General discussion

General Discussion

Cardiac Stem Cells may hold thekey for therapeutic approaches in cardiac diseases[1,2]. In the present work, two populations of CSCs were isolated, fractioned and analyzed by mass spectrometry in order to characterize their proteomes.

CSCs were isolated by Fluorescence Activated Cell Sorting, using fluorescent probes for the c-kit and Sca-1 membrane markers. c-kit bearing cells were much more difficult to isolate, as described by literature, as these cells are not so common in mice. Sca-1$^+$ CSCs were isolated in larger amounts from smaller samples, as expected, since these cells have previously been described as the most abundant CSCs in mice[3]. FACS seems to be a good method for CSCs isolation, although in the future optimization of the protocol is needed to reduce the amount of mice used for obtaining samples.

Fractioning of CSCs to obtain membrane and nuclei enriched fractions was successful for both populations, as 80% of the proteins identified from c-kit$^+$ CSCs membrane enriched fraction and 62% of the proteins identified from Sca-1 CSCs membrane enriched fraction belong to the membrane, and all of the proteins identified in c-kit$^+$ CSCs nuclei enriched fraction and 62% of the proteins identified in Sca-1$^+$ CSCs membrane enriched fraction are nuclei proteins. The method used, an adaptation of the methods described in *Intoh, 2009*[4] and *Dormeyer, 2008*[5], is well suited for the fractioning of stem cells. However, the most suitable velocity for centrifugation could not be used (46000 g, 1h) due to technical issues. The velocity used seems to be sufficient when combined with a greater period of time, but in the future the method described in the literature should be implemented, as it is more efficient and economic.

The c-kit$^+$ CSCs sample was very small and so, to ensure a better analysis of this cell population proteome, a whole cell sample was prepared. This sample yielded identifications belonging to different cell compartments, such as nuclei, membrane, mitochondria, lysosome and endoplasmatic reticulum. This sample cannot be directly compared with the Sca-1$^+$ CSCs fractions, but there are some common proteins with interesting roles in the stem cell biology, specially the uncharacterized ones, which seem to be very abundant.

Proteome analyses by MALDI-FTICR allowed the total identification of 122 proteins (48 for the c-kit$^+$ population, and 74 for the Sca-1$^+$ population). Most of the proteins identified for the Sca-1$^+$ CSCs membrane and nuclei fractions were identified in triplicate. For the c-kit$^+$ CSCs membrane and nuclei fractions, only one replicate was made, due to the difficulty of isolating this cells by FACS, except for the whole cell sample that was analyzed in triplicate.

Membrane proteins are encoded by 20-30% of the genes in the vertebrate genome[4]. These membrane integral and associated peripheral proteins are involved in the central cellular processes and also in the mechanisms of stem cell proliferation and differentiation[4]. Membrane proteins identified for the c-kit$^+$ CSCs membrane enriched fraction are present in the Sca-1$^+$ CSCs membrane enriched fraction. However, most of the proteins identified for Sca-1$^+$ CSCs membrane enriched fraction are not common to either c-kit$^+$ CSCs membrane enriched fraction or c-kit$^+$ CSCs whole cell sample. This is a very important observation, since differentially expressed proteins can have meaningful roles in stem cell biology. However, as the c-kit cell sample preparation was not as successful as expected, further replicates must be made in order to obtain a real data comparison, as any differences visible at the moment may be due to the method limitations with small sample amounts.

An important area of study of the stem cell biology is the mechanism involved in stem cell migration, as the present paradigm states that stem cells exist in niches and only migrate to the injured tissue in response to signals. Proteins identified in the c-kit whole cell fraction have variable functions (Chapter 4, Table 4.2), including proteins involved in cell mobility (MARKS, Calgranulin-A), which can be important factors in the homing processes of CSCs.

Other proteins identified in the c-kit whole cell fraction are common proteins involved in the cell metabolism (Prolyl 4-Hydroxalase α-1, 3-Mercaptopyruvate Sulfurtransferase), in the biosynthesis processes (Seryl-tRNA, 3-Hydroxy-3-Methylglutaryl-Coenzyme A synthase 1, Adenylosuccinate Synthase isoenzyme 2, Uridine Monophosphate Synthetase, ATP Synthases) and in the glycolysis pathway (LDH-A, Phosphoglycerate Kinase-1, Alpha-Enolase).

An interesting finding in this sample is the presence of proteins that may be involved in the recognition of the self by the immunity system (Calgranulin-A, Cofilin-1). Stem cell transplant is one of the therapy possibilities that these cells offer, and if the immune system is capable of recognizing these cells, than the risk of rejection diminished, along with the possibility of auto-transplant[6].

