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PhD Thesis:

CARDIOMYOGENIC DIFFERENTIATION OF HUMAN MULTIPOTENT STROMAL CELLS: A NEW APPROACH FOR REGENERATIVE MEDICINE IN CARDIOVASCULAR DISEASE

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Abstract

Cardiovascular disease (CVD), which include hypertension, coronary heart disease (CHD), stroke, and congestive heart failure (CHF), are the leading and most common causes of mortality in both developing and industrialized countries, accounting for one third of all deaths globally.
Cardiomyocyte regeneration is limited in adult life and therefore the identification of a putative source of progenitors is of great interest to provide usable *in vitro* models and new perspectives for regenerative therapies. This appears to be a challenge due to the peculiar functional and anatomical heart properties. The goal of this project was to differentiate adult human Adipose tissue-derived multipotent Stem Cells (ASCs) into cardiomyocytes or their close progenitors. Thus, the ultimate aim is to develop stem cell-based therapies for the cure of cardiovascular disease. More specifically, we investigated the application of two innovative approaches: the use of human growth factors in a well defined serum-free culture medium and the application of electrical and mechanical stimuli to mimic the physiological environment of the heart.

First of all, we developed a new protocol for the isolation of ASCs according with the standard GMP-procedure, which allows to extract a substantial number of cells for clinical application. The starting material was obtained from subcutaneous adipose tissue collected during an elective liposuction procedure. The isolated ASCs were characterized by flow cytometry and expanded until second passage. Several different cocktails of growth factors, cytokines and several culture conditions were tested for their ability to induce cardiomyogenesis. The goal was to identify the right combination of those factors and their time of application. One of the cardiogenic cocktails tested was indeed very effective in inducing the selected cardiac markers (e.g. NKX2-5 and MEF2C) and was chosen for the second part of this research work. The differentiation obtained was highly reproducible, as it was observed in 15 out of 15 samples from different patients. Interestingly, non induced ASCs expressed half of the selected cardiac markers (i.e. TBX5, GATA4 and BAF60C), suggesting the high

intrinsic potential and the inherent propensity of ASCs to differentiate into cardiac precursors. We further analysed the morphological changes that ASCs underwent during their differentiation by use of electron microscopy and immunofluorescence staining. By treating the cells with our selected induction cocktail, clear changes in their morphology were observed: the cells assumed a fusiform shape and became oriented.

The last milestone was to understand if the combination of the cardiac induction cocktail with a mechanical (and eventually electrical) stimulation on a scaffold would increase the efficiency of cardiogenic differentiation. For this, a customized device has been developed by the Swiss Stem Cell Foundation in collaboration with Scuola Universitaria Professionale della Svizzera Italiana (SUPSI) for the application of controlled mechanical and/or electrical stimuli to a scaffold over a desired lap of time. The scaffolds used were developed and characterized by Prof. Ciardelli's Research Group (Materials in Bionanotechnology, Department of Mechanical and Aerospace Engineering, Politecnico di Torino). The preliminary results showed that the combination of growth factors, cytokines and mechanical stimulation for 10 days led to an at least 2 fold increase in mRNA expression of NKX2-5, MEF2C, HAND2 and MHC when compared to the induction with cardiogenic cocktail alone on a simple 2D culture condition.

In conclusion, in this work we present a new protocol for the extraction of ASCs starting from adipose lipoaspirates, a new serum-free based cardiogenic induction cocktail for ASCs and preliminary data about the differentiation in scaffold enhanced by mechanical stimulation opening a new perspective to cardiac regeneration.

Abbreviations

5-AZA: 5-azacytidine
ACTC1: Cardiac α-actin
ASCs: Adipose derived stem cells
AT: Adipose tissue
βFGFR: Basic fibroblast growth factor receptor
BM: Bone marrow
BMPs: Bone morphogenetic proteins
BV: Blood vessel
CAL: Cell-assisted lipotransfer
CAMTA: Calmodulin binding transcription activator 2
CDs: Clusters of differentiation
CFU-Fs: Colony-forming unit-fibroblasts
CHD: Coronary heart disease
CHF: Congestive heart failure
CPCs: Cardiac progenitors cells
CSCs: Cardiac stem cells
CVD: Cardiovascular diseases
DAPI: 4', 6-diamidino-2-phenylindole, dihydrochloride
DC: Differentiated support cells
DKK1: Divkkopf-1
ECM: Extracellular matrix
EGFR: Epidermal growth factor receptor
ESCs: Embryonic stem cells
FACS: Fluorescence-Activated Cell Sorter
FGFs: Fibroblast growth factors
FOG: Friend of Gata
G-CSF: Granulocyte colony stimulating factor
Glu: Glutamine
GvHD: Graft-versus-host disease
HLA: Human leukocytes antigen
HSCs: Hematopoietic stem cells
ICM: Inner cell mass
IFNγ: Interferon-γ
IGF: Insulin like growth factor
IL: Interleukin
iPS: Induced pluripotent stem cells
ITS: Insulin, Transferrin and Selenium
IWR: Inhilits Wnt-induced accumulation of β-catenin

klf4: Krupple-like family transcription factor 4
LIF: Leukemia inhibitory factor
LVEF: LV ejection fraction
LVR: Left ventricle remodelling
MEF2C: Myocyte enhancer factor 2 c
MI: Myocardial infarction
MNCs: Mononucleated cells
MSCs: mesenchymal stem cells
MYL2: Myosin light chains
MYL6: α-myosin heavy chain
Nanog: Nanog homeobox
NCX: Sodium-Calcium Exchanger
NOD: Non-obese diabetic
Oct4: Octamer-4
PCL: Polye caprolactone
PDGF: Platelet –derived growth factor
PGA: Polyglycolide
PLA: Polylactide
PURs: Polyurethanes
RT: Room temperature
SEM: Scanning electron microscopy
SERCA-2: Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
Sox2: Sox determining region Y-box 2
SR: Sarcoplasmic reticulum
SSEA-4: Stage specific embryonic antigen 4
SVF: Stromal vascular fraction
TBX: T-box
TEM: Transmission electron microscopy
TERM: Tissue engineering/regenerative medicine
TGF-β: Transforming growth factors b
TGFR1: Trasforming growth factor receptor
TNFa: Tumor necrosis factor-a
TNNI3: Cardiac Troponin III
UCB: Umbelican cord blood
VEGF: Vascular endothelial growth factor
Wnt: Wingless-related protein
α-SA: α-sarcomeric actin
β-MHC: β myosin heavy chain

1. Introduction

1.1 Stem Cells

Stem cells are defined as unspecialized cells with the ability to self-renew through cell division or to differentiate to become tissue or organ specific cells with specialized functions (Stanford and Julie, 2009). To ensure self-renewal, stem cells undergo two types of division. Symmetric division gives rise to two identical daughter cells, both endowed with stem cell properties, whereas asymmetric division produces only one stem cell and a progenitor cell with limited self-renewal potential. Progenitors can go through several rounds of cell division before finally differentiating into a mature cell. It is believed that the molecular distinction between symmetric and asymmetric division lies in differential segregation of cell membrane proteins between the daughter cells. A classification of stem cells has been compiled by the National Institute of Health (National Institutes of Health (U.S.), 2001). Until the 8-cell morula stage, the cells in the embryo are totipotent because they can give rise to all the tissue of the organism, including extra-embryonic tissues, such as the placenta, the umbilical cord and the yolk sac [Fig. 1]. The cells from inner cell mass (ICM) of the blastocyst are pluripotent, with the capacity to differentiate into all cell types of the body but not in the cells that compose the extra-embryonic tissue. Multipotent stem cells are able to specialize only in certain cell types and the unipotent stem cells can generate only one type of specialized cell [Fig. 1]. The progress in stem cell research has raised hopes for novel cell therapy treatments of severe diseases such as diabetes, neurological disorders and cardiac failure.

1.2 Pluripotent Stem Cells

1.2.1 Human embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of mammalian blastocysts, and mouse ESCs were first isolated in 1981. Human ES cells derived from human blastocysts were first established by Thomson and co-workers in 1998 (Thomson et al., 1998).

The embryo at the blastocyst stage is composed of three structures: the

outermost cell layer called trophoblast surrounds the blastocyst and will give rise to the extra-embryonic tissues, the internal cavity or blastocoel and the inner cell mass, a group of approximately 30 cells within the blastocoel, destined to differentiate and give rise to the embryo or to become source of ESCs. Therefore ESCs are pluripotent cells capable of generating all the different cell types of the body; ESCs are unspecialized cells and can replicate indefinitely, if kept under appropriate conditions. ESCs have been also genetically manipulated. Thanks to these characteristics, these cells could be particularly useful for therapeutic use in an attempt to treat certain human diseases in which cellular functions are altered or destroyed.

When undifferentiated hESCs are capable of proliferating extensively *in vitro*, they have the ability to differentiate towards all three germ layers and they can give rise to all cell types of the body. Furthermore, hESC express several transcription factors and surface markers associated with the non-differentiated phenotype, such as Octamer-4 (Oct4), Nanog homeobox (Nanog), Sox determining region Y-box 2 (Sox2) and Stage specific embryonic antigen 4 (SSEA-4) (Boyer et al., 2005). Telomerase and alkaline phosphatase activities of hESCs are high and the karyotype should be normal and remain unaltered during extended culture periods (Hoffman and Carpenter, 2005).

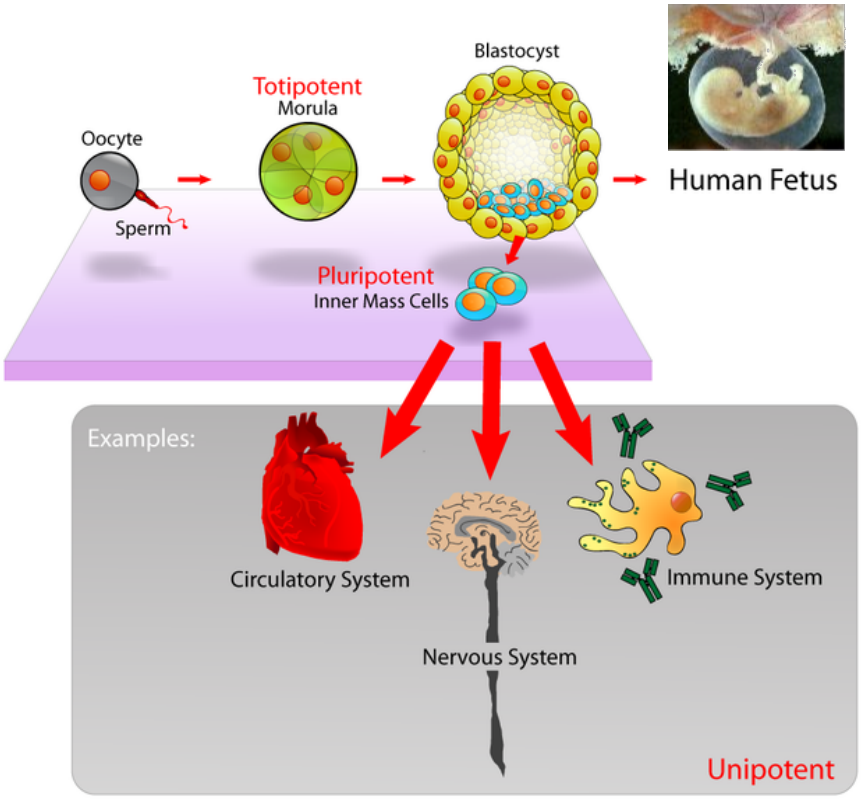

Figure 1. Hierarchy of Stem Cells. A schematic representation of the levels of staminality of stem cells. The cells in the embryo until the 8-cell morula stage are totipotent; the cells from inner cell mass (ICM) of the blastocyst are pluripotent; multipotent stem cells are able to specialize only in certain cell types and the unipotent stem cells can generate only one type of specialized cell (from *Wikipedia.org*).

1.2.2 Induced pluripotent stem cells (iPS cells)

The induced pluripotent stem cells (iPS cells) were first established in 2006 by Takahashi and Yamanaka by retrovirus-mediated transduction of four transcription factors (Oct4, Sox2, c-myc and Krupple-like family transcription factor 4 - klf4) into mouse fibroblasts (Takahashi and Yamanaka, 2006). These reprogrammed cells, which were selected by the expression of a beta-geo cassette (a fusion of beta-galactosidase and neomycin resistance genes) driven by the mouse Fbx15 promoter, failed to contribute to adult chimeras. To obtain high-quality iPS cells, reprogrammed cells were selected for their expression of either Nanog or Oct3/4 expression, both of which are involved in pluripotency. These cells successfully contributed to adult chimeras and also showed germline transmission (Fukuda and Yuasa, 2013).

Human iPS cells were established in 2007 (Takahashi et al., 2007) by the transduction of either the same set of transcription factors or another set of transcription factors (i.e. Oct3/4, SOX2, Nanog, Lin28) into human fibroblasts (Yu et al., 2007). These human iPS cells are similar to human ES cells in their morphology, gene expression and epigenetic status of pluripotent cell-specific genes. Furthermore they can differentiate into the cell types of the three germ layers *in vitro* and *in vivo* (Takahashi et al., 2007). Human iPS cells have been established from skin fibroblasts (Park et al., 2008), keratinocytes (Aasen et al., 2008), mobilized CD34+ hematopoietic stem-progenitor cells (Loh et al., 2009) and differentiated T cells from peripheral blood (Loh et al., 2010). Human iPS cells provide a chance to develop new treatment modalities in the field of regenerative medicine (Yoshida and Yamanaka, 2011).

1.3 Multipotent stem cells

1.3.1 Fetal stem cells

Fetal stem cells can be obtained either from fetus or from extra-embryonic structures such as the umbilical cord blood, the amniotic fluid, the Wharton's Jelly, the amniotic membrane and the placenta. Umbilical cord blood (UCB) is collected at the time of birth and provides a pool of adult-

like stem cells. Transplantation of UCB has been clinically successful for hematopoietic stem applications resulting in high degree of engraftment, favourable immunotolerance and limited evidence for graft-versus-host disease compared to adult bone marrow stem cell transplantation. Reconstruction of the adult hematopoietic system was the initial rationale for UCB-based applications.

1.3.2 Adult stem cells

Adult stem cells are multipotent undifferentiated cells, characterized by continuous self-renewal and located, together with the specialized cells typical of a tissue, within the same organ. Adult stem cells comprise a wide range of progenitors derived from non-embryonic, non-fetal tissue such as bone marrow, adipose tissue and resident stem cell pools. Scientific interest in adult stem cells is focused on their ability to generate all the cell types of the organ from which they originate, potentially being able to regenerate an entire organ starting from a few cells. Unlike embryonic stem cells, the use of adult stem cells in research and therapy is not considered to be controversial, as they are derived from adult tissue samples rather than left over human embryos. Adult stem cells are therefore a leading candidate for clinical application in regenerative medicine based on their accessibility, autologous status and favourable proliferative potential. In addition, adult stem cells have no ethical reservations and are easy to isolate.

Adult stem cells have been known since the sixties, when two different types of stem cells in the bone marrow were discovered: the hematopoietic stem cells (HSCs), that give rise to all types of blood and the mesenchymal stem cells (MSCs) or stromal cells that can differentiate into multiple mesenchymal tissue cell types such as bone, cartilage, adipose and muscle cells [Fig. 2].

Bone marrow-derived hematopoietic stem cells represent the earliest example of cell-based regenerative medicine, pioneered to address the needs of patients treated with total body irradiation against leukemia that developed life-threatening infections and irreversible tissue destruction. Those stem cells are defined by the expression of the CD34 surface marker and can also be obtained via peripheral blood leukapheresis for clinical

engraftment (Nelson et al., 2009). Hematopoietic stem cells did provide the basis for autologous and allogeneic stem cell transplantation and offer novel treatments for patients with cancer, autoimmune diseases, genetic diseases such as severe combined immunodeficiency and thalassemia, spinal cord injury (Mackay-Sim and Féron, 2013), liver cirrhosis (Takami et al., 2012) and peripheral vascular disease (Subrammaniyan et al., 2011). Transplant studies have also revealed engraftment of non-hematopoietic cell lineages derived from donor bone marrow, unmasking subpopulations capable of a diverse range of lineage specific differentiation.

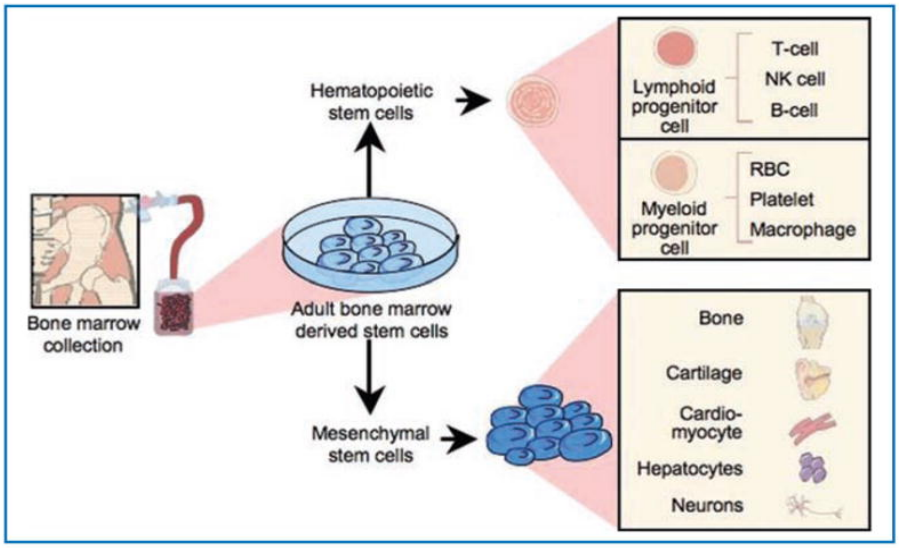

Figure 2. Adult stem cells. Adult stem cell are considered multipotent, as illustrated for bone marrow-derived stem cells that contain both hematopoietic progenitors (HSCs) and mesenchymal stem cells (MSCs); HSCs give rise to lymphoid-derived T cells, B cells, natural killer cells and myeloid-derived red blood cells, platelets and macrophages. HSCs provide the standard of care for bone marrow reconstruction. MSCs also have a diverse spectrum of differentiation that includes bone, muscle, cartilage, cardiomyocytes, hepatocytes and neurons (Nelson et al., 2009).

1.3.3 Cardiac stem cells (CSCs)

Recent evidence suggests that the heart contains a small population of endogenous stem cells that most likely facilitate minor repair and turnover-mediated cell replacement. These cells have been isolated and characterized in the hearts of rats, mice, dogs and humans (Beltrami et al., 2003). Thus, the heart's own regenerative potential opens up a new paradigm in cardiology.

The cells can be harvested in limited quantity from human endomyocardial biopsy specimens and can be injected into the site of infarction to promote cardiomyocyte formation and improvements in systolic function (Messina et al., 2004). Separation and expansion *ex vivo* over a period of weeks are necessary to obtain sufficient quantities of these cells for experimental purposes.

Experimental results suggested that CSCs are able to differentiate into three major cardiac cell populations, including cardiomyocytes, smooth muscle cells and endothelial cells (Beltrami et al., 2003; Zhao and Huang, 2013). All together, CSCs may be the optimal stem cells to use in the regeneration of the myocardium for the treatment of heart failure (Messina et al., 2004).

1.4 Cells of our interest: Adult stem cells

Adult stem cells have been found in almost all organs: brain, bone marrow, blood vessels, skeletal muscle, skin and liver but their number is very low. Stem cells reside in specific niches in each organ, remain quiescent for a long time and, when activated in case of injury, they begin to proliferate, migrate out of the niche and undergo differentiation. Such niches contain also specialized cells that secrete several growth factors and organize the extracellular matrix for the maintenance of the ability of stem cells to self-renewal.

Adult stem cells include a large population of progenitor cells derived from non-embryonic tissues such as bone marrow, adipose tissue as well as a variety of other organs. Stem cells exhibit three main properties. First, they possess high proliferation capacities. Second, they exhibit self-renewal

abilities, which allow them to preserve a population of undifferentiated stem cells while proliferating and differentiating. Third, they are able to differentiate towards multiple lineages and to repair damaged tissues. Our body harbours a limited number of stem cells that may differentiate into one or several mature cell types when the surrounding tissue is damaged or lost (Ong and Sugii, 2013). However, in many clinically relevant cases, the tissue damages exceeds the body's natural repair potential, requiring cell-based medical intervention involving sufficient amounts of repair-competent cells. In this regard, due to their accessibility and to their differentiation and proliferative potential, adipose derived adult stem cells (ASCs) are currently the best candidates for clinical applications in regenerative medicine involving autologous cells.

1.5 Adipose Tissue

Almost every animal species has a way to store excess energy in the form of fat. This is mainly achieved in a mesodermal tissue termed “white adipose tissue” in which mass triglyceride storage takes place under hormonal control (Cousin et al., 2006). However, white adipose tissue not only acts as a fat reservoir tissue, but is also responsible for mechanical support and thermal insulation, and it works as the largest and most efficient endocrine organ, releasing a wide range of hormones and cytokines capable of regulating immune response, blood pressure, angiogenesis or bone mass (Cousin et al., 2006). At the histological level, adipose tissue (AT) appears to be mostly composed of adipocytes (large cells with a peripheral nucleus and a cytoplasm occupied by a single drop of lipid that are held together in a framework of collagen fibres (Mazo et al., 2011). The white adipose tissue makes up most of the fat in humans and can be classified into visceral and subcutaneous AT (Oedayrajsingh-Varma et al., 2006). Several studies have pointed out that subcutaneous AT contains a greater number of multipotent cells than visceral tissue. Although the subcutaneous fat is present in all parts of the body, selected anatomical sites appear to harbour larger numbers of stem cells. In fact, higher quantities of stem cells were isolated from subcutaneous abdominal region as compared to thighs and hips (Aust et al., 2004).

From an embryological point of view, human adipose tissue is derived from the mesodermal germ layer and contains a supportive stromal vascular fraction (SVF) that can be easily separated from the surrounding tissue (Baer and Geiger, 2012). The SVF is composed of a heterogeneous mixture of cells that can be isolated by enzymatic digestion or density gradient centrifugation among which are cells interesting for adipogenesis and regenerative medicine, including the precursors of adipocytes, endothelium, pericytes and adipose-derived stromal cells. Hence, the SVF is not homogeneous, but rather a mixture of several cell populations that can be characterized by flow cytometric analysis of cell-surface clusters of differentiation (CDs). We previously demonstrated that the SVF includes not only multipotent stem cells (CD44, CD73 and CD90), but also hematopoietic (CD11b, CD34 and CD45) and endothelial cells (CD31, CD133) (Tallone et al., 2011).

Adipose Stem Cells (ASCs) are a subpopulation of stromal cells found in subcutaneous and visceral fat tissues. The main advantage in using AT compared to bone marrow, is that AT can be obtained in much larger amounts and with a far less-invasive surgical operation (i.e. liposuction). More importantly, the number of ASCs in harvested fat is approximately 40-times higher than that of bone marrow MSCs. Furthermore these cells exhibit a high proliferation capacity, self-renewal and the ability to differentiate into different cell types and repair damaged tissue. All these features have attracted the attention of many researchers involved in regenerative medicine, particularly in the field devoted to restoring function of the damaged heart.

Furthermore, many groups have shown that adult stem cells derived from white adipose tissue can differentiate along multiple pathways raising great hope in regenerative medicine as AT can be an abundant source of therapeutic cells (Schäffler and Büchler, 2007). Recently, human adipose derived stem cells were reprogrammed successfully into embryonic stem cell-like colonies (induced pluripotent stem cell, iPS) faster and more efficiently than adult human fibroblasts using the strategy developed by Yamanaka and co-workers (Amabile and Meissner, 2009; Sun et al., 2009).

As a result, ASCs represent major candidates for tissue engineering application.
ASCs are very appreciated in the plastic and reconstructive surgical procedures, where it is apparent a shift toward tissue-engineering strategies using stem cells (Tanzi and Farè, 2009).
Available reconstructive surgery using synthetic materials or autologous fat transplants is often unsatisfactory, due to the unpredictability of volume maintenance at long term. Transplanted ASCs may overcome the problems.
Similar applications of ASCs are also widely desired for cosmetic purposes. So, the clinical use of autologous ASCs has been reported across the globe. For example a novel transplantation strategy, termed cell-assisted lipotransfer (CAL), which involve the enrichment of ASCs in graft, has been used with promising results by Yoshimura and co-workers (Yoshimura et al., 2008, 2009). In Europe, Rigotti and co-workers reported the use of ASCs to treat the late side effects of radiotherapy (Rigotti et al., 2007) and in USA Coleman and co-workers reported the use of fat grafting for soft tissue repair (Coleman and Saboeiro, 2007).

1.6 Mesenchymal Stromal Cells (MSCs)

More than 40 years ago Friedenstein and colleagues demonstrated that bone marrow (BM) contains a population of hematopoietic stem cells (HSCs) and a rare population of plastic-adherent cells (1 in 10000 nucleated cells in BM), initially referred to as stromal cells and are now called mesenchymal stromal cells (MSCs). MSCs are spindle-shaped, fibroblast-like multipotent stem cells. MSCs are a subpopulation of SVF and can be identified by the criteria proposed by The International Society for Cell Therapy. They include:

1. the ability to adhere to plastic in standard culture condition and proliferate *in vitro*;
2. the expression of the surface molecules CD73, CD90 and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 surface molecules as assessed by fluorescence-activated cell sorter analysis;

3. the ability to form quantifiable colony-forming unit-fibroblasts (CFU-Fs), round colonies resembling fibroblastoid cells;
4. the capacity for *in vitro* differentiation into osteoblasts, adipocytes, chondroblasts and other tissues of mesodermal origin [Fig. 3].

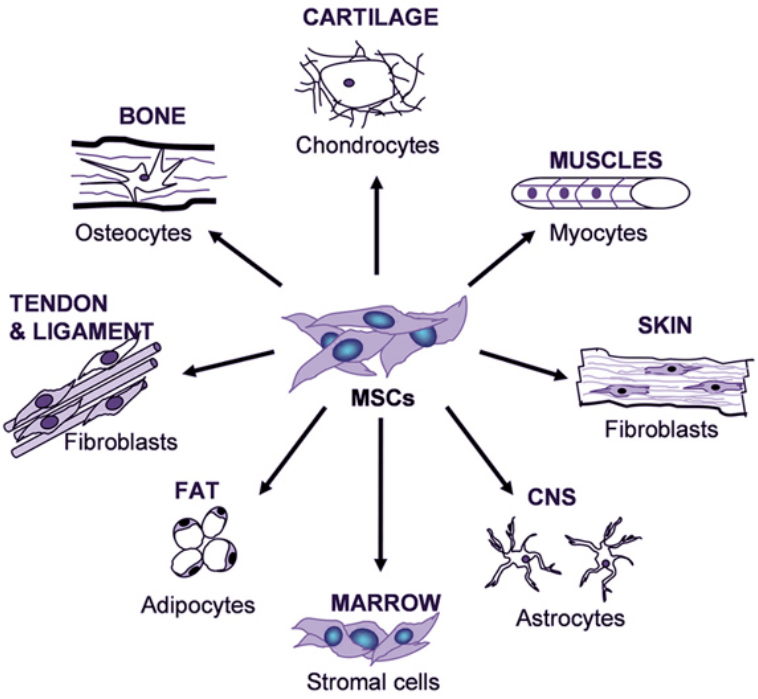

Figure 3. Mesenchymal Stem cells. A representation of the ability of these cells to differentiate into different cell types (*from sci-therapies.info*).

MSCs are distributed throughout the body. The major limitation in their use is the difficulty to identify them. In the literature there are papers that describe MSCs growth characteristics and their ability to differentiate into different cell types (Dominici et al., 2006), but this functional characterization is not sufficient to uniquely identify MSCs as required by the protocols used in the medical field. Furthermore, several studies have attempted to determine the expression of specific markers (CDs: clusters of differentiation) on MSCs (Dominici et al., 2006). Despite conflicting data, it

is interesting to note that the MSCs isolated from various tissues have all a similar immunophenotype: MSCs do not express CD11 (i.e. marker of immune cells) or CD45 (i.e. marker for cells of erytropoietic lineage). Similarly, CD31, expressed by endothelial cells, is not expressed by MSCs.
Several markers have been reported in the literature and different combinations have been used by each research group to identify MSCs populations. Stro-1 has been the best known marker to identify MSCs for several years; cells negative for this marker were unable to form colonies and a preparation of bone marrow enriched with a population of Stro-1 positive cells, increased the frequency of CFU-F. Despite this, it is not possible to use Stro-1 as a general marker for the identification of MSCs anymore, as it has been shown that it is not expressed by this cell type and is lost during *in vitro* expansion. Presently CD73, a protein involved in vascular adhesion, seems to be a promising marker for MSC.
The MSCs were found in a variety of tissues: mesodermal tissues such as adipose tissue and bone marrow, and more recently also in non-mesodermal tissues such as brain, synovial tissue, umbilical cord blood, peripheral blood, kidney and lungs. It is important to note that MSCs have variable growth potential, but all have similar surface and mesodermal differentiation potential. Many attempts have been made to determine specific cell-surface antigen profiles (immunophenotype) for more specific identification of MSCs. Particularly relevant is the question of whether MSCs isolated from different tissues can be identified by the same immunophenotype, i.e. whether all these cells have common ancestors or at least share similar properties. Researchers showed that the morphology and the phenotype of MSCs from different sources are in fact rather similar. This has led to develop a theory according to which in every tissue there are microenvironments (niches) sharing similar characteristics. These niches are isolated from the surrounding environment and harbour MSCs that are kept in an undifferentiated state until they are required to exit the niche. The niche consists of various extracellular matrix elements and additional cells that surround the MSCs and keep them in their undifferentiated stage, until a stimulus occurring at the level of niche drives the MSCs to proliferate and differentiate (Kolf et al., 2007). Figure 4 shows

schematically a hypothetical stem cell niche. A niche is constituted by a precise number of mesenchymal cells that are located in a specific perivascular microenvironment. These cells are closely associated with differentiated support cells (DC) and an extracellular matrix (ECM). The supporting cells communicate with mesenchymal cells through cell-cell interactions and secrete paracrine factors. It seem that also a slight hypoxia is favorable to the MSCs to maintain the undifferentiated stage.

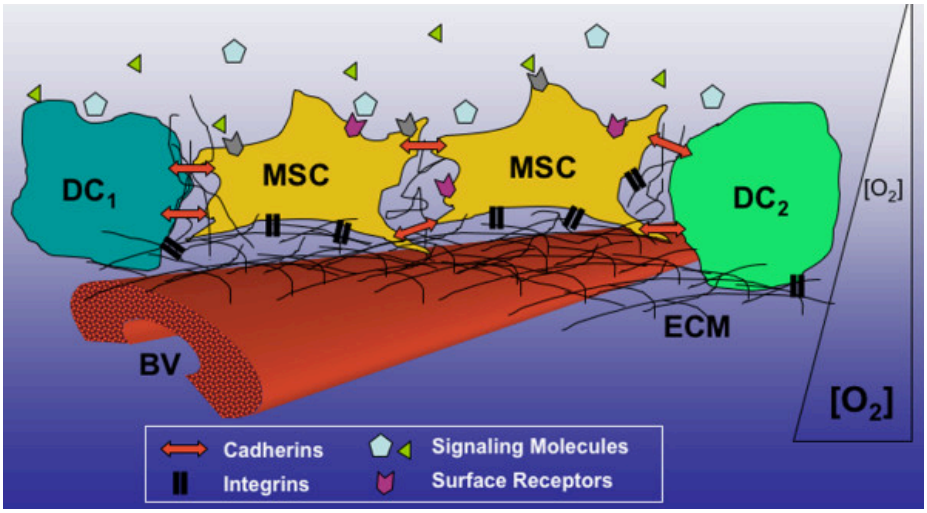

Figure 4. Mesenchymal Stem cell Niche. A niche is constituted by a precise number of MSCs located in a specific perivascular microenvironment (BV, blood vessel). These cells are associated with differentiated cells (DC) and extracellular matrix (ECM) (Kolf et al., 2007).

Again, MSCs are not a homogenous population, but rather a heterogeneous mixture of cells with variable proliferation and differentiation potentials and with different morphology and expression of surface antigens correlates with their functions.

The identification of specific signalling networks and “master” regulatory genes that govern unique MSCs differentiation lineages remains a major challenge. The ability to modulate biological effectors that drive a desired differentiation program, or that possibly prevent spurious differentiation of

MSCs, is needed for effective clinical applications, as for tissue engineering and regeneration.
MSCs can be influenced via a multitude of growth factors and, accordingly, a number of growth factor receptors have been identified on their surface. Indeed, membrane receptors and their ligands are relevant for a number of metabolic cell functions. EGFR, βFGFR, TGFRI and TGFRII were reported to be important for MSCs self-renewal and differentiation. MSCs express a wide set of cytokine receptors: IL-1R, IL-3R, IL-4R, IL-6R, IFNgR and TNFI and IIR. Recent evidence demonstrates that MSCs stimulated by TNFa and IL-6 are able to invade and migrate through basement membrane-like matrigel. Furthermore, some researchers reported expression of Notch 1, 2 and 3 in MSCs. The Notch pathway is particularly relevant in this context because it is considered the gatekeeper of stemness. Activation of Notch by its ligand, triggers proteolytic cleavage and release of the Notch intracellular domain, which enters the cell nucleus and alters gene expression (Niessen and Karsan, 2007).
Experimental evidence suggests that ASCs are located at the level of vascular precursors. It is well known that the SVF contains progenitor cells that can differentiate in endothelial cells thus participating in the formation of blood vessels.
Immunohistochemical studies have shown that CD34 positive cells are predominantly associated with blood vessels of small and medium caliber (50-150 μm) present in adipose tissue; the expression of this marker remains the same along the section of the vessel from intima tunica to inside of the adventitia tunica, while the expression of CD31 decreases. Many papers have shown that the very high CD34 expression in SVF decreases rapidly during the first passages of cell culture. However, this loss of expression is due to a negative regulation of the expression, not to the death of CD34 cells (Tallone et al., 2011).

Human MSCs express moderate levels of human leukocytes antigen (HLA) major histocompatibility complex class I, lack major histocompatibility complex class II expression and do not express co-stimulatory molecules B7 and CD40 ligand. Tolerance of MSCs in an allogeneic transplant is due to

this unique immune-phenotype coupled with powerful immunosuppressive activity via cell-cell contact with target immune cells and secretion of soluble factors, such as nitric oxide and heme oxygenase-1. MSCs have immune-modulatory functions by interacting with both innate and adaptive immunity. The innate immune cells (neutrophils, dendritic cells, natural killer cells, eosinophils, mast cells and macrophages) are responsible for a nonspecific defence to infarction and MSCs have been shown to suppress most of these inflammatory cells.
A remarkable property of MSCs is their powerful ability of regulating immune response. As a result, current MSCs-based therapy has mainly been applied to alleviate immune disorders. Various studies have evaluated the therapeutic effect of MSCs in preclinical animal models and demonstrated great clinical potential (Ren et al., 2012).

Graft-versus-host disease (GvHD) is a severe complication following bone marrow and HSC transplantation. It has been shown that more than 40% recipients of bone marrow transplantation develop GvHD and many of them are hard to treat even with steroids (Kernan et al., 1993). In recent years, MSCs have been successfully applied to treat GvHD in mouse models. One or two infusions of MSCs after bone marrow transplantation improved the survival rate of GvHD mice and dramatically reduced immune cell infiltration in various organs. These studies also demonstrated that pro-inflammatory cytokines are critical in MSCs-mediated immunosuppression *in vivo*, as elimination of interferon-γ (IFNγ) signalling diminishes the therapeutic effect of MSCs (Polchert et al., 2008; Ren et al., 2008).

In the treatment of autoimmune type I diabetes, MSCs significantly delayed diabetes onset in non-obese diabetic (NOD) mice. MSCs infusion protected islets from destruction, as evidenced by insulin staining, lymphocyte infiltration and islet morphology.

1.7 Cardiovascular disease (CVD)

Cardiovascular disease (CVD), which include hypertension, coronary heart disease (CHD), stroke, and congestive heart failure (CHF), are the leading and most common causes of mortality in many developing and industrialized countries, accounting for one third of all deaths globally (Thom et al., 2006). The American Heart Association reported that cardiac failure was responsible for 38% of all deaths in recent years. Nearly half of all deaths associated with CVD are the result of heart failure due to ischemic cardiomyopathy events such as myocardial infarction (MI), a pathology characterized by clot formation within a coronary artery, which prevents blood flow to the region of the heart distal to the site of occlusion. Acute myocardium infarction (MI) and all heart injuries can lead to immediate death, due to loss of oxygenation in ventricular muscle, usually by occlusion of a coronary artery, which quickly results in ischemia/reperfusion injury and necrosis of the tissue. In the best case, living patients face progressive deterioration of their condition over several years, ultimately resulting in heart failure. This failure stems from a lack of intrinsic regenerative responses able to replenish the vast amounts of lost cardiomyocytes and results in scar formation and rapidly compromised heart function (Rosenthal and Harvey, 2010).

The prevalence of CVD is expected to increase to three fourths of all deaths by 2020 in part due to an increasing prevalence of risk factors related to coronary disease, such as obesity and sedentary lifestyle (metabolic syndrome), making CVD an even greater public health concern. Both of these conditions result in death of cardiomyocytes by apoptosis and/or necrosis. Since cardiomyocytes rarely divide, contractile cells cannot repopulate the infarct area, while non-contractile cells (fibroblast) gradually replace them (Holmes et al., 2005; Leor and Cohen, 2004; Sun and Weber, 2000). Moreover healthy myocytes close to the infarcted area may also die, causing an increase of the necrotic tissue area. Dead cardiomyocytes are replaced by fibroblasts that divide and migrate into the damaged area to form scar tissue [Fig. 5]. This process involves the left ventricle and it is called left ventricle remodelling (LVR). During LVR, the ventricle wall becomes thinner and structural changes occur (Holmes et al., 2005; Leor

and Cohen, 2004)(Zhu et al., 2009). Changes in size and shape of left ventricle can be observed: an enlargement of the heart chamber takes place and the sphericity index (ratio of the long-axis length divided by LV short-axis length, both during systole and diastole) increases. LVR renders the heart more inclined to dysfunctions and to failure. LVR may also be associated with an increased risk of ventricular arhythmias (Zamilpa and Lindsey, 2010).

The persistence of scar tissue following myocardial infarction suggests that the heart has little if any capacity to generate new cardiomyocytes.

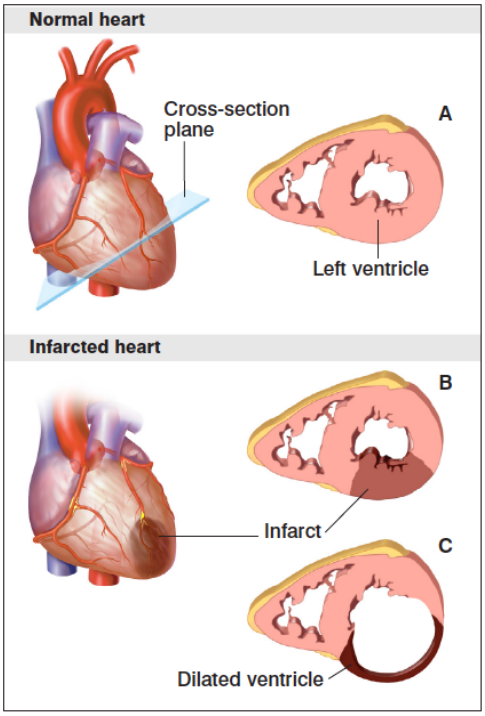

Figure 5. Normal vs Infarcted Heart. The left ventricle has a thick muscular wall, shown in cross-section in A. After a myocardial infarction (heart attack), heart muscle cells in the left ventricle are deprived of oxygen and die (B), causing the ventricular wall to become thinner (C) *(from Regenerative Medicine - Prometheus)*.

The design of future cell based therapies for CVDs appears to be a challenge due to the peculiar functional and anatomical properties of the heart. Cardiomyocytes merely represent approximately 30% of the ventricular cell population. Transmission and scanning electron microscopy have identified five types of non-muscle cells within the adult myocardium:

endothelial cells, fibroblasts, pericytes, smooth cells, and macrophages, which comprise approximately 65-70% of ventricular cells. In addition to these cells types, the 3D extracellular matrix (ECM) of the myocardium plays an important role in its function. The myocardium ECM consists of collagen, fibronectin, elastin and laminin as well as various growth factors, proteoglycans and glycosaminoglycans. Collagen is the most abundant ECM component in the heart and contributes to structural stability and the integration of cardiomyocytes and myofibrillar bundles. The various components of the myocardium, cells and matrix, play important and very complex roles in both the healthy and diseased heart.

Unlike most other cell types, cardiomyocytes, the functional components of the heart muscle, are incapable of replication, or do so on a very small scale. As a result, when the heart is damaged, as in the case of a myocardial infarction, there is a limited internal mechanism for repair and the patient is left with decreased cardiac function that rarely improves over time.

The adult heart has been generally considered as a terminally differentiated organ (Beltrami et al., 2001). Cardiomyocytes were assumed to be fully post mitotic cells. They possess a very complex and well-developed cytoskeleton consisting of hundreds of sarcomeres, which physically impede mitotic spindle formation and tight cell-cell junctions necessary for synchronous beating (GAP junction). Adult cardiomyocytes are often multinucleated and polyploid. Beltrami an co-workers in 2001 demostrated that the adult heart possesses a population of cardiomyocytes with proliferative potential (Beltrami et al., 2001). This pool of cells was clearly insufficient to heal the heart after myocardial infarction and seemed to be more relevant for long-term homeostatic maintenance of heart function. These observations gave impetus to the efforts focusing on finding and activating cardiac-resident stem cells, on the transplantation of *in vitro* stem cell-derived cardiomyocytes, and on the administration of growth factors and cytokines, aimed at improving vascularization, preventing scar formation and inducing cardiomyocytes proliferation.

Conventional therapies are largely palliative in nature and do not significantly contribute to cardiac repair, as they only help to alleviate or

prevent additional damage by factors that contributed to the initial damage, such as hypercholesterolemia and atherosclerosis (Palpant and Metzger, 2010). An alternative for cardiac diseases is total heart replacement or mechanical implants. Heart transplantation and artificial hearts are effective treatments for loss of organ and tissue function. They are associated, however, with serious problems such as a critical shortage of donor organs, rejection, the need for life-long immunosuppression, and incomplete biocompatibility. These limitations have led to the exploration of alternative biological options through stem cell-based therapy for repair of the damaged cardiac tissue.

One of the main long-term goals is the *de novo* cardiomyocyte production to provide a source of autologous cardiomyocytes for cell replacement in damaged heart. Many forms of heart disease, including congenital defects and acquired injuries, are irreversible because they are associated with the loss of non-regenerating, terminally differentiated cardiomyocytes.

Developmental biology and available data on the differentiation of embryonic and adult stem cells clearly indicate that the process of forming a heart requires many carefully orchestrated signals, some of which originate outside the pre-cardiac mesoderm and some of which originate in the developing heart. During gastrulation the heart is generated from a small number of mesodermal progenitor cells. These progenitors undergo complex and dynamic processes: they migrate, proliferate and differentiate to form a linear heart tube, the primitive form of a functional heart. During these processes, cells initially acquire generic cardiogenic phenotypes typical of cardiac progenitors and later exhibit more specific properties reflecting their final differentiation into for instance atrial and ventricular cells.

Cardiomyocytes regeneration is limited in adult life and therefore the identification of a putative source of cardiomyocyte progenitors is of great interest to provide usable *in vitro* models and new perspectives for regenerative therapies.

1.8 Clinical Trials based on the use of stem cells

The use of stem cells to improve recovery of the injured heart after myocardial infarction (MI) is an important emerging therapeutic strategy. In recent years, several clinical trials that use stem cells have been developed using different approaches.

The majority of clinical trials based on stem cell therapy for heart disease used Bone Marrow cells (BM), in particular mononucleated cells (MNCs). In other trials other types of stem cells were tested and used as source to be delivered by intracoronary infusion, including specific bone marrow CD34+ or CD133+ and umbilical cord blood cells (Hoover-Plow and Gong, 2012).

Another approach is based on the use of "induced pluripotent stem" (iPS) cells. This source relies on *in vitro* de-differentiation of adult cells to embryonic-like stem cells and their reprogramming using specific culture conditions to induce cardiac lineage cells including cardiomyocytes, smooth-muscle cells and endothelial cells. In the past, the most commonly used adult cells for iPS were fibroblasts derived from a variety of tissues such as epidermis, liver, stomach, pancreas and neural as well as hematopoietic cells. Nowadays, in the new trials, instead, other types of stem cells are tested, including mesenchymal stem cells, adipose tissue derived stem cells and cardiac progenitors stem cells.

There are two clinical trials that use freshly isolated adipose-derived cells (ADCs) in patients with ischemic heart failure: the PRECISE study (Barnett and Van den Hoff, 2011) and, in patients with acute myocardial infarction, the APOLLO Trials (Mazo et al., 2012). Preliminary data obtained from these studies showed an improvement in LVEF, reduced infarct size and improved myocardial perfusion.

A step ahead of these trials is MyStromalCellTrial that uses culture-expanded-adipose-derived stromal cells (ASCs) in patients with chronic ischemic heart disease and refractory angina (Qayyum et al., 2012).

Although it has been believed for a long time that cardiac myocytes were terminally differentiated, dividing myocytes found in the heart implied that

there are resident or non-cardiac cardiomyocytes progenitor cells. There have been intensive efforts to identify the cardiomyocyte stem and progenitor cells in the last 10 years. At present, there are four ongoing clinical trials to test autologous cardiac progenitors cells (CPCs): one study (ALCADIA) uses cardiac-derived SCs to treat ischemic cardiomyopathy and two studies take advantage of the cardiosphere-derived stem/progenitor cells (derived from cell outgrowth of autologous cardiac biopsy) for patient with a recent MI (CADUCEUS) or heart failure (TICAP). In the SCIPIO trial, patients with ischemic cardiomyopathy are treated with c-kit+lin- CPCs derived from the right atrial appendage.

It is thus clear that there are many ongoing clinical trials that use different methods [Fig. 6]. Although the use of cardiac progenitor cells (CPCs) and bone marrow derived stem cells represent a good therapeutic choice, their retrieval requires an invasive surgical intervention.
Due to easily accessible anatomical location and the abundant existence of subcutaneous adipose tissue in the abdominal region, ASCs hold the advantage of a simple and less invasive harvesting technique. Adipose tissue normally allows the extraction of a large volume of tissue with a limited morbidity under local anesthesia. Moreover preclinical studies indicate that ASCs can differentiate into endothelial cells, thus promoting angiogenesis, or into cardiomyocytes, thus replacing the injured myocardium.
Beside the hypothesis of replacement and regeneration by trans-differentiation of freshly ASCs into tissue specific cells, the paracrine hypothesis has gradually gained credibility, where paracrine stimuli of stem cells lead to the replacement of injured tissue.

We believe that a viable approach to myocyte regeneration has to be safe and feasible and could use expanded ASCs already committed to cardiomyogenic fate. This strategy using committed stem cells could also improve the quality of life and increase life expectancy in patients.

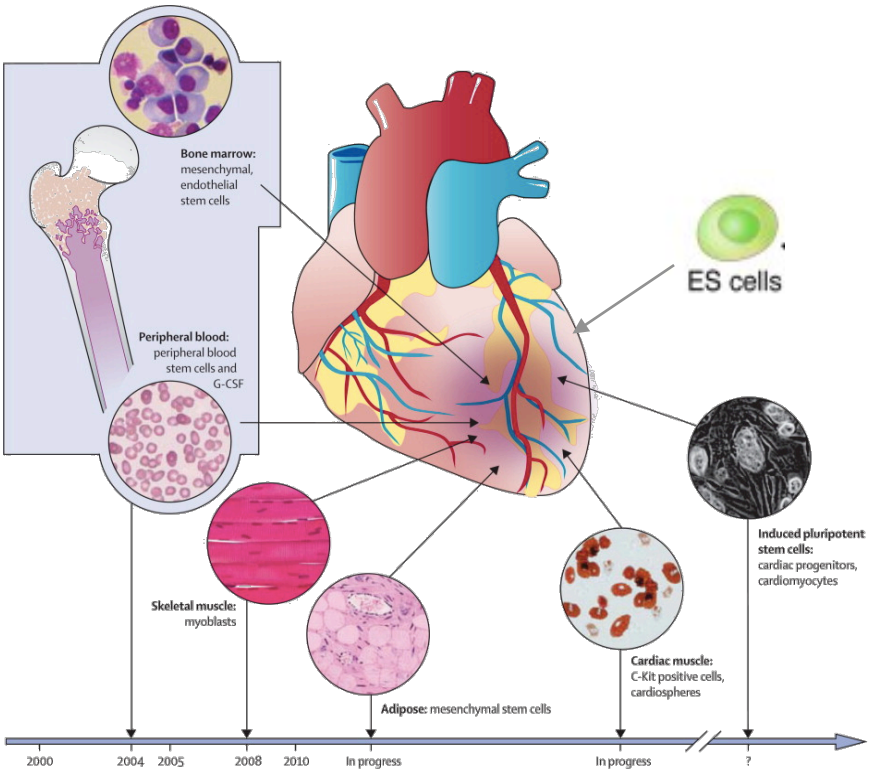

Figure 6. Clinical Trials. The timeline describes the main cells source used in clinical trials in cardiovascular disease (*from peixun114.net*).