Proteins involved in the proliferation processes were also found the c-kit whole cell fraction (Stress-Induced Phosphoprotein-1, Alpha-Enolase, Protein Chibby Homolog 1), and their role in stem cell biology should be further studied, along with the role of the uncharacterized proteins (Gsn protein, Transmembrane protein C2orf18, Uncharacterized protein LP9056, Hypothetical Protein), as these proteins can play a major role in the differentiation and proliferation processes of CSCs.

Other proteins found in this sample have general functions that maintain stem cell homeostasis, such as proteins involved in oxidative phosphorylation (NADH dehydrogenase, Inorganic Pyrophosphatase), intracellular protein breakdown (Cathepsin D, Proteasome subunit beta type-2, Chaperonin containing Tcp1), transcription (Heterogeneous Nuclear Ribonucleoprotein A3, Cleavage stimulation factor), cell structure (Col1a1,Flotilin-1), motor activity (Myosin regulatory light chain 2, Cytochrome bc1 complex subunit 1) and membrane transport (Cytochrome b561).

In the c-kit$^+$ CSCs membrane enriched fraction, some new proteins are found when comparing to the c-kit whole cell sample, mostly proteins involved in membrane transport (Na^+/K^+ transporting ATPase subunits), cell signaling and recognition of the "self" by the immune system (CD59 glycoprotein precursor, Membrane cofactor protein precursor) and cell-to-cell communication (Rab13).

All proteins identified in the c-kit$^+$ CSCs membrane enriched fraction were also identified in the Sca-1$^+$ CSCs membrane enriched fraction, and were discussed in Chapter 4. They seem to have rather ubiquitous roles, apart from the uncharacterized ones, that may have important functions, and should be targeted in further studies.

Proteins identified for the Sca-1$^+$ CSCs membrane enriched fraction were mostly different from the ones found in the c-kit$^+$ CSCs membrane enriched fraction, as previously stated. This difference is probably due to the differences in the samples,

and therefore further studies will be needed in order to accurately discuss the proteome differences between the two stem cells populations. The identified proteins were discussed in Chapter 4, and the study of the function of the uncharacterized proteins and of some other relevant proteins will be held in future projects to better understand their impact in stem cell differentiation, proliferation and homing processes.

Proteins involved in the proliferation mechanisms were common to all the CSCs population, as is the case of the Protein Chibby Homolog 1, or specific of c-kit$^+$ population (Alpha Enolase) or to the Sca-1$^+$ population (47 kDa heat shock protein). This is a good starting point for further comparison of the two populations' proteome.

In the nucleus, we expected to identify transcription factors and other proteins involved in signal transduction that could indicate which genes were activated, and also chromatin-remodeling enzymes. In the c-kit$^+$ CSCs nuclei enriched fraction, only a small number of proteins were identified, all involved in regulation processes and ubiquitous. In the Sca-1 CSCs nuclei enriched fraction, more proteins were identified, mostly involved in chromatin activation processes and transcription regulation, but also some interesting proteins involved in cell proliferation and aging (Stress-70 protein, Nucleophosmin) and signaling (Serine/Threonine-protein kinase ATR).

Further analyses of all the identified proteins using computational methods to understand which pathways are activated in CSCs will contribute to a better comprehension of the biological processes underlying the differentiation, proliferation and homing processes of CSCs.

Future Directions

The present study of CSCs proteome provides the fundamental basis for subsequent functional and structural studies of the uncharacterized proteins, as well as for the studies of proteins involved in the differentiation, proliferation and homing processes of CSCs. Further characterization of c-kit$^+$ CSCs membrane and nuclei fractions should also be held, as the present results are not complete. Also, bioinformatics studies should be held in order to identify the active signaling transduction and metabolic pathways in the CSCs.

References

1. Singla, D.K., *et al*, *Stem cells in the infarcted heart*, Int J Cardiovasc Imaging, Springerlink.com, 2010
2. Jun Wu, FaquanZeng, Richard D. Weisel and Ren-Ke Li, Chapter: "*Stem Calls for Cardiac Regeneration by Cell Theraphy and Myocardial Tissue Engineering*", in "Engineering of Stem Cells", Springler Berlin/ Heidelberg, Vol 114, 2009
3. Beltrami, A., Anversa P., *et al*, *Adult cardiac stem cells are multipotent and support myocardial regeneration*, Cell,114, 763-776, 2003
4. Intoh, A., Asashima, M., *et al*, *Proteomic analysis of membrane proteins expressed specifically in pluripotent murine embryonic stem cells, Proteomics*, 9, 126-137, 2009
5. Dormeyer, W., Heck, A., *et al*, *A practical guide for the identification of membrane and plasma membrane proteins in human embryonic stem cells and human embryonal carcinoma cells*, Proteomics, 8, 4036-4053, 2008
6. Boyd, A., Higashi, Y., Wood, K. *Transplanting stem cells: Potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantationI*, Advanced Drug Delivery Reviews, 57, 1944-1969, 2005