1.9 Embryonic cardiac development

The heart is generated from a number of progenitors cells that are derived from mesoderm during gastrulation. These cells undergo complex and dynamic processes, migrating, expanding and diversifying to acquire a linear heart tube that is the primitive form of functional heart. During this process, progenitors acquire cardiogenic phenotypes. There are a lot of factors that are important during early cardiac specification.

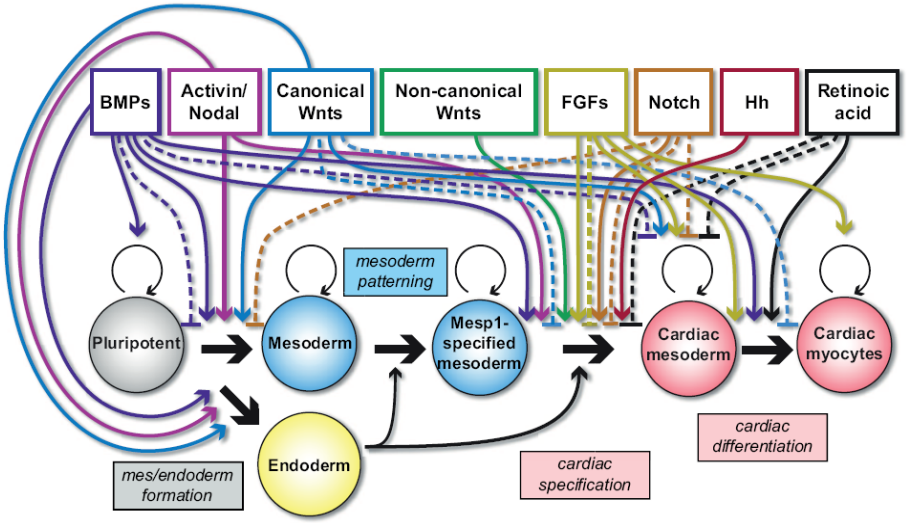

Figure 7. Crucial biochemical pathways involved in cardiac muscle formation. Signaling pathways involved in cardiogenesis were identified using the power of experimental embryology. This picture underlines the pioneering work performed identifying the pathways involved in cardiac specification and development (*Noseda et al., 2011*)

The picture in figure 7 shows the crucial biochemical pathways involved in cardiac muscle formation during embryogenesis and stem cell differentiation. Indeed, this chart helps to identify growth factors and cytokines that, acting on the main cardiac differentiation pathways, could be used in the induction cocktail to obtain functional cardiomyocytes physiologically indistinguishable from cardiomyocytes derived from cardiac tissue.

In the developing embryo, the cardiac precursors are specified by a tightly controlled environmental signalling. Understanding how different signalling pathways dictate cardiac precursor specification is critical for the *in vitro* generation of such cells and for the prediction of how they will behave after transplant. In many developmental systems, Nodal, Activin, TGF-β, bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) promote cardiogenesis (Behfar et al., 2008).
Three main families of peptide growth factors have been identified to be essential for heart development and speciation. They are equally important in the mammalian heart development, acting either as positive or negative regulators. These factors are the bone morphogenetic proteins (BMPs), members of the transforming growth factors b superfamily, the wingless-related proteins (Wnt) and the fibroblast growth factors (FGFs). In fact, several members of all of these families or their inhibitors are expressed in the endoderm. A disrupted expression of these ligands, of their receptors or of their downstream target genes has a dramatic and distinct effect on cardiac development.

BMPs control several major cardiac specific transcription factors, including NKX2-5. BMP2 and BMP4 are co-expressed spatially and temporally with GATA4 and NKX2-5 in the pre-cardiac mesoderm, suggesting again that they may control the expression of cardiac transcription factors.
Fibroblast growth factors (FGFs) signalling are involved in the induction and formation of the mesoderm in all vertebrate species. FGF1, 2 and 4 are secreted by the portion of the developing embryo that is necessary for cardiac development, although their exact role is not clear. However, it is clear that they cooperate with BMPs to induce cardiomyogenesis.
Another family member, FGF8, is expressed in the cardiac mesoderm. Again, BMPs seem to cooperate with FGF8 in heart induction and morphogenesis.
Wingless (Wnt) proteins in vertebrates are involved in highly complex networks of developmental pathways that include cardiac specification. Wnt were initially considered as suppressors of heart formation, but both induction and inhibition were lately reported. The “canonical” Wnt acts

upon inhibition of GSK3, thereby allowing the nuclear localization of β-catenin, and appears to inhibit the cardiac differentiation. Instead, the "non-canonical" Wnt involves protein kinase C and seems to enhance the cardiac differentiation. Supporting this, the inhibition of the "canonical" Wnt signaling increases the cardiac differentiation and conditional deletion of β-catenin from the definitive endoderm of the mouse results in the formation of multiple ectopic hearts. The presence of Wnt antagonists induces cardiomyocyte differentiation in non-heart-producing mesoderm. Wnts 3a and 8 block NKX2-5 and TBX expression and therefore cardiomyocyte differentiation. Recent findings showing that β-catenin signalling must be blocked for heart valve formation in zebrafish also demonstrate a negative role for Wnt in the development of the heart.

Recent findings have demonstrated that canonical Wnt signalling plays distinct time-specific roles during cardiogenesis, regulating the specification, maintenance and differentiation of cardiac progenitors cells (CPCs) distinctly. According to one study, a canonical Wnt activity blocks the specification of CPCs, promote CPCs proliferation and stops the differentiation of CPCs into cardiomyocytes during ESCs differentiation. Taken together these informations demonstrate divergent time-dependent roles for canonical Wnt signaling.

Multiple signalling pathways are integrated and cross-regulated during cardiac differentiation. By specifically activating and blocking their activity, Activin, Nodal and Wnt activities were shown to be essential for cardiac specification of mesoderm, whereas BMPs (bone morphogenetic proteins) promote mesoderm formation through a Wnt/Activin/Nodal-dependent mechanism(Alexander and Bruneau, 2010; Willems et al., 2011a).

1.10 Main growth factors and molecules that controls cardiogenic cell fate

Development is defined as a process by which a body is formed from a cell by repeated cell divisions, called mitoses, and by the differentiation of the cells derived therefrom, that acquire the typical structures of their species (Noseda et al., 2011). The development depends at a large extent on the effects of a group of cells over another one. Those effects are exercised

during embryonic induction, which involves the release of a factor that induces a signalling centre and that acts on a group of responding cells. There are several families of such molecular factors that are known as important growth factors, cytokines or hormones.

To direct the differentiation of MSCs to the cardiac lineage we have to follow two specific steps:

1- induction and specification of cardiac mesoderm
2- expansion of the cardiovascular lineages

To achieve this, we selected and used a specific combination of different factors that are known to play a role in the development steps and examined several components of Transforming Growth Factors-β family (TGF-β) for their ability to promote expression of the principal cardiac transcription factors. This family includes:

- ✓ TGF-β
- ✓ Activin/Nodal
- ✓ BMP (bone morphogenetic proteins)

TGF-β starts the signal transmission by binding to receptor serine/threonine kinases type I and type II on the cell surface. Signalling propagates through intracellular Smad protein phosphorylation. Smad is translocated into the nucleus where it regulates the transcription of target genes. TGF-β family play evolutionarily conserved roles in inducing mesoderm and ectoderm (Yang et al., 2008).
Nodal has a graded influence at this time: high levels induce definitive endoderm and anterior mesoderm, including cardiac mesoderm, while lower levels favour more lateral and posterior derivatives. Thus, specification of cardiogenic cells requires a particular dose in a temporal window (Brennan et al., 2001)(Mercola et al., 2011). Similarly, biphasic effects exist for BMPs and Wnts.
BMPs, in particular BMP2 and BMP4, are needed during mesodermal patterning but not subsequently for differentiation of committed precursors. In short, exposure to these factors must be tightly regulated.

Recent studies have shown that the combination of BMP4 and Activin A promotes cardiac development in human ESCs (Kattman et al., 2011; Teo et al., 2012).
Extracellular inhibitors of BMPs and Wnts are the principal inducers that commit multipotent progenitors to a cardiac fate, as marked by heart-forming transcription factors NKX2-5 and MEF2C.
Because studies have shown that stage-specific inhibition of canonical Wnt signalling is required for cardiac development (Marvin et al., 2001), we investigated the role of this pathway in the emergence of the cardiac lineage from MSCs.

Willens and co-workers, by screening approximately 550 pathway modulators, identified a small-molecule inhibitor of the β-catenin-dependent canonical Wnt pathway and showed that Wnt inhibition was sufficient to drive the hESCs-derived mesoderm to a cardiac fate in the absence of other signalling modulators (Willems et al., 2011b). Furthermore, Wnt inhibition drove cardiomyocytes specifically and did not increase other mesoderm lineages, which suggests that Wnt inhibition specifically drives a mesoderm progenitor toward cardiomyocytes. Willens and co-workers investigated also diverse Wnt inhibitors that target different cellular components of the pathway. All of the small molecules were much more cardiogenic than the natural inhibitors Divkkopf-1 (DKK1).
In particular, they tested IWP, an inhibitor that prevents cells from producing Wnt and IWR-1 a recently published inhibitor of the canonical Wnt signalling response.

Once the heart compartment is defined, proliferation and fate of cardiac progenitors are controlled by signals such as FGFs and Notch. The latter is particularly fascinating because it acts at different times in nearly all tissues within the developing cardiovascular system. During specification of cardiogenic cells, Notch suppresses cardiogenesis, as seen in ESCs cultures (Mercola et al., 2011; Rones et al., 2000). Members of the fibroblast growth factors (FGFs) family play a critical role in the induction of the mesodermal

germ layer during the early stages of embryogenesis. FGFs are also required for the development of certain dorsal types of mesoderm. Therefore, bFGF could be added to support the continued expansion of the developing cardiovascular lineages.
A diaminopyrimidine (i.e. Cardiogenol C) could also be tested in the cocktail because it is the most potent inducer of MHC expression. To confirm that this compound is also a cardiomyogenesis inducting agent, Wu and co-workers analysed its effect on undifferentiated R1 mouse ESCs that are maintained in a pluripotent state with the addition of leukemia inhibitory factor (LIF) in the culture medium (Wu et al., 2004). After 7 days, the presence of beating cardiac muscle was visible under a microscope and cells expressed the cardiac muscle cell specific transcription factors MEF2C and NKX2-5.

1.11 Markers for cardiogenic differentiation

1.11.1 Transcription factors

Cardiomyocyte-specific transcription factors are relevant tools to assess the degree of differentiation achieved in our *in vitro* system.
Members of the Nk family of transcription factors are expressed in all animals with contractile vascular cells and hence are crucial for myocardial development (Bartlett et al., 2010); NKX2-5 is specifically required for left ventricular chamber development (Zhao et al., 2012).
Gata is another family of transcription factors that interacts with Nk factors to promote differentiation of cardiomyocytes, smooth muscle cells and endoderm, and plays an important role in cardiac development. Furthermore, GATA4 regulates myocardial expression and is required for fusion of the heart tubes in the ventral midline. GATA4 gene knockout studies demonstrate the essential role of this factor in cardiac development and coordination of the expression of downstream cardiac genes (Armiñán et al., 2010). Gata5 is required for endocardial differentiation.
The implication of NKX2-5 and GATA4 transcription factors is well documented (Armiñán et al., 2009). These factors trigger the cardiac genetic program in both embryonic stem cells (ESCs) and adult stem cells,

recapitulating the molecular mechanism that operates during embryonic heart development. During embryonic heart development, the expression and activity of the transcription factor NKX2-5 is regulated by members of the BMPs and TGF-β signalling molecule families. This signalling mechanism is reproduced when stem cells are treated with a cocktail of differentiated factors. The outcome of this signalling machinery is the activation of several terminal cardiac differentiation genes, including gap junction, the α-sarcomeric actin (α-SA), the β myosin heavy chain (β-MHC).

T-box genes also play an important role in cardiac morphogenesis and TBX5 is required for atrial septation. Another important transcription factor that should be considered is BAF60C a subunit of the BAF complex. It is expressed in the developing heart and it is required for cardiac morphogenesis and establishment of left-right asymmetry. The addition of BAF60C to a cocktail of cardiac transcription factors (GATA4, NKX2-5 and TBX5) resulted in induction of beating cardiomyocytes from non-cardiogenic mesoderm (Ohtani and Dimmeler, 2011; Takeuchi and Bruneau, 2009). Finally, BAF60C reinforces the function of cardiac transcription factors such as GATA4 and TBX5 by acting as a bridge between transcription factors and target genes.

The myocyte-specific enhancer factor 2C (MEF2C) is also involved in cardiac morphogenesis, myogenesis and vascular development. Mice without a functional copy of the MEF2C gene die before birth and have abnormalities in the heart and vascular system (Bi et al., 1999). Other important genes are (1) Mesp1 (Bondue et al., 2008), which has been described as "the master regulator" of cardiac progenitor specification, (2) FOG2 (Friend of GATA4), a basic helix-loop-helix transcription factor transiently expressed in mesodermal populations that is physiologically associated with the N-terminal zinc finger of GATA4 and modulates its transcriptional activity and last (3) CAMTA (Calmodulin binding transcription activator 2), that is a co-activator associated with NKX2-5.

1.11.2 Structural and functional genes

Contractility is a hallmark of cardiac myocytes. Many contractile proteins are uniquely or predominantly expressed in cardiac myocytes and not in other muscle cells. These proteins include several troponins, cardiac actins and myosins. Troponin is a complex of three regulatory protein (Troponin C, T and I) that is integral to contraction in myocardium. Cardiac troponin I (TNNI3) and II are very sensitive and specific indicators of damage to the heart muscle. Actins and myosins constitute contractile fibres. Cardiac α-actin (ACTC1), myosin light chains (MYL2) and α-myosin heavy chain (MYL6) are predominantly expressed in the heart but not in others muscles.

1.12 Tissue Engineering

Tissue engineering is a field that combines principles of engineering and of life sciences for the development of biological substitutes that restore, maintain or improve the function of damaged tissues and organs. Langer and Vacanti were the first to promote the concept that cells may be seeded on artificial matrices to support formation of functional tissue-like structures that may be used to repair diseased organs (Langer and Vacanti, 1993).

The goal of this technology is therefore to expand cells *ex vivo* on polymeric biocompatible three-dimensional matrices (named scaffolds) for the repair of injury and for tissue regeneration; the full colonization of such scaffold will give rise to a pseudo-tissutal construct *in vitro*, which can be implanted *in vivo* in humans.

Particularly, the perspective to use engineered tissues as organ replacements in patients has intrigued scientists and clinicians. There is a great medical necessity to develop alternatives to current organ transplantation, given the worldwide mismatch between patients waiting for organ transplants and the availability of donor organs (Zimmermann et al., 2006).

The standard engineering methodology for the engineering for implantations in humans involves the isolation of a specific cell line using a biopsy, performed directly on a patient or on a compatible donor, and a subsequent seeding of these cells on the scaffold in order to cultivate the

construct in specific and reproducible conditions. Finally, the construct (scaffold and cells) will be placed in the patient in order to reconstruct or repair the damaged tissue. These structures represent the second bioartificial generation of replacement systems of organs and tissues. It is very important to understand and characterized the interaction of the cell with the substrate, the biomolecules transport within the matrix and the development of technologies for the control of chemical-physical and mechanical parameter (bioreactors). Furthermore, it is important to have a biodegradable and bioresorbable scaffold to allow a gradual absorption of the support after a period of time from its contact with the biological environment during the formation of new tissue engineered (Dutta and Dutta, 2009; Kawakami, 2008; Rezwan et al., 2006).

Tissue engineering/regenerative medicine (TERM) approach could be a really promising tool to induce cardiac infarcted tissue repair, avoiding heart transplantation drawbacks and risks. One of the most promising approaches in myocardial TERM is the design and fabrication of scaffolds, which are realized with synthetic and natural materials. TERM techniques based on scaffold implantation are more appropriate for the regeneration of tissues that require a mechanical support to the reparative process, such as the cardiac one, while cell therapy is more suitable for tissues with a relatively simple structure and in case of small injuries (Leor and Cohen, 2004; Obradovic et al., 2010). The use of 3D scaffolds provides some advantages over cell injection into damaged area: such a scaffold temporarily replaces the injured tissue, provides a transitional support to cell colonization, migration and proliferation and degrades with time as the need of an artificial aid decreases; scaffold implantation allows control over graft dimension, shape, mechanical properties and composition; since a myocardial infarct (MI) generally causes the loss of more than one billion cells, cell therapy would require the injection of a large number of cells. When cells are injected intra-coronary, about 90% of them are lost in the blood circulation and 90% of the successfully injected ones dies within a week (Leor and Cohen, 2004)(Curtis and Russell, 2009). Several studies have demonstrated the capability of heart patches to alleviate LV remodelling and improve cardiac function when implanted on the infarcted

region immediately or within two weeks after the MI (Fujimoto et al., 2007). More recently Liao and co-workers (Liao et al., 2010) demonstrated the capability of heart patches to attenuate LVR and improve cardiac performances (LV ejection fraction improvement) even if implanted in chronic conditions (eight weeks after the MI). Scaffolds can be used as heart patches, in order to create the optimal platform for living cells to grow and then degrade over time as the cell form their own external support structures. These substrates can be designed to enhance recruitment and differentiation of cardiac progenitors cells (CPC), by introducing bioactive molecules such as growth factors and extracellular matrix (ECM) molecules. Patches can be pre-seeded with cells or not, in approaches that can be classified *in vitro* or *in vivo*. To guide myocardial regeneration and give a mechanical support, the scaffold must have the following characteristics: long-term elasticity that matches the mechanical demands of force-generating contractile tissue; biodegradation at rates compatible with tissue regeneration; ability to support cell proliferation, migration and differentiation; porous structures to attain the desired mass transport (Engelmayr et al., 2008; de Mulder et al., 2009; Silvestri et al., 2013).

Biomaterials for TERM must satisfy requirements from a biological, chemical and mechanical point of view. These materials not only need to be cell and tissue compatible, with no inflammatory response after implantation, but also need to support cell attachment, differentiation and proliferation. One of the main physical requirements that a biomaterial has to meet is the biodegradability. Materials have to degrade *in vivo* at rates compatible with tissue regeneration, with the release of non-toxic, easily metabolized, and secreted products from the body, not inducing inflammatory response.
They have also the possibility to be processed into porous scaffolds for cell penetration, tissue growth, vascularization and nutriment delivery. Also the mechanical characteristics are important, since the heart patch should restore heart geometry, provide a mechanical support and accommodate to the rhythmic movements of the heart without plastic deformation or

failure (Christman and Lee, 2006). Since the most abundant cardiac ECM protein is collagen, Chen and co-workers (Chen et al., 2009) supposed that cells could find the most suitable microenvironment for their proliferation into a matrix with analogue mechanical properties to those of myocardial collagen.

1.12.1 Biomaterials and polymers in regenerative medicine

Biomaterials should respond to the need of functionally and metabolically replacing a no more active human tissue or organ, damaged by pathological or traumatic events. The number of their applications has grown significantly in recent years.

The performance of materials used in medical field is evaluated according to their bio-functionality and biocompatibility. The bio-functionality refers to the property that a device must have to play a specific physical and mechanical function. The biocompatibility is the ability of the device to perform the same function during the useful life of the system and refers to the interactions between the biomaterials and the tissues. The starting parameters include: cytotoxicity, cytostasis, lysis of the cell membrane, mutagenesis and carcinogenesis.

Secondary tests are designed to analyse the inflammatory and immune response. The materials are implanted for a defined period (i.e. from weeks to years) and, at the end of the established time any possible damage has to be evaluated. The materials are implanted in large animals and only after animal testing are approved for humans.

Polymers consist of very long and flexible molecules, composed by the repetition of simple units (called monomeric units) linked together through covalent bonds. Long chains are joined together by Van der Waals forces, by hydrogen bonds or by covalent bonds. A fundamental parameter of the polymer is the degree of polymerization (average number of repetitive units within a molecule), that determines the relative mobility of the chains and the mechanical characteristics of the polymer.

1.12.1.1 Natural Polymers

Natural Polymers are highly biocompatible, stimulating low *in vivo* inflammatory response and providing a natural substrate for cell attachment, proliferation and differentiation in their native state. They favour cell attachment and proliferation due to their adhesion specific sites (Jawad et al., 2007). Despite these positive features, natural polymers have poor mechanical properties, poor workability and rapid degradation kinetics, which limit their clinical application. To overcome these problems a strategy consists in blending them with synthetic polymers (Sui et al., 2011). Among natural polymers, collagen, gelatin, fibrin alginate have been mainly investigated (Silvestri et al., 2013)

1.12.1.2 Synthetic Polymers

Synthetic materials are widely used in myocardial tissue regeneration, due to some excellent characteristics. First they show superior mechanical properties than natural polymers that make them suitable for mechanical support (Jawad et al., 2008; Sui et al., 2011). These properties, together with hydrophilicity and degradability, can be controlled in a reproducible manner. Drawbacks regard their poor interaction with the cells and the biological environment. The main synthetic polymers are: polyesters, polylactide PLA, polyglycolide PGA, PLGA that is a combination of polylactide PLA and polyglycolide PGA, polye caprolactone PCL and polyurethanes PURs (Silvestri et al., 2013).

1.12.1.3 Polyurethanes (PURs)

Polyurethanes are a large group of polymers widely used in the biomedical and industrial field. Materials with varying chemical, mechanical, biological and degradation properties can be achieved (Li et al., 2013) by proper selection of different starting reagents (polydiols, diidocyanates and chain extenders). In cardiac TERM, degradable PURs, based on polyester polyols, have been studied for a long time because of their biocompatibility and elastomeric properties that fit the requirements of materials for cardiovascular disease (Silvestri et al., 2013).

1.13 Aim of the project

The goal of this project is to differentiate adult adipose tissue-derived mesenchymal stem cells into cardiomyocytes progenitors. On the long term, these studies could contribute to the development of stem cell-based therapies for the cure of cardiovascular diseases. The first part is focused on developing protocols for isolation, characterization, *in vitro* culture and identification of optimal conditions for long-term storage of Adipose Tissue-Derived Multipotent Stromal Cells (ACSs). For that, isolated ACSs has been extensively characterized by flow cytometry, and genetically profiled by a reverse-transcription and PCR-based approach [Fig. 8].

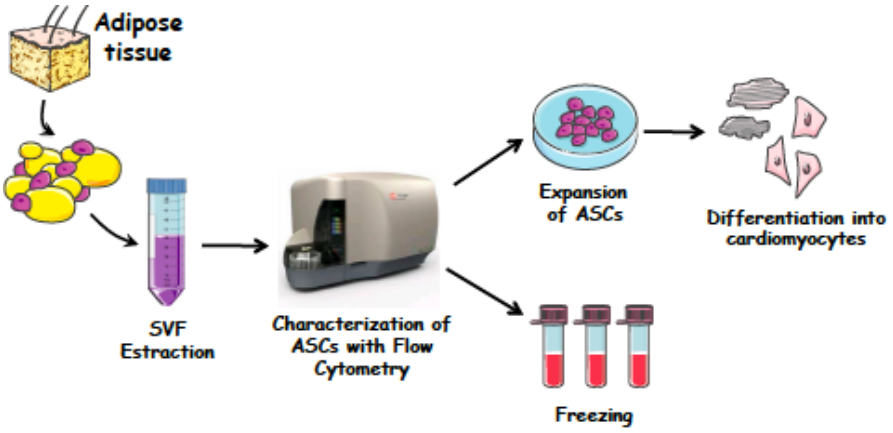

Figure 8. Aim and strategy of the project. The first aim focused on developing protocols for the isolation, *in vitro* culture, characterization and freezing of ACSs for long-term storage in liquid nitrogen.

After the achievement of this initial phase, we have tried to differentiate ASCs into cardiac progenitor cells following two innovative approaches: the use of human growth factors in a defined serum-free culture induction medium and the application of mechanical stimuli to mimic the heart environment [Fig. 9]. Since these protocols are ultimately aimed at producing cells for applications in humans, we developed protocols compliant with the strict European regulations concerning the Advanced

Therapy Medicinal Product. Therefore, from the very first steps of the project (i.e. the isolation of ASCs), we focused on the use of "clinical grade" reagents, consumables and techniques.

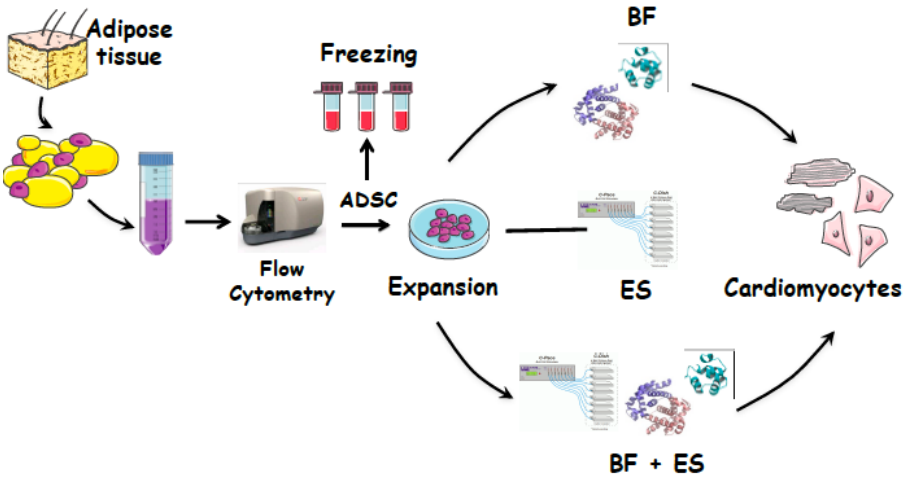

Figure 9. Aim and strategy of the project. The second goal is to find strategies for cardiogenic differentiation. We tested three different approaches, the first based on the use of growth factors and cytokines, the second based on the application of an mechanical stimuli, and the last one will be a combination of the first two. ES: Electro-mechanical stimulation, BF: Biochemical factors.

1.14 Our approach

We are particularly interested in investigating the cardiogenic potential of ASCs. In the past, different approaches have been used to induce the differentiation of ASCs toward the cardiac lineage such as methylcellulose media (Sliwa et al., 2009), culturing the cells with nuclear and cytoplasmic extracts from rat cardiomyocytes (Gaustad et al., 2004), co-culturing with neonatal rat cardiomyocytes (Madonna et al., 2008)(Zhu et al., 2009), or using of the chemical agent 5-azacytidine (5-AZA). Because the use of animal derivatives or chemical agents may affect the genomic stability of

the cells (5-AZA) (Madonna et al., 2008; Palpant et al., 2007), none of the above cited methods is compatible with the use of ASCs in the clinical practice. Moreover the heart should be considered as a complex structure organ in which the electrical conduction and mechanical stimuli play a very important role. This particular aspect has been poorly investigated and may significantly contribute to the differentiation of the cardiac progenitors during embryonic development.

2. Materials and Methods

2.1 Isolation of Stromal Vascular Fraction (SVF)

We designed the isolation protocol around the use of a 100ml syringe (Omnifix 100ml with Luer Adaptor, B. Braun AG, Melsungen, Germany) as a separation funnel (Patent pending; Minonzio et al., submitted manuscript). The protocol is based on the fact that adipose tissue and hydrophilic fluids spontaneously separate in two phases without need of centrifugation. The piston of the syringe is used to take in or to expel the solutions used to wash the sample, to dissociate the suctioned fat, or to extract the cells from the dissociated adipose tissue. The syringe is hold in a vertical position using a laboratory support stand with support rings. Therefore, all the necessary manipulations for the extraction of ASCs are performed inside the syringe and last about 70 minutes. The first step is to wash the sample with 40 ml Dulbecco's PBS (PBS, with Ca^{2+} & Mg^{2+}, PAA Laboratories, Pasching, Austria) by gentle agitation. The syringe is hold vertically in the support stand for few minutes to allow the separation of the phases and the lower aqueous phase is discarded by pushing the piston. The sample is washed twice. To free the cells in the aqueous phase the washed adipose tissue is digested with the appropriate amount of Liberase MTF-S (Roche Applied Science, Basel, Switzerland) at a final concentration of 0.28 Wünsch U/ml [10] diluted in 10 ml DPBS (with Ca^{2+} & Mg^{2+}). The sample is incubated 45 minutes at 37°C under constant but gentle agitation. Enzymatic reaction is stopped by aspiration of 30 ml of injectable 5% human albumin solution (CSL Behring AG, Bern, Switzerland) in the syringe. The syringe is then put back in vertical position to allow the separation of the phases. The lower layer, which contains now the SVF cells, is carefully poured out into a conical 50 ml centrifuge tube (TPP, Trasadingen, Switzerland). Extracted adipose tissue is washed again with 40 ml 5% human albumin solution to increase cell yield. Finally, after filtration through 100 and a 40 μm sieve (Cell Strainer, BD Falcon, Basel, Switzerland), SVF is centrifuged 400g 5 min RT and the pellet resuspended in DPBS (without Ca^{2+} & Mg^{2+}, PAA Laboratories, Pasching, Austria) or in tissue culture medium. The SVF was

analysed for cell count and the number of nucleated cells was established using an electronic cell counter (Hemocytometer – AxonLab ABX Micros60).

2.2 Cellular characterization by Fluorescence-Activated Cell Sorter (FACS) analysis.

The Fluorescence-Activated Cell Sorter (FACS) allows the measurement, characterization and separation of cells suspended in a fluid medium, based on the physical characteristics of the cell and on the presence of fluorescent molecules bound to the cell.
In this work cytometric analysis was performed using a Navios machine, 10 channel flow cytometer (Navios, Beckman and Coulter, Nyon, Switzerland), and the data were analysed with a Kaluza software.

2.2.1 Stromal Vascular Fraction characterization

Fresh SVF cells were characterized by FACS following standard procedures. All antibodies and reagents mentioned below have been provided by Beckman and Coulter.
Briefly, 550.000 cells from fresh SVF preparation were taken and centrifuged 5 min 400 g at RT. The pellet was resuspended in 220 µl of FACS buffer (i.e. PBS without $Ca2^{+}/Mg2^{+}$ with 1% human converted AB serum). Two test tubes were prepared for staining with the following antibodies:

1) control tube

- ✓ IgG2a-PE - A12695
- ✓ IgG1-KRO - A96415
- ✓ IgG1-APC-A750 - A71120
- ✓ Syto 40 - S11351 (Invitrogen)

2) sample tube:

- ✓ CD146-PE - A07483
- ✓ CD45-KRO - A96416
- ✓ CD34-APC-A750 - A89309
- ✓ 7-AAD - A07704

100 µl of the resuspended cells were then added to the tubes containing the antibodies and incubated for 25 min at RT in the dark. 1 ml of Versalyse Lysing solution was added to both tubes that were thereafter incubated for 10 min at RT in the dark. 100 µl of flow count Fluorospheres were added to the sample tube just before the measurement.

2.2.2 Characterization of cultured ASCs cells

At the second passage all cells were characterized for the expression of cell surface molecules and mesenchymal stem cell markers with the following anti-human monoclonal antibodies:

- ✓ CD105-PE (A07414) Beckman Coulter
- ✓ CD73-FITC (561254) BD
- ✓ CD90-PC5 (IM3703) Beckman Coulter
- ✓ CD31-FITC (IM1431U) Beckman Coulter
- ✓ CD45-KRO (A96416) Beckman Coulter
- ✓ CD34-APC-A750 (A89309) Beckman Coulter

And the proper controls:

- ✓ IgG1-FITC (A07795) Beckman Coulter
- ✓ IgG1-PE (400113) BioLegend
- ✓ IgG1-PC5 (A07798) Beckman Coulter
- ✓ IgG-APC AF750 (A71120) Beckman Coulter
- ✓ IgG1-KRO (A96415) Beckman Coulter

2.3 Colony-Forming Unit (CFU-F) Assay.

The CFU-F assay was performed as already described elsewhere (Casteilla et al., 2011; Pittenger et al., 1999) and used to evaluate the frequency of mesenchymal progenitors in the SVF fraction. Freshly extracted nucleated cells were plated at two cell concentrations (5000 and 10000 cells) in standard 100 x 20 mm tissue culture dishes (growth area 58.95 cm^2, BD Falcon, Basel, Switzerland) and cultured in MEM/5% converted human serum/1% antibiotics for 14 days. The plates were then washed with DPBS, fixed in 2% formaldehyde (Sigma-Aldrich, Buchs, Switzerland)/0.2% Glutaraldehyde (AppliChem, Darmstadt, Germany) for 5 minutes and

stained with crystal violet solution (Sigma-Aldrich, Buchs, Switzerland) for 10 minutes. After washing the plates with water, the number of colonies in each dish was counted. A colony consisting of more than 50 cells was defined as a CFU-F.

2.4 Cryopreservation and thawing of SVF.

Fresh SVF cells were centrifuged for 5 min at 400g, resuspended in ice-cold solution of injectable 5% human albumin solution with 10% DMSO (WAK-Chemie Medical GmbH, Steinbach, Germany), and transferred into a freezing cryovial. The cells were frozen using a programmable freezer (Consartic GmbH, Schoellkrippen, Germany) under standard "controlled-rate" conditions from 4°C to -100°C and then finally stored in liquid nitrogen. For thawing, the cryovial was put in ice for 5 min and immersed in a 37°C water bath usually 2-3 min. Immediately after being thawed the cells were carefully aspirated, mixed with an equal volume of injectable 5% human albumin solution in a 15 ml TPP conical tube and centrifuged at 400g for 5 min. The supernatant was discarded, the pellet resuspended in fresh tissue culture medium and used for the subsequent analyses.

2.5 Cell culture and standard differentiations.

Cells were cultured until passage 2 in basal Ham's F12/IMDM (1:1) medium supplemented with various growth factors and referred to as *serum free control medium* (Patent pending). For osteogenic differentiation cells were then plated at a density of 3 x $10^3/cm^2$ onto multi well plates (PureCoat ECM Mimetic Cultureware, BD Biosciences, Bedford, USA) and put in osteogenic differentiation medium for 21 days. Non-induced cells were incubated in serum free control medium. Osteoinductive medium was the serum free control medium supplemented with Sr^{2+} and Vitamin D (Patent pending). Morphological changes appeared during the second week of culture and cell differentiation was confirmed at day 21 by Alizarin Red staining (see 2.5.2). Briefly, the cells were fixed in 10% formalin for 30 min

at RT and incubated 30 min at RT in Alizarin Red staining. The formation of red calcium deposits is a marker of osteogenic differentiation.
The adipogenic potential was assessed by plating cells at a density of 3 x $10^3/cm^2$ onto multi wells plates (BD PureCoat ECM Mimetic Cultureware, BD Biosciences, Bedford, USA) in serum free control medium or in adipogenic induction medium for 21 days. Adipogenic induction medium was the serum free control medium supplemented with EGF (cyt-217, ProSpec-Tany Technogene Ltd., East Brunswick, USA) and Rosiglitazone (Sigma-Aldrich, Buchs, Switzerland) (Patent pending). Adipogenesis was assessed by Oil Red staining (see 2.5.1 for details). Briefly, cells fixed in 10% formalin for 30 min at RT were incubated in fresh Oil O Red water solution for 5 min at RT. Induced cells should be visible as cells containing consistent red deposits in vacuoles.
Chondrogenic differentiation was assessed by induction of ASCs using the micromass method. Briefly, ASCs were gently centrifuged in 15 ml conical tube to form small pellets and cultured for 21 days in the serum free control medium supplemented with sodium pyruvate, BMP-6, TGF-beta3, beta-FGF and PGE2 (Patent pending). Chondrogenic pellets were fixed in 10% formalin for 30 min at RT. Samples were then embedded in paraffin and sections stained with Alcian Blue.

2.5.1 Oil-Red O staining

The presence of lipid granules in cell cultures induced to adipogenic differentiation was determined with Oil-Red-O. The cells were fixed in fixing solution and washed in H20 and incubated for 15 minutes with a solution of isopropanol 0.5% Oil-Red-O (Sigma), filtered and diluted to 60% in distilled H20. Subsequently the cells were washed in H20.

2.5.2 Alizarin Red Staining

The formation of red calcium deposits is a marker of osteogenic differentiation and it is shown by the use of Alizarin Red staining.
After the induction the medium was discarded, cells were then washed with PBS without Ca and Mg and fixed in formalin 10% for 30 minutes. The plate was washed again with distilled water, Alizarin Red O solution added and the plate incubated in a gentle agitation for 20 minutes. The samples

were washed 3 times with distilled water to remove the excess of Alizarin Red O. Calcium deposits were observed at the microscope.

2.6 Cardiac Induction - cell culture

Cells were cultured until passage 2 in basal Ham's F12/IMDM (1:1) medium supplemented with various growth factors and referred as to *serum free control medium* (Patent pending). Cells were then detached with Tryple Select (Gibco, Switzeland) and then replated at a concentration of 15 x 10^3 Cells/cm^2 and maintained with serum free culture medium in 6-well plates (PureCoat ECM Mimetic Cultureware, BD Biosciences, Bedford, USA) in a controlled CO_2 cell incubator.
Twenty-four hours later, the culture media was removed, the cells washed with PBS solution, and each well submitted to a specific protocol aimed at obtaining cardiogenic differentiation, as shown in Table 2. Plates were incubated in 5% CO_2 incubator at 37°C for 21 days. During this period, cultures were observed and evaluated regarding their morphological changes on an inverted optical microscope.

2.7 Cardiogenic Differentiation Analysis

2.7.1 RNA extraction and PCR reaction

Total mRNA was extracted from ASCs cultivated in serum free medium at passage 1 or from human heart biopsies (positive control, see section 2.7.1.1), using TRI Reagent (Sigma) and following manifacturer's instructions. Total mRNA was then reverse-transcribed using Maxima H Minus First Strand cDNA Synzhesis Kit Reverse Transcriptase (Thermo Scientific). The resulting cDNA was used as a template for PCR amplification with Taq DNA Polymerase (Qiagen), using primer pairs specific for cardiac-specific genes and transcription factors [Tab. 1]. All primer sequences were original and determined through established mRNA GenBank sequences. β actin was used as a control for PCR efficiency. Reactions were performed in a CFX Connect Real Time System (BIO-RAD) for 35 cycles, consisting of 94°C for 30 seconds, 94°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds with a final extension at 72°C for 5 minutes. The PCR products

were size-fractionated by 2% agarose gel electrophoresis in a TAE buffer (Invitrogen). Signals with gel red staining was detected and quantified using a CCD camera-based instrument. Experiments were repeated at least three times.

Table 1. Primers used in standard and real time PCR.

Gene	Sequence
β-Actin	Quantiltect Primer Assay (Cat n° QT01680476) - Qiagen
HPRT	5'-GTC AAG CAG CAT AAT CCA AAG ATG-3' 5'-GTC TGG TCT ATA TCC AAC ACT TCG-3' (Microsynth)
GATA4	Quantiltect Primer Assay (Cat n° QT00031997) - Qiagen
TBX5	Quantiltect Primer Assay (Cat n° QT00056574) - Qiagen
TBX18	5'-GCC TGC CTC GGG GGA GAC TT - 3' 5'-TGA CGT GCA CTC GCG GTT GG - 3' (Microsynth)
CX43	5'-AGG AGT TCA ATC ACT TGG CG -3' 5'-GAG TTT GCC TAA GGC GCT C - 3' (Microsynth)
BAF60C	Quantiltect Primer Assay (Cat n° QT00069181) - Qiagen
NKX2-5	5'-ACC TCA ACA GCT CCC TGA CTC-3' 5'-ATA ATC GCC GCC ACA AAC TCT CC-3' (Microsynth)
MEF2C	5'-AGA TAC CCA CAA CAC ACC ACG CGC C-3' 5'-ATC CTT CAG AGA GTC GCA TGC-3' (Microsynth)
HAND1	5'-AAC TCA AGA AGG CGG ATG G-3' 5'-TCG GCT CAC TGG TTT AAC TC-3' (Microsynth)
HAND2	5'-GCA GGA CTC AGA GCA TCA AC-3' 5'-CAT GAG GTA GGC GAT GTA GC-3' (Microsynth)
FOG	5'-ATG AAG ACA GTG CCC ATC AG-3' 5'-TCA CGG AAT CAG CAG CAG TGT AG-3' (Microsynth)
CAMTA	5'-ACT TAG AGC AGG AGG TTG AC-3' 5'-GGA ATG AGC CAC AGA CAA TG-3' (Microsynth)
CKIT	5'-GAG TCC AAG AGG GTC CTT TAG -3' 5'-ACT GAT GCC TTC CAC TTC C-3' (Microsynth)

SIRPA	5'-TAC TCC GTG TGG TTG TTG-3' 5'-CAG GGC AAGVCAG ATG TCG CAA G-3' (Microsynth)
NCX	5'-GAA GGG CAA TCA TGT TGT TGG TG-3' 5'-AAG GAT AAG GAT GGG TTA G-3' (Microsynth)
SERCA-2	5'-ACC GAA GGC ATG CAT TAC-3' 5'-TGC TGC AAG AGG AAT GAG-3' (Microsynth)
MHC	5'-GAA GCC CAG CAC ATC AAA AG-3' 5'-GAT CAC CAA CAA CCC CTA CG-3' (Microsynth)

2.7.1.1 Cardiac Biopsy

Cardiac biopsy specimens were obtained with informed consent from patients undergoing open-heart surgery. In total we used the tissue from 3 patients. Mean ages were 67 years. Atrial cells were obtained from the atrial appendage. The heart samples were taken in conjunction with the insertion of the cannula for venous drainage of the right atrium through the atrial appendage, a necessary procedure for the institution of cardiopulmonary bypass (extracorporeal circulation). Individual specimens were immediately transferred into tubes containing ice-cold Ca^{2+} free Krebs-Ringer saline solution; specimens were transported to the cell culture laboratory within 30 minutes. For total RNA extraction, the tissue was placed in cryovial tubes and dipped into liquid nitrogen for 3 minutes and then minced with sterile scissors. TRI Reagent (Sigma) was added and mRNA isolated according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using Thermostable reverse transcriptase. cDNA samples were subjected to PCR amplification with primers for cardiac-specific genes.

2.7.2 Immunofluorescence

After differentiation induction, cells grown on coverslips, were washed with PBS solution and fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. After washing twice with PBS, fixed cells were permeabilized with PBS + 0.1% Triton X-100 (Sigma) 3 times for 5 minutes and then non-specific binding was blocked using 2% bovine serum albumin (BSA) (Sigma) at 37°C for 30 minutes. Then the cells were incubated with

the primary antibody in 0.2% BSA + 0.2% Triton X-100 solution for 1 hour at RT. The cells were then washed 3 times in PBS + 0.1% Tween (Sigma) + 0.2% BSA and incubated with the secondary antibody. At the end they were washed 3 times for 5 minutes with PBS + 0.1% Tween + 0.2 % BSA for 30 minutes at RT. To provide a quick assessment of cell distribution, 4', 6-diamidino-2-phenylindole (DAPI, Sigma - Italy) was used to stain cell nuclei. Coverslips were mounted with one drop of Hydromount (National Diagnostics, USA) and protected from light. Samples were analysed using fluorescent microscopy (Olympus BX50). Cells incubated with only secondary antibody were used as negative controls.

Table 2. List of primary and secondary antibodies used in immunofluorescence analysis.

Primary Antibody	Secondary Antibody
Anti-sarcomeric-α-actinin (Santa Cruz Biothechnologies)	TRITC; 1:200 dilution (Jackson, Immuno Research Laboratories, West Grove, Pa. - USA)
Anti-actin (Santa Cruz Biothechnologies)	TRITC; 1:200 dilution (Jackson, Immuno Research Laboratories, West Grove, Pa. - USA)
Anti - Myosin heavy chain (Santa Cruz Biothechnologies)	TRITC; 1:200 dilution (Jackson, Immuno Research Laboratories, West Grove, Pa. - USA)
NKX2-5 goat polyclonal antibodies (Santa Cruz Biothechnologies)	Rabbit F (ab')2 – Anti-Goat IgG (H+L) Human FITC (Beckman Coulter)

2.7.3 Optical, transmission (TEM) and scanning (SEM) electron microscopy

These analysis were performed by Prof. Magda de Eguileor's group (Department of Biotechnology and Life Sciences, University of Insubria - Italy).

After 10 days or 21 days of induction the cells were collected and fixed with 4% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2).

Pellets were washed in 0.1 M Na-cacodylate buffer (pH 7.2) and post-fixed for 20 minutes with 1% osmic acid in cacodylate buffer (pH 7.2). After standard dehydration in an ethanol series, samples were embedded in an

Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsin) and were observed with a light microscope (eclipse Nikon). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan).

Cells for SEM were fixed and dehydrated as described above, treated with hexamethildisilazane and mounted on polylysinated slides. Next, the samples were air-dried and covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech, Baltimore, MD, USA). Specimens were then examined with a SEM-FEG Philips XL-30 microscope (Philips, Eindhoven, Netherlands).

2.7.4 Characterization of induced cells by FACS Analysis

At the end of the induction of cardiac differentiation, a representative aliquot of the cells was characterized for the expression of particular cell surface cardiac markers using the following anti-human monoclonal antibodies:

- ✓ CD140a (PDGFR)-PE (323505) BioLegend
- ✓ CD184 (CXCR4)-BV 421 (306517) BioLegend
- ✓ CD117 (cKIT)-PC7 (IM3698) Beckman Coulter
- ✓ CD309 (VEGFR)-AF 647 (338909) BioLegend
- ✓ IgG1-PC7 (737662) Beckman Coulter
- ✓ IgG1-PE (400113) BioLegend
- ✓ IgG2a-BV 421 (400259) BioLegend
- ✓ IgG1-AF 647 (400130) BioLegend

2.8 Tissue Engineering

The scaffolds used in this thesis to produce cellularized matrices for application in cardiac tissue engineering/regenerative medicine (TERM) were developed and characterized by Prof. Ciardelli's Research Group (Materials in Bionanotechnology, Department of Mechanical and Aerospace Engineering, Politecnico di Torino -ITALY-). The scaffold-forming material is an elastomeric polyester urethane developed and synthesized by the same research group. This biodegradable polyurethane with acronym K-BC2000 was synthesized as previously described (Rechichi et al., 2008; Sartori et al., 2013) starting from poly(ε-caprolactone) (PCL) diol, 1,4-budandiisocyanate and L-lysine ethyl ester dihydrochloride.

2.8.1 Fabrication of PUR porous scaffolds

PUR porous scaffolds were fabricated by thermally induced phase separation (TIPS) and subsequent solvent extraction. The applied method was similar to that described by Guan et al. (Guan et al., 2005, 2008). Briefly, the polyurethane was first dissolved at 60 °C in dimethyl sulfoxide (DMSO) (Sigma Aldrich -ITALY-) at 12 %w/v; then, the obtained solution was poured in a stainless steel parallelepiped mold (50 x 25 x 30 mm) and cooled down at -20 °C for 5 hours. The quenching was performed under application of a thermal cooling gradient to induce DMSO crystal growth, i.e. pore formation, in a preferred direction. All mold walls were insulated using polystyrene and cotton wool except one. In order to extract DMSO, the frozen scaffold was placed for three days in a water/ethanol solution (30/70 v/v) refreshed twice a day. The scaffold was finally freeze-dried (Martin Christ ALPHA 2-4 LSC) and cut to obtain matrices with a thickness of about 1 mm.

2.8.2 Scaffold characterization

2.8.2.1 - Morphological and structural properties

- Scaffold morphology

The morphology of the produced scaffolds was evaluated by Scanning Electron Microscopy (SEM; LEO 1450VP). Micrographs were taken with a beam voltage of 20 kV and instrument magnification of 100x. All samples were gold-coated before analysis. Image data were analyzed by ImageJ software. The average pore size and pore size distribution were obtained by measuring the diameter of 80 pores chosen at random throughout the central section of the samples.

- Scaffold porosity

Scaffold porosity was determined using a liquid displacement method similar to that reported by (Zhang and Ma, 1999) and (Hsu et al., 1997). Ethanol was used as the displacement liquid. A scaffold sample was immersed in a glass cylinder containing a known volume of ethanol (V_1). The sample was pressed to force air out of the scaffold until no further air bubbles were seen, allowing the ethanol to fill the pores. The total volume of ethanol and the ethanol-impregnated scaffold was recorded as V_2. The ethanol-impregnated scaffold was then removed from the cylinder and the residual ethanol volume was recorded as V_3. The percentage of porosity (p%) of the scaffold was determined by:

$$p\% = \frac{(V_1 - V_3)}{(V_2 - V_3)} \cdot 100$$

2.8.2.2 Mechanical properties

- Tensile tests

Scaffold rectangular samples (25 x 7 x 1 mm) were analyzed by stress-strain tests, performed using a MTS QTest/10 Elite Controller equipped with a 10 N load cell. The cross-head speed was 1 mm/min. The samples were tested in dry and wet conditions at room temperature. For instance, the samples were immersed in water overnight before test, when analyzed in wet conditions. Tensile tests were conducted in triplicate.

- Durability tests

Durability tests were conducted to evaluate the capability of the developed matrix to sustain the mechanical stress it will be subjected to during dynamic cell culture. For this reason, these tests were conducted in the dynamic cell culture system "Stimiote".

2.8.2.3 Statistical analysis

Tensile test results are expressed as a mean ± standard deviation calculated using Microsoft Excel (Redmond, WA, USA) software. Statistical analysis was performed using GraphPad Prism. One-way ANOVA followed by post hoc analysis (Bofferoni) was used to compare the results. A P value of less than 0.05 was considered as statistically significant.

2.8.3 Stimiote

Stimiote is a novel device (patent pending), engineered, developed and built by the Swiss Stem Cell Foundation in collaboration with the Scuola Universitaria della Svizzera Italiana (SUPSI) Lugano (Switzerland) for the application of controlled mechanical and/or electrical stimuli that are applied on a scaffold over a desired lap of time [Fig. 10].

Figure 10. Stimiote. Photograph of the dynamic cell culture system device engineered and built for application of controlled mechanical and/or electrical force.

The main components of the system [Fig. 11] are: (i) a mechanical structure to support an actuator that makes it possible to apply a controlled longitudinal force to the sample, (ii) an endless screw actuator, (iii) two displacement detectors to monitor patch deformation, (iv) four flexible blades with one degree of freedom to limit friction and support the movable part of the device, (v) a flask containing the culture medium, (vi) a system of movable rigid beams to transmit movement to the patch and record information about patch position and the applied force, (vii) a clamp system to fix the patch to the movable rigid beams.

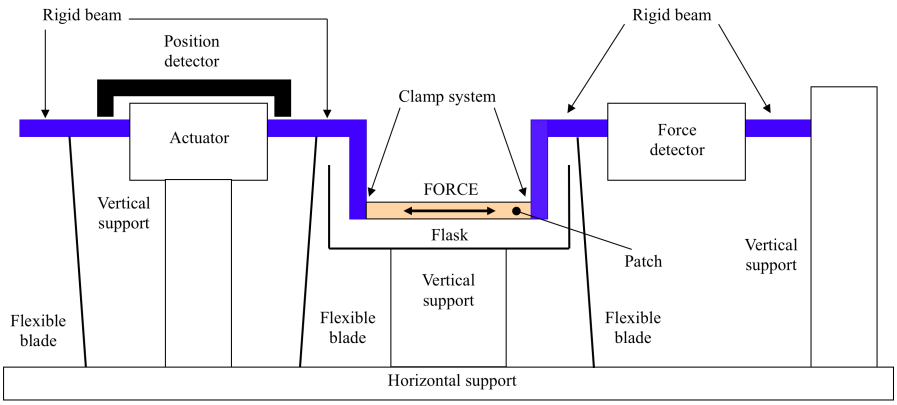

Figure 11. The main component of Stimiote. Schematic diagram of the main components of Stimiote dynamic cell culture system.

The Stimiote system is a stress-controlled bioreactor that makes it possible to apply a force up to 3 N to a bio absorbable patch. It is characterized by a force and strain accuracy of 0.66% and 0.1 mm, respectively. Moreover, it allows the application of a sinusoidal mechanical stress with frequency ranging between 0 and 1 Hz. In order to test durability, the patch (30x25x10 mm) has been subjected to the same mechanical pattern it will be subjected during in vitro cell culture tests: applied force 0.4 ± 0.2 N, frequency 1 Hz, wet conditions, 12 days. All samples were preconditioned in cell culture medium for 24h before analysis. Stimiote equipment allows to record sample position and applied force over time. A specifically developed Matlab routine makes possible to analyze data. Tests were

conducted in triplicate.

2.8.4 Seeding the scaffold and stimulation with Stimiote

Before cell seeding, scaffolds must be coated with collagen I (Coating Matrix Kit, Gibco) overnight to enhance cell attachment. Sterilized membrane (30 x 12.5 mm) were placed into 60 mm petri culture dish and seeded with $1x10^6$ cells. The cell suspension was pipetted directly on the membrane surface (6 drops of 25 μl each). Cells were allowed to attach to scaffold for 2 hours before adding the culture medium. Cells were maintained in humidified 37°C incubator containing 5% CO_2 for 5 days. At day 6 the scaffold was assembled on the device with a sterile forceps under laminar flow. The device was placed in a sterile container and the induction started using cardio induction culture medium and setting the device on a force of 0.4N, amplitude 0.2N, frequency 1 Hz [Fig. 12]. In this study Alamar Blue assay was used to assess cell viability 5 days after seeding and at the end of the mechanical stimulation. The duration of the experiment was 10 days.

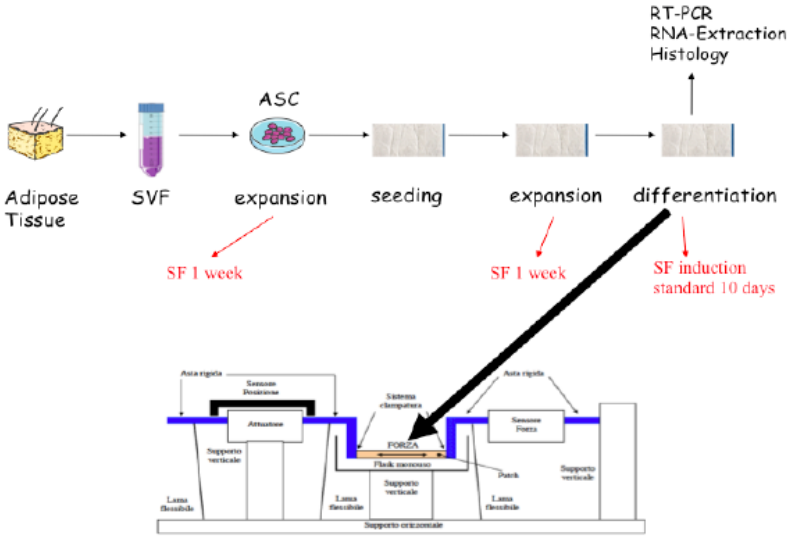

Figure 12. A schematic representation of seeding scaffold. After expansion the cell suspension was pipetted directly on the membrane surface. Cells were maintained in SF in incubator for 5 days. After this period the scaffold was assembled on the device with a sterile forceps under laminar flow for 10 days with cardioinduction medium.

2.8.5 Alamar Blue Assay

Alamar Blue is an assay to monitor cell health. Plasma membrane integrity, DNA synthesis, DNA content, enzyme activity, presence of ATP, and cellular reducing conditions are known indicators of cell viability and cell death. The Alamar Blue cell viability reagent functions as a cell health indicator and by using the reducing power of living cells to quantify the proliferation of MSCs. When the cells are alive they maintain a reducing environment within the cytosol of the cell. Resazurin, the active ingredient of Alamar Blue reagent, is a non-toxic, cell permeable compound that is Blue in colour and non-fluorescent. Upon entering cells, Resazurin is reduced to Resorufin, a red and fluorescent compound. Viable cells continuously convert Resazurin to Resorufin, increasing the overall fluorescence and color of the media.

Alamar Blue cell viability reagent is used to assess cell viability by simply adding the 10x ready-to-use solution to the culture medium and incubating at 37°C o/n. There is no requirement to remove media from cells or to place cells in minimal media. The absorbance of Alamar Blue was read on a spectrophotometer at 540 and 630 nm. Results were analysed by plotting the absorbance versus the compound concentration.

3. Results

3.1 Stromal Vascular Fraction (SVF) Extraction

Our first milestone was the preparation of the SVF from adipose tissue and the subsequent isolation of ASCs fraction. The starting material for our study was obtained, after informed consent of healthy female donors, from subcutaneous adipose tissue collected during an elective liposuction procedure. The lipoaspirate (generally 70 to 150 g) was transferred to the lab, and SVF cells were then isolated by our standard GMP-optimized procedure, consisting of an initial washing step to remove blood and any other contaminant (e. g. the anaesthetic used during sampling), followed by the digestion of the adipose tissue with the enzyme collagenase (for details see 2. Materials and Methods). The enzymatic digestion was required to separate the mesenchymal progenitors that are trapped in the extracellular matrix and located within the tissue. After such enzymatic digestion, we performed a series of extraction steps using a solution that blocks the effect of the enzyme, helping to reduce stress and increase cell viability [*Fig. 13*]. The SVF was analysed for cell count, vitality, as well as for the expression of surface markers by flow cytometry. The number of nucleated cells was established using an electronic cell counter (hemocytometer).

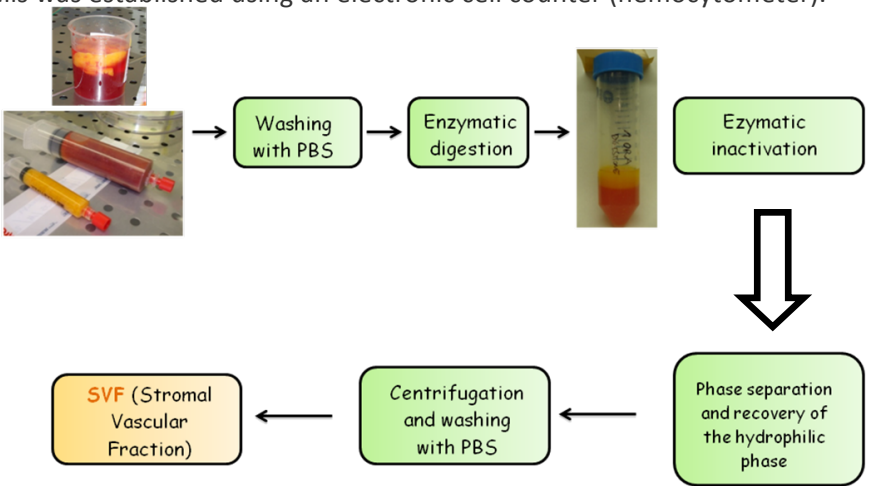

Figure 13. Isolation of the Stromal Vascular Fraction (SVF). A schematic representation of the isolation of the SVF from lipoaspirates using an enzymatic digestion designed to dissociate the cellular components from human tissues.

3.2 Characterization of the SVF

3.2.1 Immunophenotypisation of SVF from liposuction aspirate

An important milestone of this project was to establish a detailed immunophenotype of ASCs, a somewhat challenging task due to a still incomplete knowledge of the cellular and developmental biology of these cells, and the consequent difficulties in identifying multipotent stromal cells based only on a phenotypical analysis.

As already explained in the introduction, the cells isolated from the hydrophilic portion of adipose tissue are called Stromal Vascular Fraction (SVF) and represent a heterogeneous population. The major problem is that, to date, there is no easy and accurate assay capable of characterizing and enumerating these cells after isolation. In fact, this problem represents a major obstacle, especially if the SVF has to be used for clinical applications that require a well-defined composition of the "cell-drug". To overcome this problem, we developed and optimized a five-colour flow cytometry protocol that is suited for the analysis of freshly extracted SVF cells. Freshly isolated SVF was immediately analysed with a no-wash flow cytometer protocol: a small amount of the cell suspension was incubated with specific antibodies, the erythrocytes were removed with a lysing solution and the sample was immediately analysed with a cytofluorimeter. In order to define the different populations we used three surface antigen markers (i.e. CD45, CD34 and CD146). CD34 was used as marker for pluripotent stromal cells, while CD146 and CD45 were used to label endothelial and hematic cells, respectively. With this approach and marker combination we could reliably define the ASCs and reproducibly identify three distinct SVF subpopulations, of which ASCs are CD146/CD45 negative and CD34 positive cells, representing 10%-25% of the total SVF cells.

The viability dye 7-AAD and the DNA marker Syto 40 were added to the analysis to remove artefacts and improve separation of the populations. The cell viability assay is very important since the ASCs cells are subjected to a long period of stress starting with the surgical procedure, through the extraction steps and finally to the seeding in a cell culture plate. This analysis allowed us to quantify living cells and to seed them at an optimal

density, which is a main factor affecting cell proliferation and maintenance of pluripotency.

3.2.2 Gating strategy

To ensure that no false-positive results occur, we developed a gate that excludes debris, cell doublets and dead cells [Fig. 14]. After exclusion from the analysis of the flow count fluorospheres used for counting the cells, the first two gates (A and B) allow to discriminate the nucleated cells (i.e. Syto 40 positive) and to avoid artefacts due to cell doublets. The Boolean gate Clean SVF (C) combines the gates A and B, with the exclusion of the fluorospheres. Finally, before analysing the surface marker expression, we used the viability dye 7-AAD to exclude death cell from the analysis D.

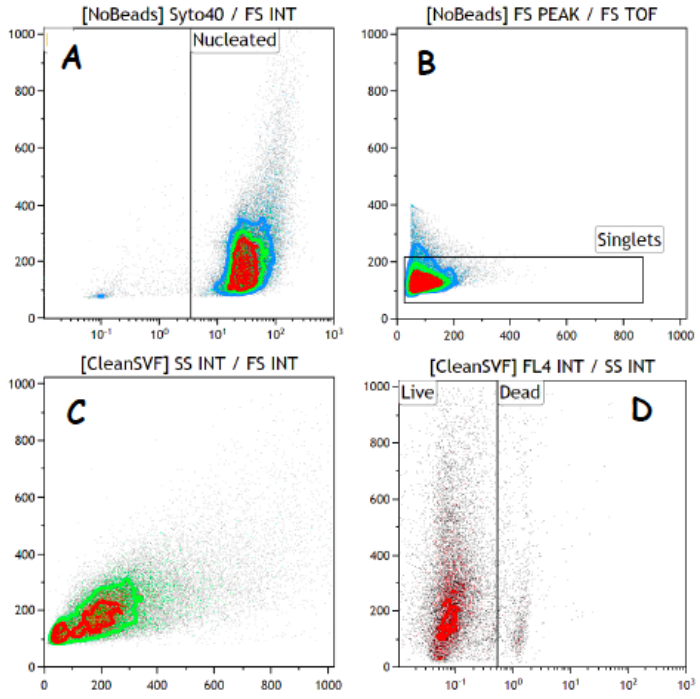

Figure 14. Gating strategy to ensure no false-positive results. To ensure that no false-positive results occur, a gate that excludes debris, cell doublets and death cells has been developed. Data were acquired with a 10 color Navios (Beckman Coulter) and were analyzed with Kaluza software (Beckman Coulter).

3.2.3 Identification of sub-populations in the SVF

In addition to the population of ASCs we were able to identify 7 different sub-populations among which we found cells of endothelial origin (i.e. CD146+, CD34+, CD45-) and a small and very interesting population of CD45 and CD34 positive cells, called "hematopoietic stem cell like" which are yet poorly characterized [Fig. 15].

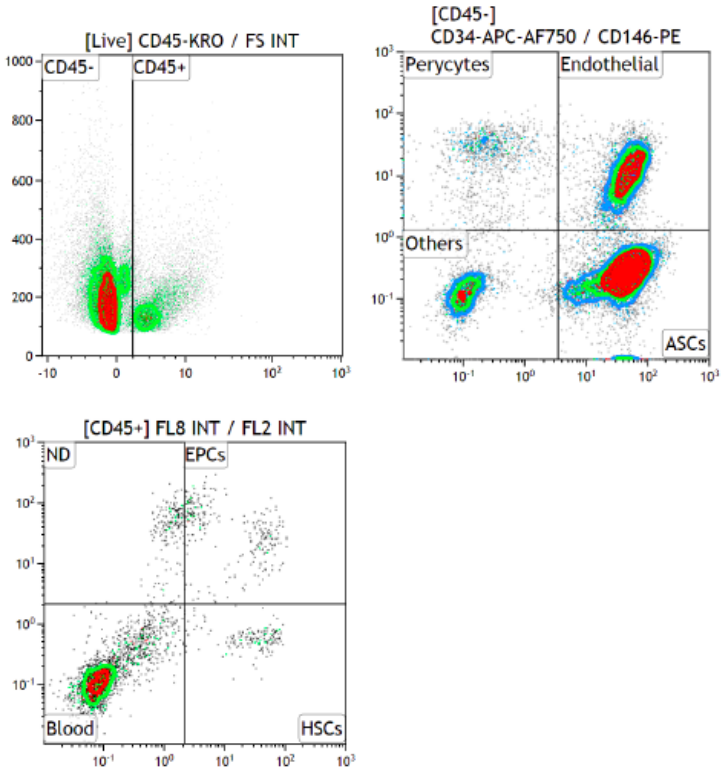

Figure 15. Identification of SVF sub-populations. A five color flow cytometer strategy allows to distinguish the following sub-populations of cells present in the SVF:

- Adipose-derived multipotent stromal cells (ASCs): CD34+, CD45- and CD146-
- Pericytes: CD34-, CD45- and CD146+
- Endothelial cells: CD34+, CD45- and CD146+
- Endothelial progenitor cells (EPCs): CD34+, CD45+ and CD146+
- Hematopoietic Stem Cells-Like (HSCs): CD34+, CD45+ and CD146-
- Blood cells: CD34-, CD45+ AND CD146-
- Other (adipocytes, fibroblasts): CD34-, CD45- and CD146-

3.2.4 ASCs selection based on adherence capacity

The SVF, that included the ASCs sub-population, was characterized to verify if cells fitted into the classical profile of MSCs. Among the properties that characterize these cells, is the adherence to plastic. After seeding the SVF in culture plastic, only a small fraction of the total cells adheres to plastic and grows. Cells were seeded and allowed to attach to the culture plate for 6 days. As the aim was to confirm the presence and identity of adherent cells, flow cytometric analysis of supernatant cells was performed. The only sub-population absent in the supernatant is the CD34+, CD45- and CD146-, corresponding to the ASCs [Fig. 16].

However, it is important to consider that not all ASCs adhere to plastic, but some of them rather loose the CD34 marker or dies, as assessed by the total amount of cells counted. Despite that, the flow cytometric analysis is very useful to quantify the amount of multipotent cells harvested from adipose tissue.

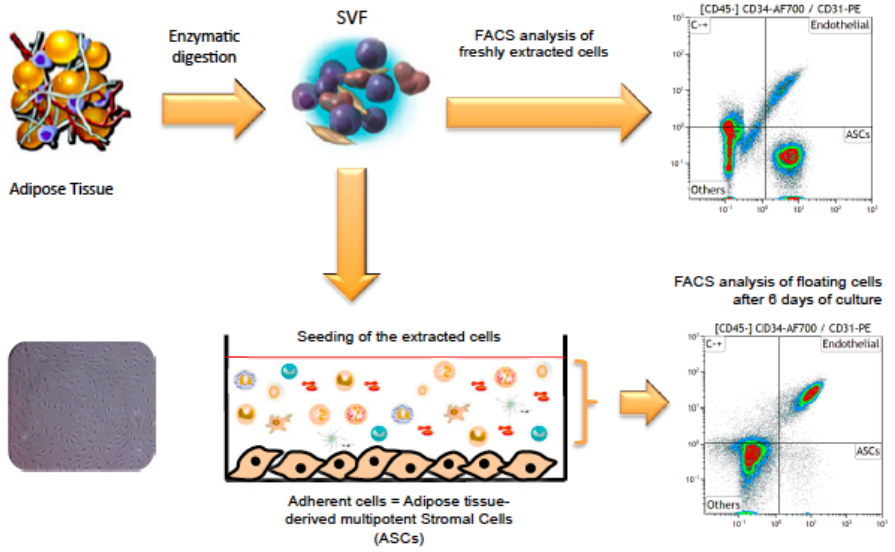

Figure 16. ASCs selection. Strategy used to select ASCs based on their adhesion capacity and cytofluorimetric analysis of SVF sub-populations. After 6 days of culture the ASCs completely disappeared from the supernatant. CD31 was used in place of CD146, because the latter quickly disappears after the seeding of the cells.

3.2.5 Clonogenic CFU-F assay

To verify the stemness of the isolated ASCs, we further performed clonogenic CFU-F assays. In this assay, every colony derives from a single cell clone, thus demonstrating the clonogenic ability of the SVF isolated with our protocol. 5.000 and 10.000 cells of SVF were seeded in two different culture plates. After 14 days of culture, the plates were fixed and colonies of more than 50 cells were then counted as positive [Fig. 17]. Even at the lower seeding density the formation of 1/70 colonies were observed.

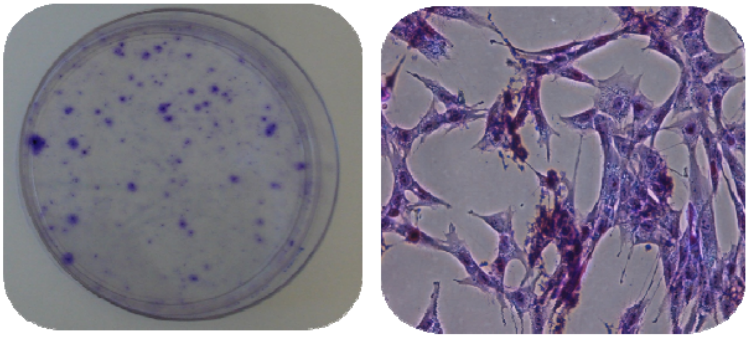

Figure 17. Colony Forming Units Fibroblast Assay. Crystal Violet-staining for CFU-F assay showing that the cultured SVF is composed of fibroblast-like cells, a typical characteristic of mesenchymal multipotent cells.

3.2.6 Ability of ASCs to differentiate in the three main lineages

Finally, we questioned the ability of ASCs to differentiate in osteogenic, chondrogenic and adipogenic cell lines. To do this we induced the cells by means of different cocktails and we analysed the cells with classical staining (i.e. Alizarin S, Oil Red O and Alcian Blue). The media and protocol used are described in Materials and Methods section 2.5. Results clearly showed that ASCs extracted with our protocol can differentiate in all the expected cell lineages [Fig. 18], thus maintaining the potential of mesenchymal stem cells.

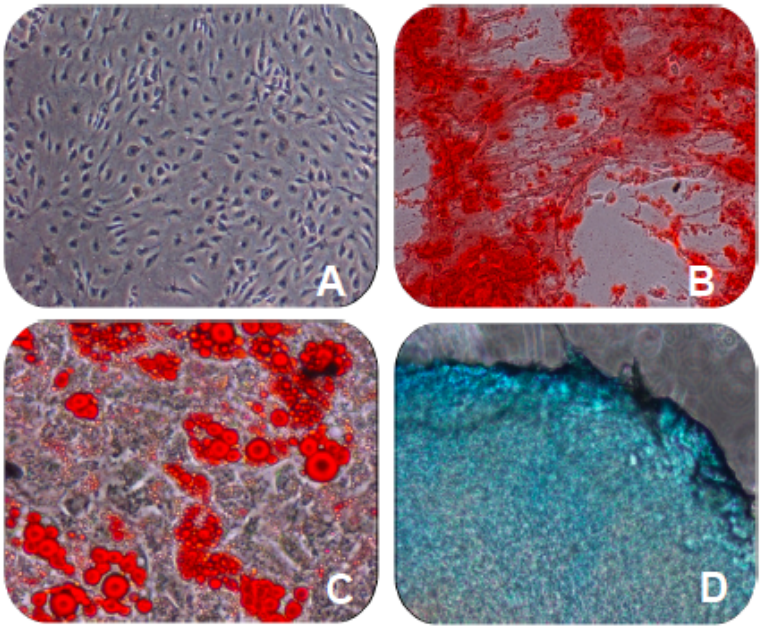

Figure 18. Differentiation potential of ASCs toward adipogenic, osteogenic and chondrogenic cell lines. A: non induced control ASCs at 10x magnification; B: Alizarin S staining to underline the extracellular calcium deposits; C: adipogenic-induced ASCs stained with Oil Red O, aimed to identify intracellular lipid droplets; D: chondrogenic differentiation stained with Alcian Blue.

3.2.7 Immunophenotypisation of expanded cells

The remaining ASCs were expanded for 6 passages and at each passage a fraction of the cells was used for immunophenotypic analysis, to confirm the mesenchymal phenotype. The expansion was carried out in a serum free medium. SVF cells were seeded onto plates that were coated with collagen I in order to allow their adhesion and promote growth. In contrast to freshly isolated SVF, *in vitro* expanded cells have been extensively characterized in the literature using different markers, and are described as $CD90^+$, $CD73^+$, $CD105^+$, $CD34^-$ and $CD45^-$ (Dominici et al., 2006). The citofluorimetric analysis of expanded ASCs showed a concordant pattern [Fig. 19]. At this stage we can therefore conclude that the isolated cells fit with the accepted definition of mesenchymal stem cell. With this in mind

we addressed the first steps involved in cardiomyogenic differentiation of ASCs.

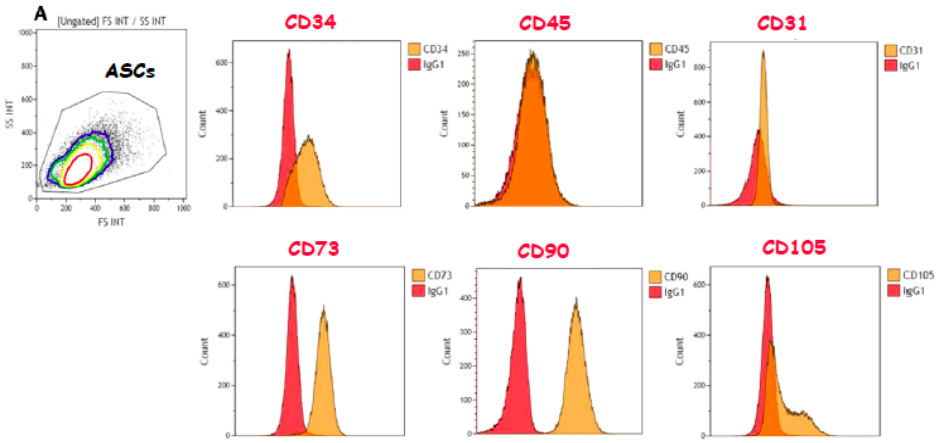

Figure 19. FACS analysis of expanded ASCs using different known cellular markers for MSC. First panel (A): gated population. Other panels: comparison of signal intensity of a given marker (orange) compared to unstained cells (red). The markers are indicated above each panel.

3.3 Expression of some cardiac-specific transcription markers in non induced human ASCs

After the selection of cardiac-specific transcription factors, as described in the introduction, total mRNA was extracted from passage 2 of ASCs cultivated in serum free medium. As positive control, RNA from a heart biopsy was used. Indeed, heart biopsy showed the expected expression of the selected transcription factors. Unexpectedly, however, non-induced ASCs also showed the expression of nearly half of the selected cardiac genes [Tab. 3].

Table 3. Expression of selected cardiac genes in heart biopsy (control) and undifferentiated ASCs, cultured in serum free.

	Heart Biopsy	ASCs (N.I.)
β-actin		
NKX2-5		
MEF2C		
GATA4		
CAMTA		
HAND1		
HAND2		
FOG		
TBX5		
BAF60C		
TBX18		
CX43		
MHC		

Legend
Green: presence of gene expression
Red: Absence of gene expression

3.4 Formulation of a cardio inducing cocktail for ASCs

Our next goal was to select the most effective combination of growth factors and cytokines, their time of application and the best culture conditions in order to obtain differentiation of ASCs into cardiomyocytes. An extensive literature search allowed to select 28 growth factors potentially involved in induction of cardiomyogenic differentiation (see introduction) that were combined in 16 different cocktails, as summarized in Table 4.

Table 4. Cardiogenic differentiation cocktails.

		1	2	3	4	5	6	7	8	9		10	11	12	13	14		15		16	
										Day1	Day 10					Day 1	Day 10	Day 1	Day 10	Day 1	Day 10
JEM																					
F12																					
Horse Serum																					
Primocin																					
Glu																					
Ascorbic Acid	1																				
ITS	2																				
bFGF	3																				
BMP4	4																				
IGF1	5																				
Activin A	6																				
TGF b1	7																				
h Thrombin	8																				
Retinoic Acid	9																				
TNF a	10																				
VEGF	11																				
FGF4	12																				
FGF8	13																				
FGF10	14																				
G-CSF	15																				
PDGF AB	16																				
PDGF BB	17																				
IL6	18																				
CARDIOGENOL	19																				
Cardiothrophin	20																				
IWR (Wnt Inh)	21																				
NOGGIN (BMP Inh)	22																				
IWP-2 (Wnt Inh))	23																				
ANYSOMICYN (JNK Act)	24																				
BEGAGESTAT	25																				
DKK (Wnt Inh)	26																				
SUBROYL (Notch Act)	27																				
XAV (Wnt Inh)	28																				

Detailed composition of the 16 cocktails tested for the cardiac differentiation of ASCs. Cocktail number 14 was chosen on the basis of the up-regulated cardiac-marker gene's panel.

Legend:

Red: fixed component

Green: variable component

All of them were tested for their cardiogenic potential. Because one of the aims of this study is to contribute to the development of stem cell-based therapies for the cure of cardiovascular diseases (CVDs), our efforts focused on the formulation of cocktails in defined serum free culture induction medium as simple as possible. In a first instance, medium containing serum was used to test the different cocktails and was substituted with serum free after the selection of the best cocktail. The time of each induction was kept constant to three weeks. However, in certain cases two distinct culture media were prepared: one used for the first 10 days of culture and the second for the next 11 days.

In this screening phase, the presence of differentiated cells was monitored by standard and quantitative RT-PCR for the expression of cardio-specific genes described in the previous section. The most informative genes were those not expressed in non-induced ASCs, like NKX2-5 and MEF2C [Tab. 3].

Cocktail no. 14 was selected because it was the most effective capacity in inducing cardiogenic phenotype compared to other cocktails, as observed by the up-regulation of NKX2-5 and MEF2C cardiospecific genes by qPCR firstly [Fig. 20] and by the induction of HAND2, FOG and CAMTA genes for the confirmation of the results by standard PCR on the second place [Fig. 21].

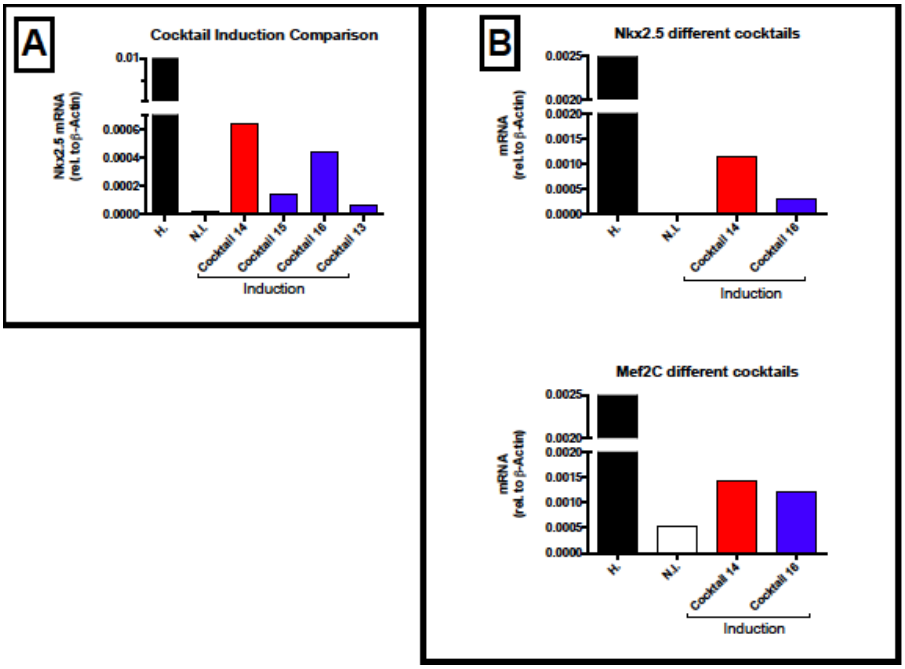

Figure 20. Expression of cardiac specific markers in cells induced with different cocktails. qPCR was used to establish the optimal cocktail to induce cardiomyogenic differentiation of ASCs. A. Expression of NKX2-5 was used to verify the best cocktail out of 4 representatives (Cocktails 13-16).
Cocktails 14 and 16 were selected due to induction of higher expression levels of Nkx2.5 trascription factor (4 and 3 fold, respectively) compared to cocktails 13 and 15.

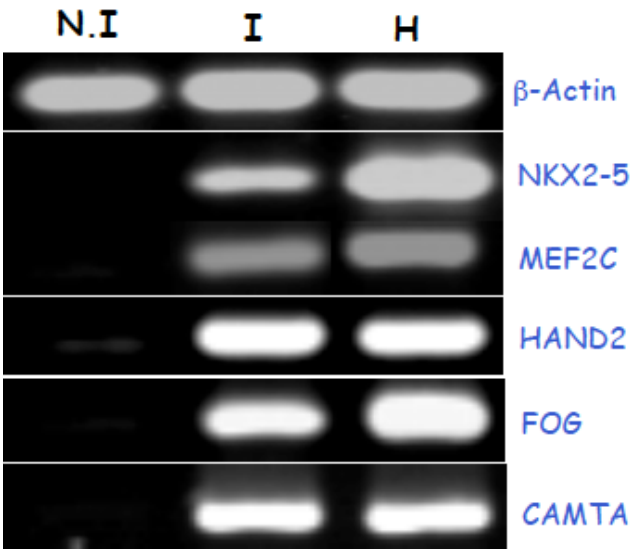

Figure 21. Expression of cardiospecific genes in induced ASCs and control cells. Up-regulation of selected transcripts that represent markers of cardiac differentiation was tested by RT- PCR in ASCs grown in culture medium containing cocktail no.14 after 21 days of culture. Beta-actin is used as positive control. Non-induced ASCs (N.I.), induced ASCs (I) and heart biopsy (H). NKX2-5, MEF2C, HAND2, FOG and CAMTA are clearly up-regulated only in induced cells and in tissue heart biopsy.

In a second phase, whose aim was to obtain a serum- and xeno-free culture medium, the previously identified cocktail no. 14 was tested without any serum but with the addition of 5% of human albumin instead, keeping constant all other culture conditions. Cocktail no. 14 was very effective in inducing cardiomyogenic conditions also in these conditions. Culture medium no. 14-SF was then challenged for the up-regulation of a panel of cardiogenic genes: NKX2-5, MEF2C, HAND2 and FOG. The results showed the up-regulation of all tested genes in induced cells also in these culture conditions [Fig. 22].

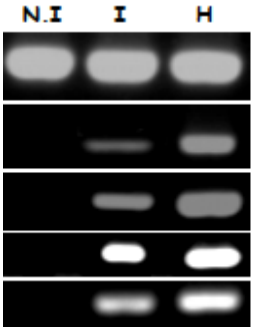

Figure 22. Expression of cardiospecific genes in induced ASCs and control cells. Gene up-regulation tested by reverse transcription PCR of culture medium composed of cocktail no.14 after 21 days of culture of ASCs without any serum component. Beta-actin represent the positive control and appears in all three lanes, non-induced (N.I.), induced (I) and heart biopsy (H). NKX2-5, MEF2C, HAND2, and FOG are clearly up-regulated only in induced cells and in tissue heart biopsy.

Table 5 summarizes the expression of cardiac markers in non-induced or induced ASCs.

Table 5. Expression of cardiac genes in Biopsy, ASCs and cells induced with Cocktail Nr.14.

	Heart Biopsy	ASCs (N.I.)	Induced Cells
β-actin			
NKX2-5			
MEF2C			
GATA4			
CAMTA			
HAND2			
FOG			
TBX5			
BAF60C			
TBX18			
CX43			
MHC			

Legend
Green: presence of gene expression
Red: Absence of gene expression

Functional genes

We further investigated the presence of functional genes in induced and non-induced cells. For instance, we evaluated the expression of SERCA-2 (Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase) and NCX (Sodium-Calcium Exchanger). Both genes are expressed in induced cells after 21 days of culture using cardiogenic cocktail nr 14. Only a faint signal is present in NCX in non-induced cells [Fig. 23]. The ability of cocktail 14-SF to induce cardiac differentiation was highly reproducible [Fig. 24].

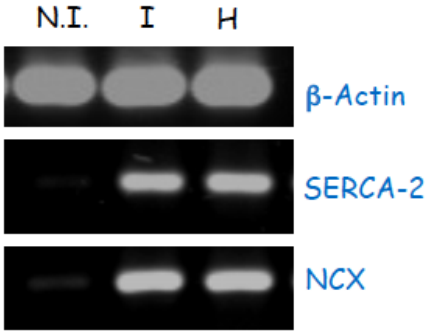

Figure 23. Expression of functional genes. Expression of functional genes (SERCA-2 and NCX) in non induced (NI), induced (I) and heart biopsy (H).

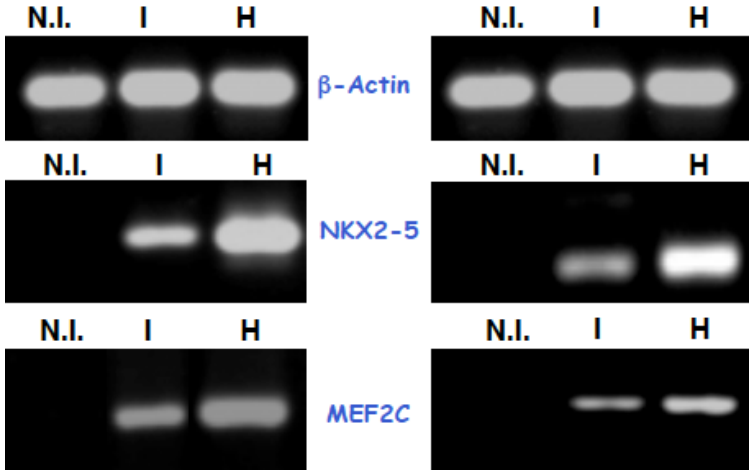

Figure 24. Reproducibility of the differentiating ability of cocktail 14-SF. Expression of Nkx2.5 and Mef2C genes in non induced (NI), induced (I) and heart biopsy (H) in two further inducing treatments of ASCs preparations. This image shows the high reproducibility of the results obtained with the selected cocktail

3.5 Morphological Characterization of induced ASCs

When ASCs were induced toward the cardiac lineage during three weeks of differentiation in the cardiogenic cocktail no. 14, they showed clear macroscopical morphological changes [Fig. 25]. In particular, when induced, the cells became more fusiform or spindle-like, whereas the non-induced ones remained more round-shaped. Results were highly reproducible, as found after studying cells obtained from 10 different patients (not shown), from which representative pictures are depicted in Figure 25.

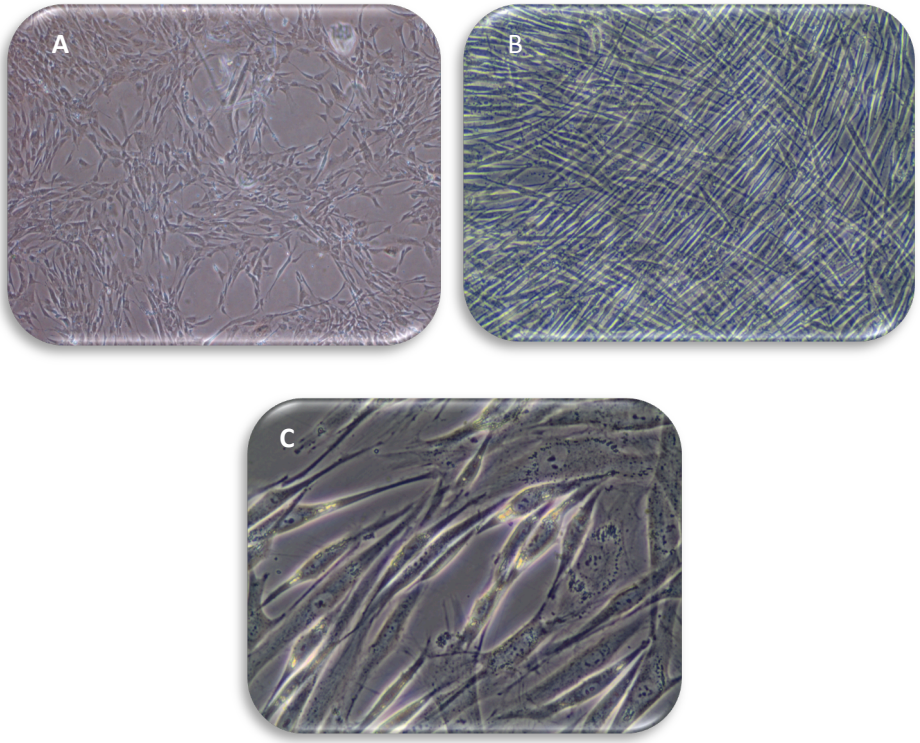

Figure 25. Morphological changes after cardiac induction of ASCs. **A.** non induced undifferentiated ASCs grown in serum free medium, 4x magnification; **B.** cardio-induced ASCs, 4x magnification; **C.** 20x magnification of induced ASCs.

Further analysis of semi-thin sections of ASCs stained with Crystal Violet and Fuchsin and observed under light microscope, showed big cells and presence of autophagosomes in non-induced cells, whereas induced cells appeared smaller and had less autophagosomes, as depicted in Figure 26.
Interestingly, further analysis by transmission electron microscopy (TEM) revealed the presence of unorganized tubular structures in the cell cytoplasm of induced cells, as shown in Figure 28. In contrast, non-induced cells displayed cytoplasm vacuolization confirming the presence of numerous autophagosomes [Fig. 27].

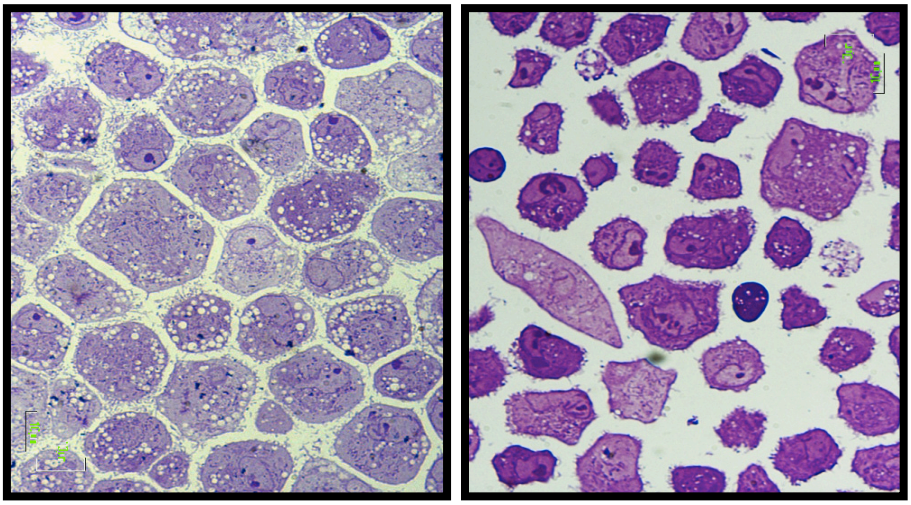

Figure 26. Light microscope images of non induced and induced cells after staining with Crystal Violet and Fuchsin. Non induced cells (left) show a regular shape and display similar size, whereas induced cells (right) are less regular in shape and uneven, generally smaller size.

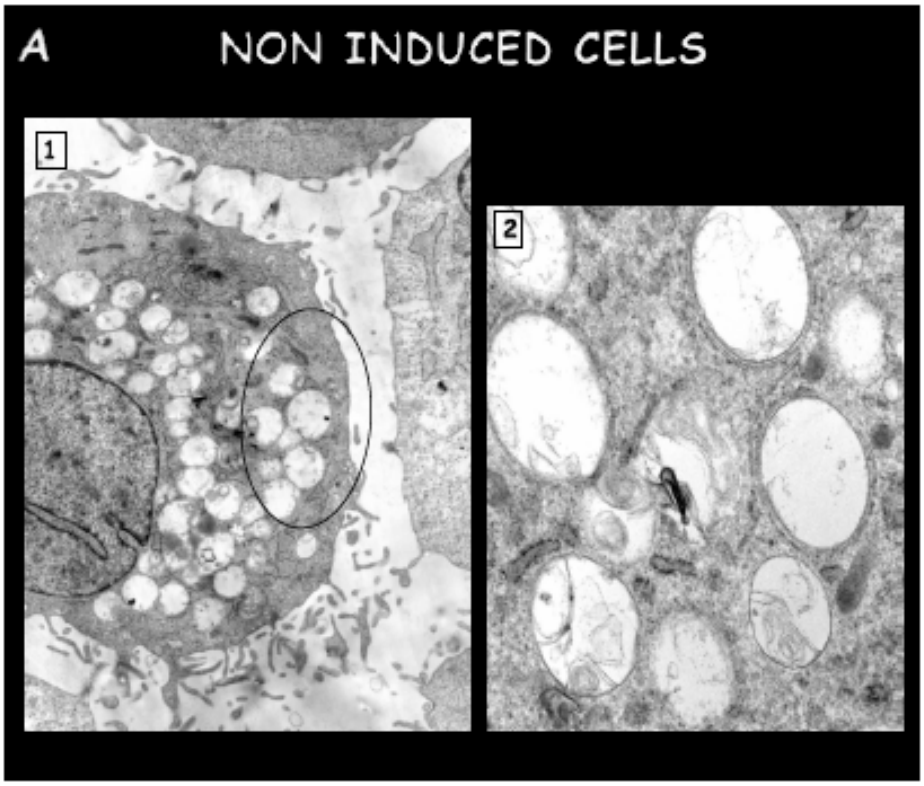

Figure 27. Transmission Electron Microscope (TEM) images of non induced cells. Non induced cells display cytoplasm vacuolization (1, encircled area) due to the presence of numerous autophagosomes (2).

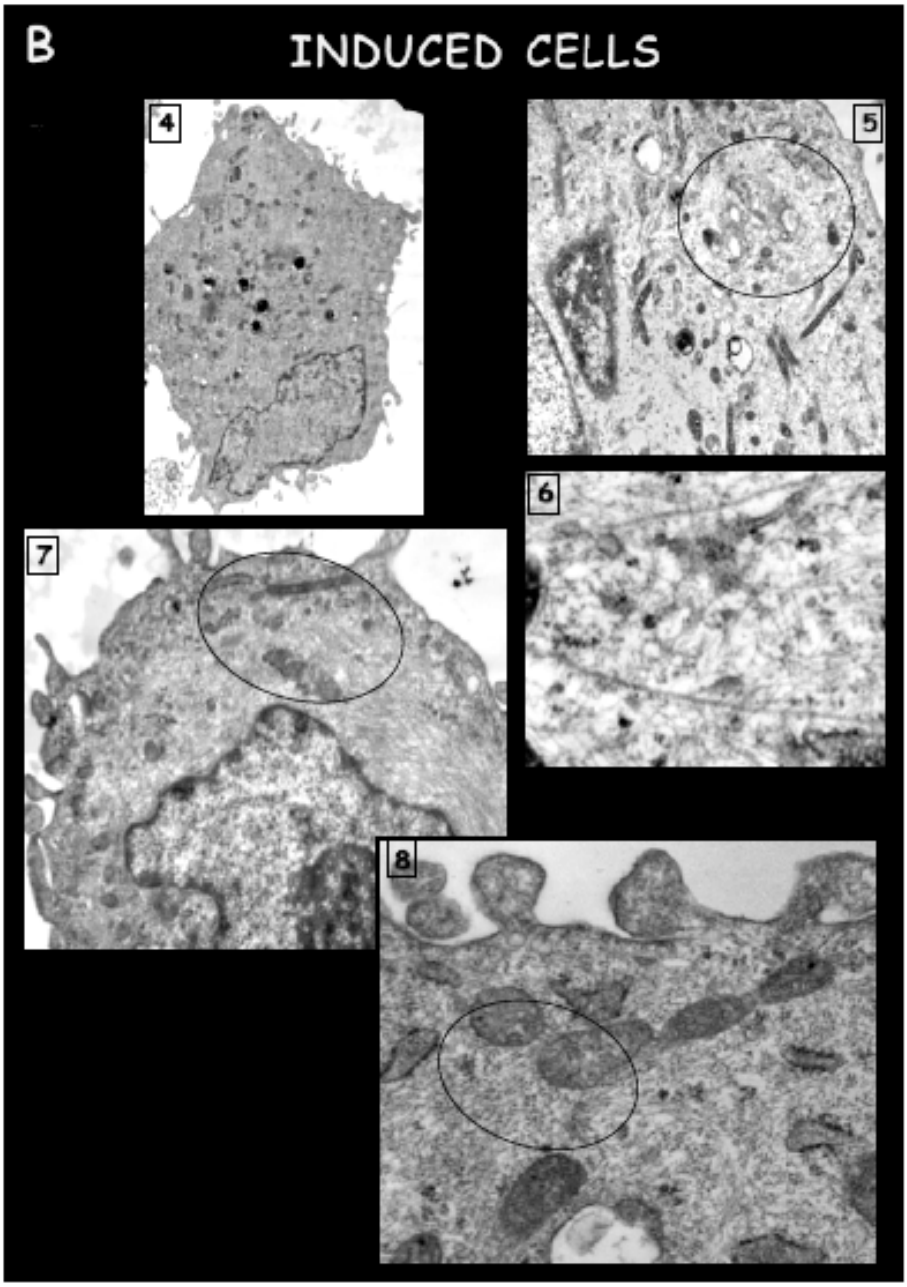

Figure 28. Transmission Electron Microscope (TEM) images of induced cells. Induced cells present a cytoplasm occupied by unorganized filaments (5-8, encircled area).

3.6 Detection of protein markers of cardiac differentiation

Finally, the presence of specific structural and functional proteins characteristic of cardiac tissue (i.e. α-actin, cardiac α-actinin and myosin light chain) and of a cardiac transcription factor (i.e. NKX2-5) was assessed by immunocytochemical analysis of induced and non-induced cells. Results highlight the differentiated status of the ASCs and are presented in Figure 29. The induction of α–actin is evident by a more prominent red staining in induced cells. Similarly, α-actinin, a more specific marker of cardiogenic differentiation was markedly increased in induced cells compared to non-induced cells, where a less intense non-specific staining is visible around the DAPI-stained nuclei. Myosin, another muscle-specific marker for myo-differentiation, could be seen in a small fraction of the induced cells, whereas it could not be seen in any other non-induced cells. The master regulator of cardiac differentiation and nuclear transcription factor, NKX2-5, was evidenced by fluorescent signal in the nucleus of all induced cells, as expected.

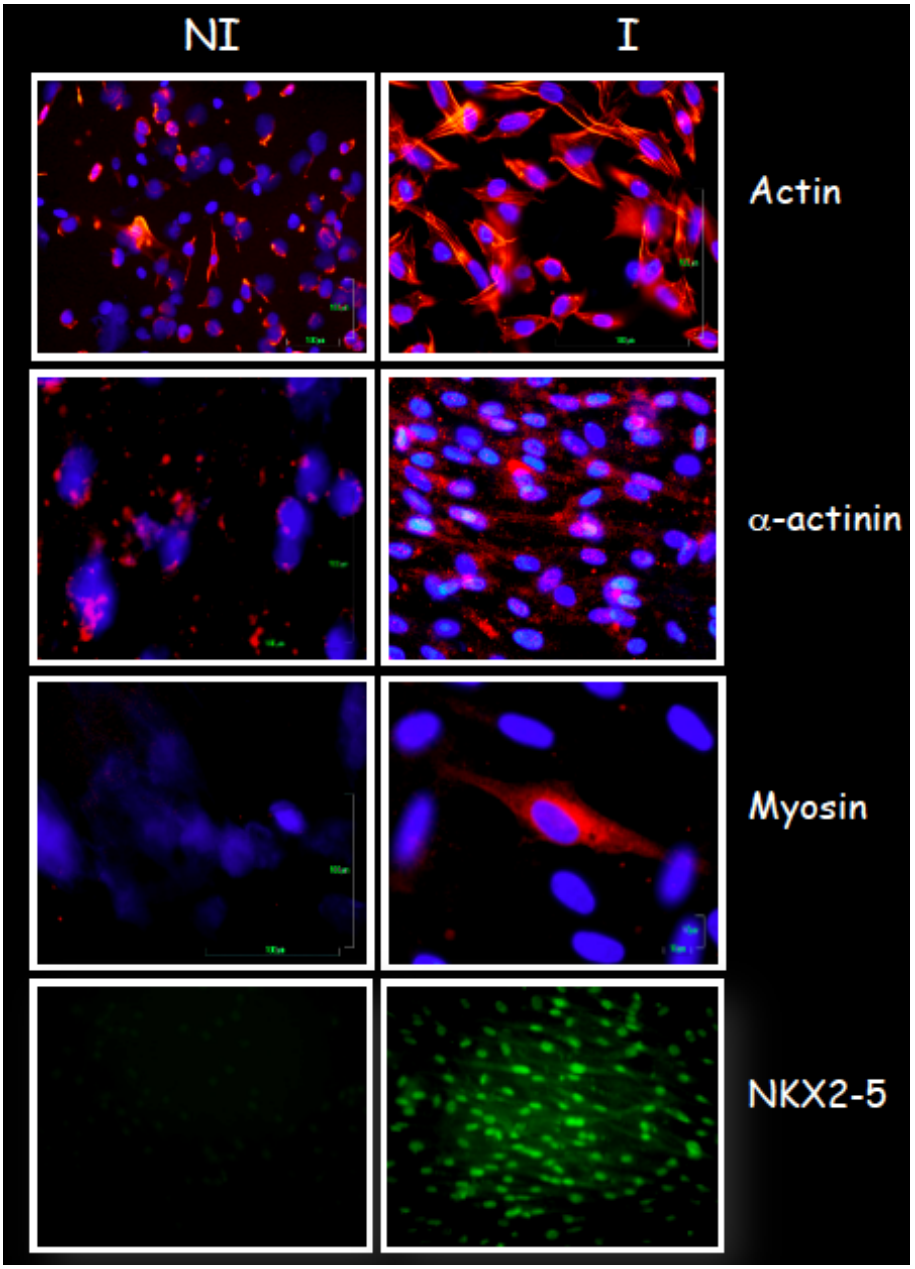

Figure 29. Immunohistochemical analysis of different markers of cardiac differentiation in induced ASCs versus controls. Non induced (NI) and induced cells (I) were stained either for Actin (red), for Cardiac α-actinin (red), for Myosin light chain (red) (*Courtesy of Prof. Magda de Eguileor's group - University of Insubria - Italy*) or for NKX2-5 (green) (from top to bottom). Nuclei were counterstained with DAPI, excepted for the NKX2-5 pictures, because the signal would overlap totally.

3.7 Immunophenotypical characterization of cardio induced ASCs

To confirm whether the cardiac differentiation of the ASCs took place by a different mean than the ones described before, we developed an immunophenotypic citofluorimetric analysis. For this, after 21 days of induction, a part of the culture was dissociated into single cells and stained for four different cardiogenic markers: CD184 (CXCR4), CD309 (VEGFR2), CD117 (cKIT) and CD172A (SIRPA). As shown the preliminary results in Figure 30, flow cytometric analysis revealed that 17.70% of the total cells expressed detectable levels of CD184, 7.39% expressed CD309, 3.50% expressed cKIT and more than 90% the marker CD172A. In contrast, non induced cells did not express any of these marker (data not shown).

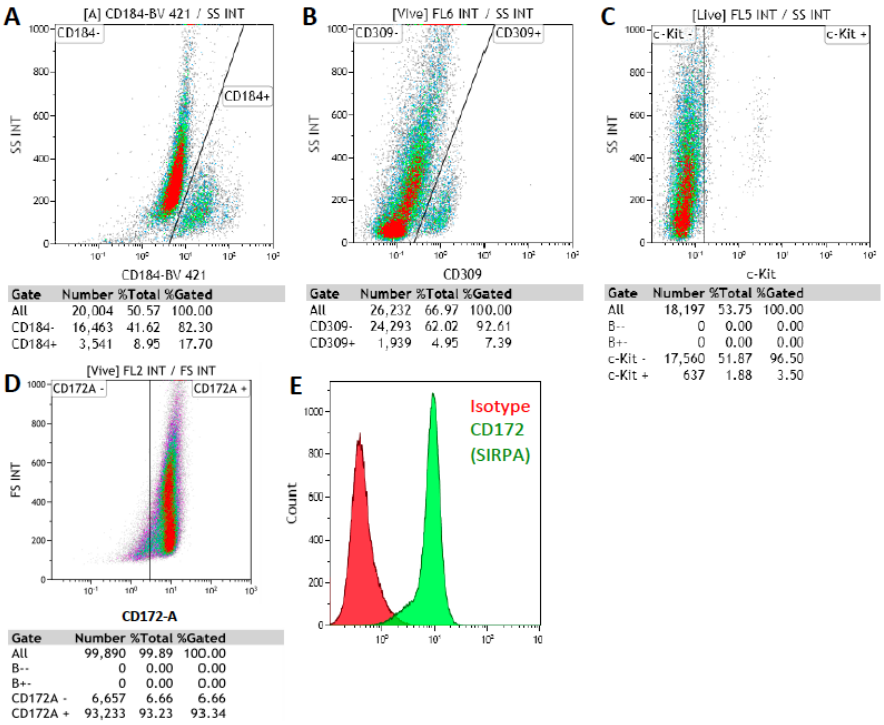

Figure 30. FACS analysis of induced cells.
After induction, cells were stained respectively for: CD184-CXCR4 (A), CD309-VEGFR (B), CD117-cKIT (C) and CD172A-SIRPA (D). In E, the comparison of signal intensity of CD172 marker (green) compared to unspecific signal (Isotype-red) is shown.

3.8 Tissue Engineering

The last important milestone of this work was to use the newly formulated cardiogenic cocktail in combination with mechanical and eventually electrical stimuli. It is a common belief that these stimuli should enhance the differentiation status of the cells by mimicking the natural niche where the cells are located (see introductory part). It was therefore interesting to test whether the combination of the two with our cocktail would further enhance the differentiation of the ASCs.
To achieve this, a device has been developed by the Swiss Stem Cell Foundation in collaboration with Scuola Universitaria Professionale della Svizzera Italiana (SUPSI) for the application of controlled mechanical and/or electrical stimuli to a scaffold over a desired lap of time (see 2.8.3). The scaffolds used were developed and characterized by Prof. Ciardelli's Research Group (Materials in Bionanotechnology, Department of Mechanical and Aerospace Engineering, Politecnico di Torino, see material and methods section 2.8 for further details). Below, preliminary experiments and results are presented.

3.8.1 Morphological and structural properties of the scaffold

We asked the researchers at the Politecnico di Torino to produce a scaffold that could be close in morphology to the human heart. As an example we looked for pictures from de-cellularized human hearts taken at the electronic microscope and compared with the scaffolds produced by Prof. Ciardelli's group [Fig. 31].
Scanning Electron Microscopy (SEM) micrograph of the produced scaffold is reported in Figure 31A and it is compared to the structure of a decellularized heart [Fig. 31B]. Despite the application of a thermal gradient to allow pore orientation in a preferred direction, the proposed scaffold does not show a highly organized microstructure. This behavior can be correlated to the concurrent effects of both the freezing conditions (-20°C for 5h) and the concentration of the starting DMSO solution (12% w/v), that do not allow solvent crystal growth in a preferred direction. The produced scaffold possesses an open and interconnected porous structure that can

promote scaffold colonization. In detail, scaffold pore size was 75 ± 57 µm and its porosity was about 82% (82 ±2 %). Figure 32 reports the pore size distribution. The proposed scaffold showed a pore size ranging from tens to hundreds µm, thus allowing both cell colonization and vascularization: small pores favor vascularization since endothelial cells are unable to fill pores larger than their diameter (8-12 µm (Christenson and Stouffer, 1996)), while larger pores are necessary to allow cell homing and migration deep in the scaffold.

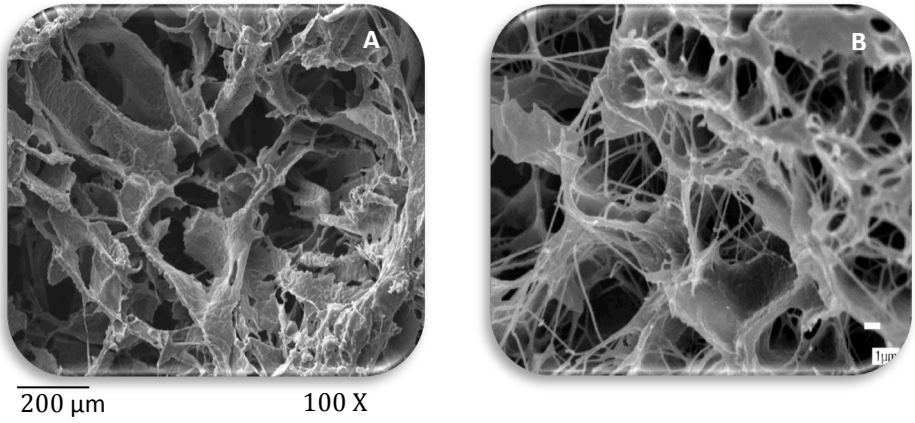

Figure 31. Morphological properties of the scaffold. Scanning Electron Microscopy (SEM) image of the proposed scaffold (A) compared to decellularized heart (B)

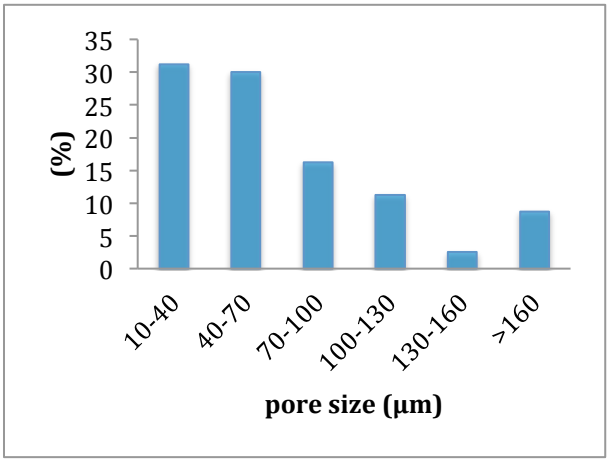

Figure 32. Structural properties of the scaffold. Pore size distribution of the scaffold as evaluated with Image J software.

3.8.2 Mechanical properties

3.8.2.1 Tensile tests

The fabricated scaffolds are characterized from a mechanical point of view by stress-strain tests in dry and wet conditions at room temperature and results are collected in Table 6. The typical stress-strain curve of the scaffold in dry and wet conditions is reported in figure 33.

Table 6. Tensile tests 1. Experimental mechanical data of the prepared scaffolds in wet and dry conditions

	Young Modulus (MPa)	Stress at break (MPa)	Strain at break (%)	Load at break (N)
DRY CONDITIONS	2.5 ± 0.3	0.4 ± 0.0	127,5 ± 4,8	5,8 ± 1,7
WET CONDITIONS*	1.1 ± 0.1	0.3 ± 0.0	171. ± 5,0	7,6 ± 1,2

*preconditioning in phosphate buffered saline fro 24h

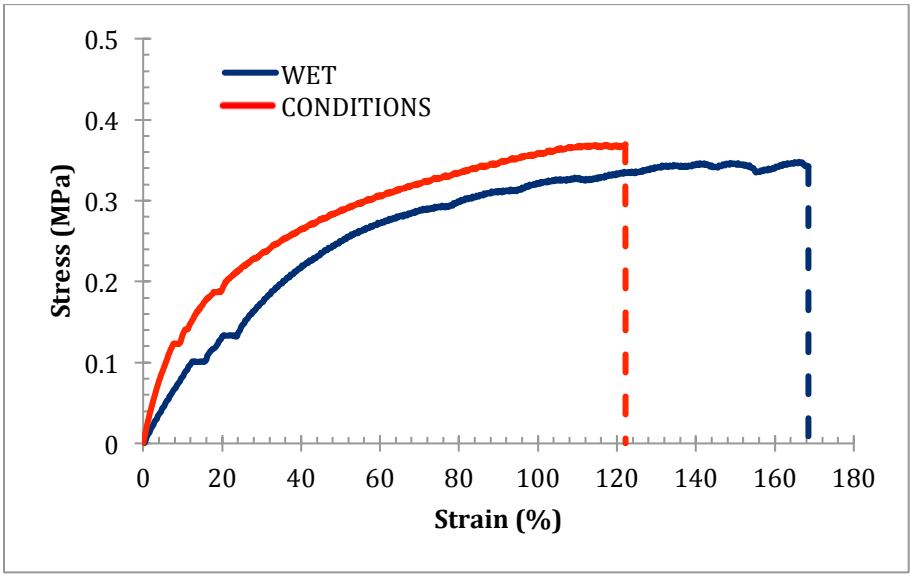

Figure 33. Tensile tests 2. Stress-strain curves of the scaffold in dry and wet conditions

The amplitude of the initial elastic region increases from about 10% to 20%, in wet compared to dry conditions, thus making the proposed scaffold suitable for cardiac TERM application (strain < 10 % at diastole initial stages: strain ≈ 15-22 % at the diastole end (Chen et al., 2008; Enderlein, 1975; Hidalgo-Bastida et al., 2007; Silvestri et al., 2013). Moreover, stress at break and young modulus in wet conditions is lower than in the dry state, while a significant ($P<0.05$) increase in strain at break was observed in wet conditions with respect to the dry ones. The changes in mechanical properties we observed in wet conditions compared to the dry state, as observed by (Klouda et al., 2008) and (Pan and Ding, 2012) for different polymer substrates, can be correlated to the plasticizing effect of the aqueous environment (Klouda et al., 2008; Pan and Ding, 2012; Zhou et al., 2005). The scaffold showed a young modulus of about 1 MPa in wet conditions. This value is in accordance with the stiffness required for scaffold application in cardiac tissue engineering/regenerative medicine (Boffito et al., 2013; Silvestri et al., 2013). Moreover, stress and strain at break are significantly higher than stress and strain at break of myocardial tissue (3-15 KPa and 22-90%, respectively) (AYGEN and BRAUNWALD, 1962; Boffito et al., 2013; Hidalgo-Bastida et al., 2007; Silvestri et al., 2013; Yamada and Evans, 1970).

3.8.2.2 Durability tests

Durability tests were conducted to evaluate the suitability of the proposed scaffolds for the intended application, i.e. the development of cellularized matrices to be applied in cardiac tissue engineering/regenerative medicine through mechanical stimulation in a bioreactor. As an example, the results of one of the three conducted analysis are reported. Figure 34 reports clamp position as a function of time. The specifically developed Matlab routine made it possible to chart the maximum and minimum clamp position and patch deformation over time, as reported in Figure 35 and 36, respectively.

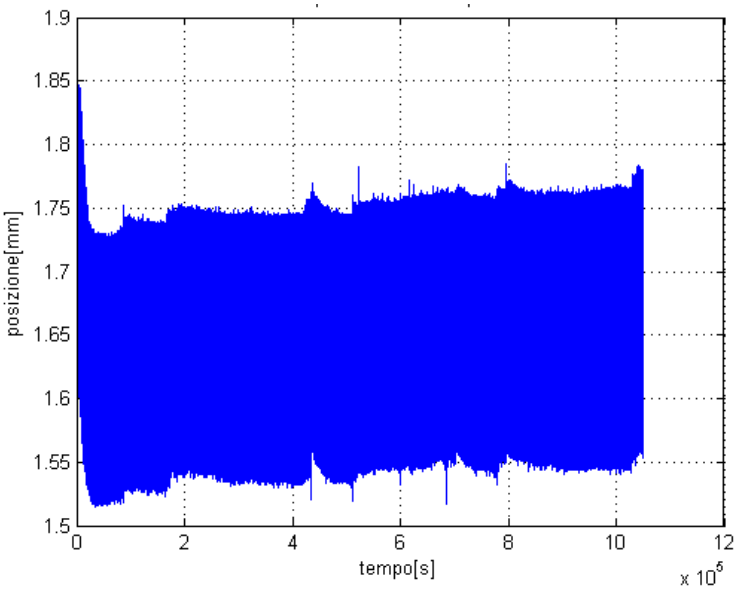

Figure 34. Durability tests 1. Clamp position as a function of time.

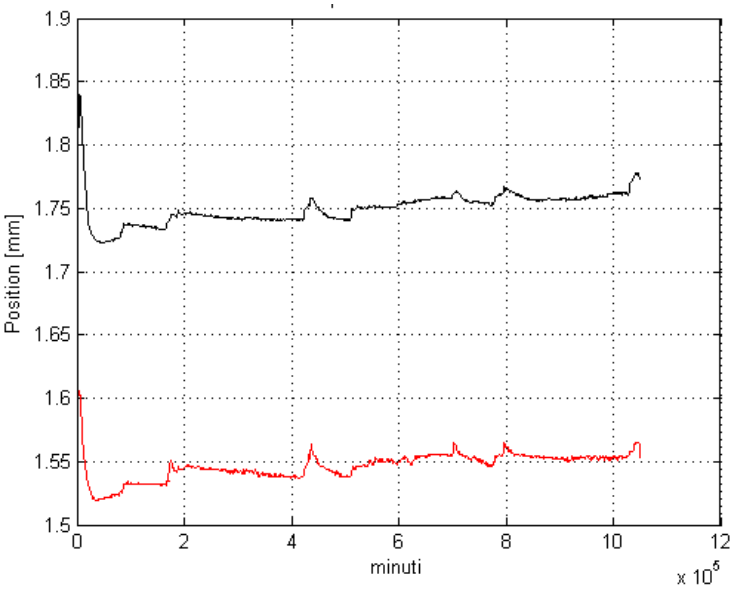

Figure 35. Durability tests 2. Maximum and minimum position as a function of time.

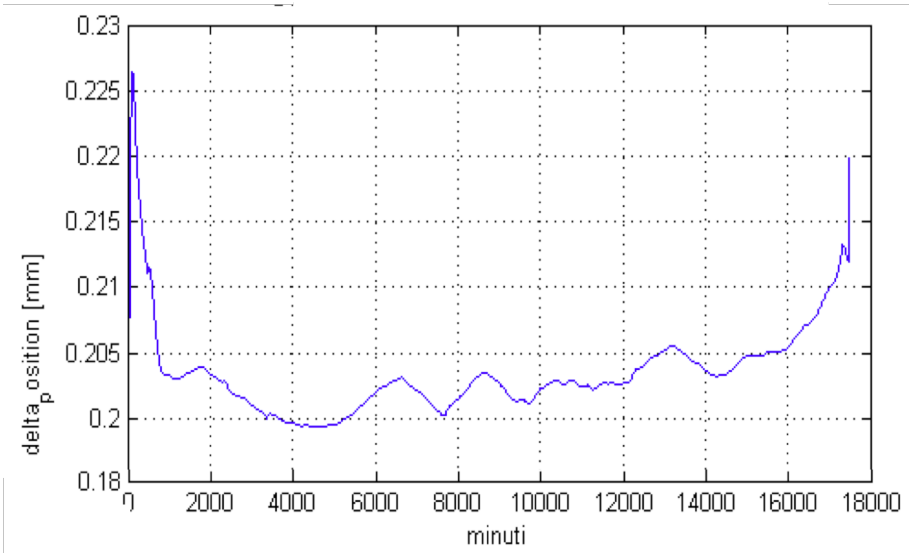

Figure 36. Durability tests 3. Patch deformation (delta position) as a function of time.

Due to the application of a sinusoidal (frequency 1 Hz) stress with amplitude 0.4 ± 0.2 N, the patch underwent a cyclic deformation of about 0.2 mm (i.e. 1%). None of the analyzed samples showed macroscopic damage or broke during the analysis. A plastic deformation was observed at the end of the experiment; each sample, indeed, showed an initial deformation followed by an elastic behavior.

3.8.3 Scaffold seeding

Before cell seeding, scaffolds were coated with collagen I to enhance cell attachment, then the scaffold was placed into a petri culture dish and seeded with $1x10^6$ cells. The cell suspension was pipetted directly on the membrane surface (6 drops of 25 μl each). Cells were allowed to attach to the scaffold for 2 hours before adding the culture medium. Cells were maintained in humidified 37°C incubator containing 5% CO_2 for 5 days. At day 5 the scaffold was assembled on the device with a sterile forceps under

laminar flow. The device was placed in a sterile container and the induction started using cardio induction culture medium and setting the device on a force of 0.4N, amplitude 0.2N, frequency 1 Hz. The duration of the experiment was 10 days. Figure 37 (A-C) shows cellular distribution on the scaffold.

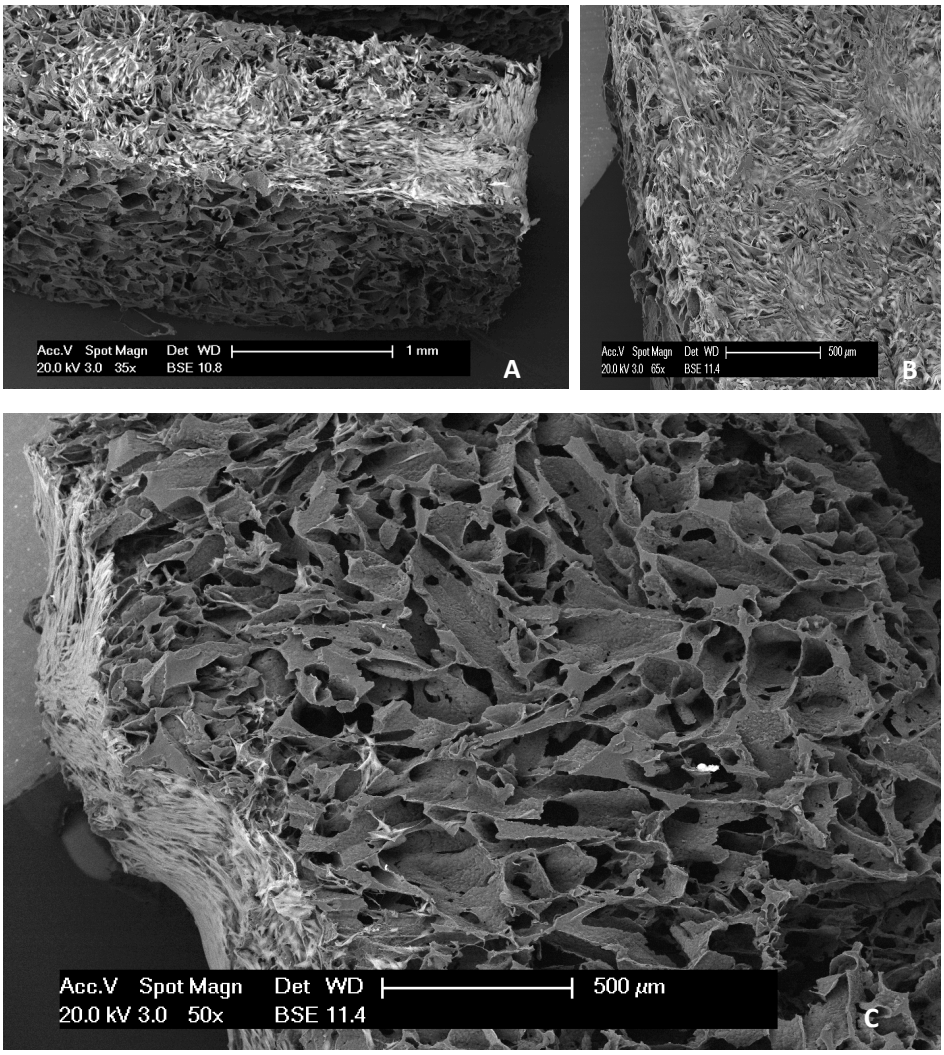

Figure 37. Scanning Electron Microscope (SEM) of the scaffold. The pictures (A-C) show a clear surface distribution of the cells on the scaffold.

3.8.4 Alamar Blue assay for the assessment of viability of ASCs in the scaffold

Due to the fact that it is extremely stable and more importantly non-toxic to the cells, Alamar Blue allows for continuous monitoring of cultures over time. We performed the assay 5 days after seeding (before assembling on the device) and at the end of mechanical stimulation. The percentage of reduction appears to be about 90% 5 days after seeding and decreases to approximately 70% at the end of the ten days of the mechanical stimulation [Fig. 38].

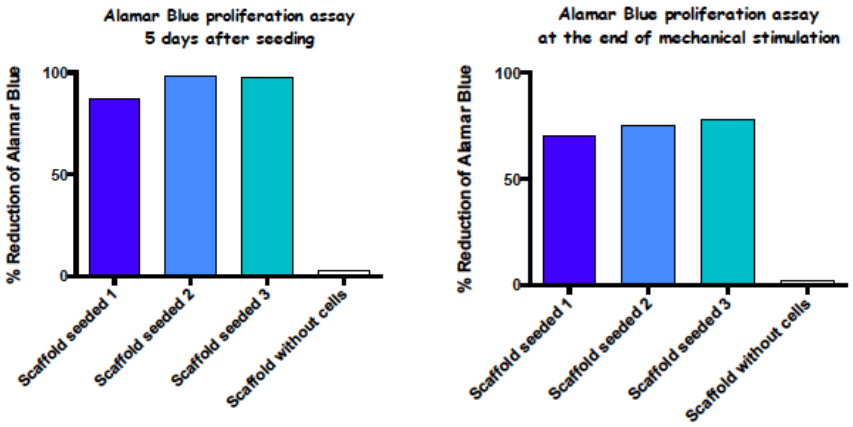

Figure 38. Cell viability assays (Alamar Blue proliferation assay) 5 days after seeding and at the end of mechanical stimulation. The graph shows the % of reduction of Alamar Blue of three different scaffolds compared with scaffold without cells 5 days after seeding (left) and at the end of mechanical stimulation (right).

3.8.5 Comparison between 2D Induction and Induction on scaffold

The final goal was to understand if the combination of cardiac induction with cocktail nr 14 and the mechanical stimulation on a scaffold would increase the expression of the most important cardiogenic transcription factors if compared to the induction with cardiogenic cocktail alone on a simple 2D culture condition. Therefore, at the end of the ten days of mechanical stimulation, qPCR was performed to confirm the expression of cardiac markers in 2D induction (on plate) and to make a comparison with the induction on scaffold subjected to mechanical stimuli. The Figure 39 shows the preliminary results: indeed the combination of growth factors, cytokines and mechanical stimulation for 10 days led to an at least 2 fold increase in mRNA expression of NKX2-5, MEF2C, HAND2, cKIT and MHC.

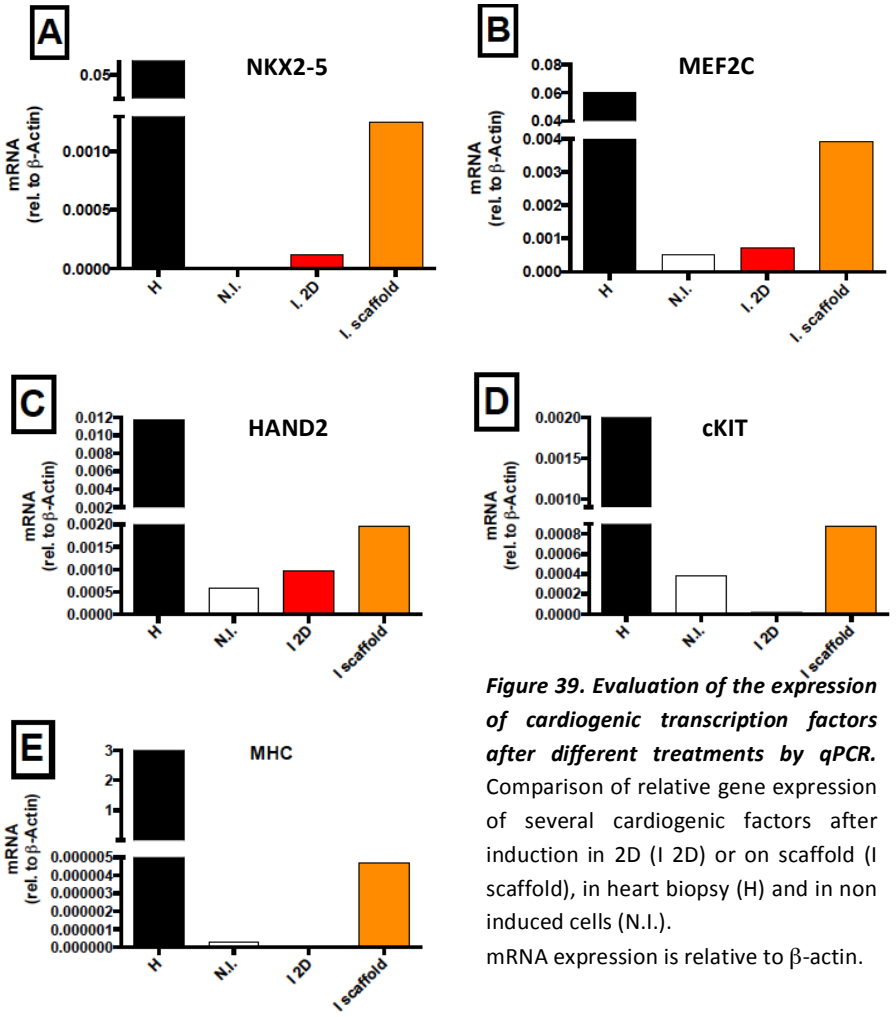

Figure 39. Evaluation of the expression of cardiogenic transcription factors after different treatments by qPCR. Comparison of relative gene expression of several cardiogenic factors after induction in 2D (I 2D) or on scaffold (I scaffold), in heart biopsy (H) and in non induced cells (N.I.).
mRNA expression is relative to β-actin.

4. Discussion and Outlook

The aim of the present thesis was to evaluate the differentiation of multipotent stromal cells to cardiomyocytes and to characterize the differentiated cells. Due to the many aspects covered in this thesis, the present discussion has been divided accordingly to the topic into three main sections: extraction and characterization of the SVF, evaluation of cardiac differentiation capability of ASCs and tissue engineering.

4.1 Extraction and characterization of SVF

Our first milestone was focused on developing protocols for the preparation of the SVF from adipose tissue and the subsequent isolation of ASCs fraction as more extensively described in our submitted paper (Minonzio et al., submitted). The cells have been extensively characterized by flow cytometry, and genetically profiled by a reverse-transcription and PCR-based approach. In the SVF we identified three distinct subpopulations of cells in which the ASCs were defined as CD146/CD45 negative and CD34 positive, representing 10%-25% of the total SVF cells. CD34 was used as marker for pluripotent stromal cells, while CD146 and CD45 were used to label endothelial and hematic cells, respectively. Dominici and co-workers extensively characterized *in vitro* the expanded cells with different markers describing them as $CD90^+$, $CD73^+$, $CD105^+$, $CD34^-$ and $CD45^-$ (Dominici et al., 2006). Therefore, our citofluorimetric analysis showed a concordant pattern. At this stage one can conclude that the isolated cells fit with the so far accepted definition of mesenchymal stem cell.

The isolated ASCs were then tested for presence of features that stem cells usually display, such as ability to adhere to plastic, CFU-F clonogenic assay for their stemness, and for their ability to differentiate in osteogenic, chondrogenic and adipogenic and finally cardiogenic cell lines.

Interestingly, all these features did attract the attention of researchers interested in regenerative medicine, in particular in the field devoted to restore the functionality of a damaged heart. In comparison to bone marrow, the main advantage in using adipose tissue is that the latter can be

obtained in much larger amounts and with a far less-invasive surgical operation. Furthermore the number of ASCs in harvested fat is approximately 40-times higher than that of bone marrow MSCs.

4.2 Evaluation of cardiac differentiation capability of ASCs.

The most important milestone and goal of this work was to investigate the cardiogenic potential of the ASCs.
Cardiovascular disease remains one of the most deadly diseases (Roger et al., 2012). One of the causes is that after a myocardial infarction, an extensive tissue damage leads to the development of congestive heart failure in many survivors. Heart transplantation is nowadays the only viable treatment for end-stage congestive heart failure, but unfortunately lack of available donors has remained an insurmountable problem (Vono et al., 2012). Therefore, in the search for alternative therapies, myocardial repair via cell therapy, that is the injection of cell suspension directly in a damaged cardiac area, has generated a great deal of enthusiasm (Vono et al., 2012). Obviously, which type of cell has to be used to generate an artificial heart tissue represents a relevant issue, since this heavily impacts on the final properties of the graft. Nevertheless, some minimal requirement must be fulfilled: the cells should at least be highly viable, be able to electromechanically integrate into the resident healthy cardiomyocytes and possibly be histocompatible. In this regard, the use of autologous stem cells into the damaged heart seems therefore on of the best solutions. It is well established that embryonic stem cells (ESCs) can generate cardiomyocytes, but ethical issues limit their clinical potential (Doetschman et al., 1985). The ability to generate induced pluripotent stem cells (iPSCs) provides another approach for the generation of autologous grafts. Nevertheless, their main limitations for the clinical use are the time that is required for the reprogramming procedure and the need to ensure that they are non tumorigenic (Mauritz et al., 2008). Resident cardiac stem cells are another promising candidate for cell therapies since they can be isolated from identifiable cardiac niches and expanded *ex vivo*. Nonetheless, the high invasiveness of the harvesting technique limits their

clinical suitability. Thus ASCs have been identified as the most suited cell type and investigated for their use in biological grafts for cardiac repair due to their relative ease and safeness of their obtainment (Beltrami et al., 2003).

In this work, after the *in vitro* expansion of ASCs in serum free medium that has been developed in our laboratory, we followed an innovative approach for the cardiogenic differentiation: the use of human growth factors in a defined serum-free culture induction medium. For this, different cocktails of growth factors and cytokines and different culture conditions were tested for their ability to induce cardiomyogenesis.

In the past years, different approaches have been used to induce the differentiation of ASCs toward the cardiac lineage. Gaustad and co-workers in 2004 investigated the possibility to use cell extracts to promote differentiation of ASCs (Gaustad et al., 2004). In particular, they reported the induction of cardiomyocyte properties in human ASCs after exposure to a nuclear and cytoplasmic extract of rat cardiomyocytes. The induced cells showed evidence of differentiation *in vitro*, they expressed cardiomyocytes markers and were induced to beat in culture. These results support the use of somatic cell extracts to elicit differentiation of MSCs towards a specific target cell lineage.

Several other groups (Makino et al., 1999; Rangappa et al., 2003) have found that mesenchymal stem cells can be transformed into cardiomycytes after the exposure to 5-azacytidine (5-aza). The latter was originally developed and tested as a nucleoside anti-metabolite for treatment of acute myelogenous leukemia (Wilhelm et al., 1999). This compound is an effective DNA hypomethylating agent and it is capable of altering the expression of certain genes and of regulating the differentiation. In addition, human ES cells and stem cell antigen-1 (Sca-1 positive) cardiac progenitor cells were differentiated into cardiomyocytes in response to 5-aza treatments. Another group (Zhu et al., 2009) investigated the role that cardiac microenvironment plays in directing the progression of stem cells into differentiated cells. Adipose tissue derived stem cells (ASCs) were cultured with cardiomyocytes directly (“direct co-culture directly”) or by cell culture insert (“indirect co-culture indirectly”). Differentiated ASCs that

experienced the co-culture presented cardiac ultrastructure and expressed cardiac specific genes, suggesting that cell-to-cell contact is important in relaying the external cues of the microenvironment and in controlling the differentiation of ASCs toward cardiomyocytes.

Because of the use of animal derivatives or of chemical agents that may affect the genomic stability of the cells (i.e. 5-AZA), none of the above cited methods are compatible with the use of ASCs in the clinical practice. Therefore, in this work, different cocktails of growth factors and cytokines and different culture conditions have thus been tested for their ability to induce cardiomyogenesis, with the goal of being compatible with a potential clinical application.

On the basis of the crucial biochemical pathways involved in cardiac muscle formation during embryogenesis and stem cell differentiation, we were able to identify and select growth factors and cytokines that were more effective in the induction cocktail. Other studies have identified three main families of peptide growth factors that are essential for heart development and speciation: the bone morphogenetic proteins (BMPs), that are members of the transforming growth factors β superfamily (TGF-β), the wingless-related proteins (Wnt) and the fibroblast growth factors (FGFs) (Behfar et al., 2010). All of them have been proven to be of similar importance in the mammalian heart as either positive or negative regulators. In our cocktails we tested several components of the TGF-β family for their ability to promote expression of the principal cardiac transcription factors. In particular, we demonstrated that BMPs are needed during mesodermal patterning but not later for the differentiation of committed precursors. The combination of BMP4 and Activin A induces the formation of primitive streak-like population (Yang et al., 2008) and promotes mesodermal fate determination (Kattman et al., 2011; Teo et al., 2012). Indeed extracellular inhibitors of BMP and Wnts are the principal inducers that commit multipotent progenitors to cardiac fate (Mercola et al., 2011; Noseda et al., 2011). For this reason after testing 16 different cocktails combining the 28 growth factors potentially involved in cardiogenic differentiation, we decided to produce two different cardiogenic media: the first was applied for 10 days to allow the creation of

mesodermal patterning and the second for the following 11 days to commit the mesodermal cells into cardiac fate.

It was also important to investigate the role of Wnt pathway in the emergence of cardiac lineage. Willens and co-workers (Willems et al., 2011b) identified small-molecule inhibitor of b-catenin dependent canonical Wnt pathway that drove ESCs-derived mesoderm to a cardiac fate. On the basis of these experiments, in our study we tested several molecules and we observed that only IWR was able to switch on the expression of the principle cardiogenic factors. Cardiogenol C turned out to be a crucial component of our cocktail, in fact it is well known to be the most potent inducer of MHC expression (Wu et al., 2004). Similarly also ascorbic acid and retinoic acid, a derivative of vitamin A, have been shown to promote cardiomyogenic differentiation of ES cells (Takahashi et al., 2003; Wobus et al., 1997).

The presence of differentiated cells was assessed by standard and quantitative RT-PCR for the expression of cardio-specific genes. Intriguingly, non-induced human ASCs unexpectedly expresses some of the cardio-specific transcription factors (GATA4, TBX5 and BAF60C) that were chosen for this first screening phase. Interestingly, a recent work demonstrates that a combination of the cardiogenic transcription factors GATA4, TBX5 and BAF60C can reprogram and drive cardiogenesis and that GATA4 and TBX5 are part of the combination that directs the differentiation of mesoderm into cardiomyocytes (Turbendian et al., 2013). These results are therefore very relevant because they suggest for the first time that, because of their particular molecular pattern, the ASCs not only have the potential but are inherently prone to differentiate into cardiac precursors.

Among all, Cocktail no. 14 was selected, because of its ability to strongly induce a cardiogenic phenotype by up-regulating the most important cardio specific genes (i.e. NKX2-5), a transcriptional factor crucial for the general myocardial development, and specifically required for left ventricular chamber formation. Since these protocols are ultimately aimed at producing cells for applications in humans, the second step was to develop a protocol compliant with the strict European regulations concerning the Advanced Therapy Medicinal Product obtaining a serum- and xeno-free

"clinical grade" culture medium. For this reason the identified cardiogenic cocktail has been tested without the addition of any serum components but with 5% human albumin instead. The results proved that Cocktail no. 14 with human albumin (patent pending) was capable of producing a similar induction also without the 1% horse serum supplement. During testing of the chosen cocktail, the expression of two functional genes in induced cells was also evaluated: SERCA-2 and NCX. SERCA-2 or sarco/endoplasmic reticulum Ca^{2+}-ATPase resides in the sarcoplasmic reticulum (SR) within muscle cells and it is a Ca^{2+}-ATPase that transfers Ca^{2+} from the cytosol of the cell to the lumen of the SR at the expense of ATP hydrolysis during muscle relaxation (Arruda et al., 2007). The sodium-calcium exchanger (NCX) is instead an antiporter membrane protein that removes a calcium ion from the cell in exchange for the import of three sodium ions. In fact, NCX is considered one of the most important cellular mechanisms for the removal of Ca^{2+}. This exchanger is usually found in the plasma membrane, mitochondria and endoplasmatic reticulum of excitable cells (Dipolo and Beaugé, 2006). Both genes were found to be expressed exclusively in induced cells further underlining the cardiac commitment of induced ASCs.
After completion of the molecular analysis, we focused on the morphological characterization of differentiated ASCs. After three weeks of differentiation in cardiogenic cocktail the cells showed a clear macroscopical changes in their morphology becoming more fusiform or spindle-like and were arranged in the same orientation, forming an ordered array. Further analysis of semi-thin sections of non-induced ASCs stained with Crystal Violet and Fuchsin showed a bigger shape and the presence of autophagosomes, whereas induced cells appeared smaller and had fewer autophagosomes. Transmission electron microscopy (TEM) analysis at day 10 of differentiation revealed the presence of unorganized tubular structures in the cell cytoplasm of induced cells, whereas non-induced cells displayed cytoplasm vacuolization due to the presence of numerous autophagosomes. Finally, the presence of specific structural and functional proteins characteristic of cardiac tissue (i.e. actin, cardiac actinin and myosin light chain) and of a cardiac transcription factor (i.e. NKX2-5) was assessed by immunohistochemical analysis in induced and non-induced

cells. Results further highlight and confirm the differentiation potential of ASCs.

In conclusion, the observation that ASCs differentiation has not reached completely could be beneficial for future cell therapy applications, since the immature, but already committed cells might better survive, could still have the capacity to further divide and be influenced by the surroundings in the recipient heart (Mummery, 2007). Therefore it would be of big interest to test the survival rate and healing potential of the ASCs induced with our serum free based cocktail no. 14 in an *in vivo* trial.

4.3 Tissue engineering

Scientific studies have shown how cell therapies are generally hampered and limited by a poor survival of the injected cells. In fact, most of them die after grafting into the injured heart mainly for hypoxia and nutrient deprivation. The generation of a tissue with complex structures and functions is not feasible by simply culturing the cells within Petri dish systems: without the appropriate chemo-physical stimuli and a 3D environment, cells cannot maintain their shapes, phenotypes and roles and lose their ability to proliferate and form organized tissues. As already discussed, the human heart has a limited capacity of self-regeneration. To investigate and guide cellular growth, differentiation and functional tissue organization, it is fundamental to recreate environments for the cells that mimic physiological conditions. Therefore, the generation of a 3D-engineered cardiac scaffold to be implanted into the injured myocardium was proposed as a more suitable approach than the direct injection of cells (Geuss and Suggs, 2013). This represents a challenging but probably also an effective and promising therapeutic strategy.

Therefore, the last important milestone of this work was to combine the newly formulated cardiogenic cocktail to mechanical and/or electrical stimuli. It is a common belief that those stimuli should enhance the differentiation status of the cells by mimicking the natural niche where the cells are located. In fact, mechanical stress has been observed to play an important role in determining cell fate during embryogenesis (Geuss and Suggs, 2013). It was therefore interesting to test whether the combination of these stimuli with our selected cocktail would further enhance the

differentiation of the ASCs. To achieve this, a device was developed by the Swiss Stem Cell Foundation in collaboration with the Scuola Universitaria della Svizzera Italiana (SUPSI) for the application of controlled mechanical and/or electrical stimuli that are applied on a scaffold over a desired lap of time. The scaffold was prepared for us at the Politecnico di Torino such that it closely resembled the morphology of the human heart. However, the proposed scaffold did not show a highly organized microstructure, despite the application of a thermal gradient to allow the pore orientation. This behavior can be correlated to the concurrent effects of freezing conditions and the concentration of the starting DMSO solution. Such result is in agreement with the data reported by Guan and co-workers, who observed that if the polymer solution concentration is high, the viscosity of the polymer/DMSO system might lead to slow crystal growth during quenching, minimizing the impact of the transient temperature gradient and resulting in random pore structures (Guan et al., 2005). In general, a good scaffold should provide a structured environment with tissue-specific mechanical properties and the ability to integrate with surrounding tissue. The Torino's scaffold showed a pore size ranging from tens to hundreds of microns, thus allowing both cell colonization and vascularization. It was characterized from the mechanical point of view by stress-strain tests in dry and wet conditions and durability tests ensuring for good stretch properties.

The concept of using a strain to induce differentiation is based on the biological function of cardiomyocytes. Experimental evidences underlines the importance of this strategy for cardiogenic differentiation: cell stretching promoted the elongation of the cell membrane and the rearrangement and orientation of actin filaments, which facilitates the connections made between cells that are necessary to promote intracellular communication (Salameh et al., 2010); Vandenburgh and co-workers showed that unidirectional mechanical stretch *in vitro* initiated a number of morphological alterations in a confluent cardiomyocyte culture, which were similar to those occurring *in vivo* during the heart growth (Vandenburgh et al., 1996). Other experiments demonstrated that, compared to unstretched controls, six days of unidirectional stretch of engineered heart tissue (made out from neonatal rat or embryonic chick

cardiomyocytes mixed with collagen I) in a custom-made device improved the cellular organization and increased expression of the atrial natriuretic factor (ANF) and α-sarcomeric actin (Fink et al., 2000). Another German group showed that after seven days of unidirectional cyclic stretch (10%, 2Hz), the engineered heart tissue displayed structural and functional features of a native differentiated myocardium and were proposed as a promising material for *in vitro* studies of cardiac function and tissue replacement therapy (Zimmermann et al., 2006). In 2008, Gwak and co-workers, reported that cyclic mechanical strain (10%, 1 Hz, for 2 weeks) promotes cardiomyogenesis in mouse embryonic stem cell (ESCs) as shown by cardiac-specific gene expression and *in vivo* tests, that showed a significant increase of grafting efficiency and cardiomyogenic potential of the implanted cells (Gwak et al., 2008).

In our preliminary experiments, we chose to apply a sinusoidal (frequency 1 Hz) stress with an amplitude of 0.4 N. With these parameters, the patch underwent a cyclic deformation of about 0.2 mm (i.e. 1%). All the cyclic deformations proposed in other mentioned studies were higher than that produced in our tests. This was due to a different approach that was based on mimicking the physiological conditions: the ASCs were in fact subjected to a mechanical force that is equivalent to the diastolic strains of the native heart as described by Hess and co-workers (Hess et al., 1979). The frequency of 1 Hz or 60 pulsations per minutes reflects the usual resting heart rate. Presence of macroscopic damage was monitored during our analysis. However, each sample underwent an initial deformation followed by an elastic behavior. These results can be correlated to the polymer chain reorganization upon the application of the mechanical stress. In fact, due to the viscoelastic nature of the polyurethane used to fabricate the scaffold, when a cyclic stress is applied, polymer chains tend to gradually lose their random coil configuration and orient themselves in the direction of the applied mechanical stress, as reported before (Guan et al., 2005).

The scaffolds were seeded with ASCs and cell viability was assessed by Alamar Blue Assay: 5 days after seeding the reduction in Alamar Blue staining was decreased by only 10% and reached 30% by the end of the mechanical stimulation. This underlines the high viability of the cells within

the scaffold after the stimulation. The final goal was to understand if the combination of the selected cardiac induction cocktail, the 3D scaffold conformation and the mechanical stimulation would enhance the expression of the most important cardiogenic transcription factors if compared to the induction with cardiogenic cocktail alone on a simple 2D culture condition. Our preliminary results suggest that indeed the combination of growth factors, cytokines and mechanical stimulation for 10 days led to an at least 2 fold increase in all mRNAs tested (i.e. NKX2-5, MEF2C, HAND2 and MHC). It would be of great interest to test whether the addition of the electrical stimuli would further enhance these effects.

Taken together our data strongly support the use of ASCs in heart regenerative medicine and underline their intrinsic propensity to differentiate into cardiomyocytes. Furthermore, as demonstrated by the molecular analyses, the entire population of cells responded to our newly selected cardiogenic induction cocktail at the level of specific expression of cardiac transcription factors. In contrast, only a small fraction of cells resulted positive for cardiac specific structural proteins. These findings therefore support the theory that the heart is a dynamic and complex organ, subjected to various mechanical signals, such as those produced by the normal contraction of heart muscle. These mechanical signals are fundamental and trigger various transduction pathways leading to gene reprogramming and enhancing synthesis of specific proteins, that determine the final cell fate. For this reason, use of a customized device and of scaffolds for cell seeding, become of crucial importance for tissue engineering: it is a more suitable approach that provides a structural environment with tissue-specific mechanical properties and the ability to integrate with the surrounding tissue. In this contest, our preliminary results demonstrate that the mechanical stimulation increases the expression of cardiac genes. Further research should first confirm these results and then investigate the effect caused by the addition of electrical stimuli. The final aim should be to obtain functional cardiac precursors in a 3D structure that is suitable for autografts regenerative therapy.

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