Mesenchymal Stem Cell-based Differentiation of Smooth Muscle Cells
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Nome: Luis Manuel Fonseca Amaral 
Endereço eletrónico: luisamaral89@gmail.com 
Número do Bilhete de Identidade: 13559292 
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Orientador: Prof. Dr. Willi Jahnen-Dechent 
Co-Orientador: Prof. Miguel Gama 

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

Braga, ____/____/______
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Abstract

Valvular heart disease is a major health and socioeconomic problem worldwide with approximately 300,000 valve replacements performed annually. Tissue-engineered heart valves with repair and remodelling capabilities could overcome the limitations of today’s valvular prostheses. One major limitation to this approach has been finding a reliable source of smooth muscle cells (SMC) because biopsies of these cells can be impractical and morbid as also present limited replicative capacity. The ideal cell source should be harvested in a non- or minimally invasive way and should deliver an initial high number of cells in order to drastically reduce the time needed for cell expansion. For these reasons there are many studies endeavoured to explore whether functional SMC could be generated from various types of adult mesenchymal stem cells (MSC): Umbilical cord (UC-MSC), bone marrow (BM-MSC), adipose derived (AD-MSC) and chorionic villi (CV-MSC) as possible sources for heart valve therapy. The MSC from different sources were isolated and expanded of healthy and different donor. In this study an isolation protocol was established for the CV-MSC, because it was never performed on this research group. Since the CV-MSC are barely reported in studies, this MSC source was characterized by flow cytometry to compare with other sources that are well-characterized in literature. After that, the MSC were differentiated by culture them in a culture medium containing transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein 4 (BMP4), based on a published 7-day differentiation protocol. The differentiation was analysed using 4 different smooth muscle markers (α-SMA, SM22α, Calponin and SM-MHC) by immunofluorescence (IF) and immunohistochemistry (IHC). A more extensively analysis was performed by flow cytometry using the smooth muscle markers and the other markers used on different studies to characterize the MSC population for a complete phenotype characterization before and after differentiation. The IF showed promising results as the smooth muscle markers stained positive for the differentiated MSC and negative for the undifferentiated MSC. On the other hand, the IHC and flow cytometry show some contradicting results for differentiation, since the expression is not consistent within the undifferentiated and differentiated MSC. These results highlight the concept that MSC represent an easily accessible, novel cell source for heart valve therapy, but despite of the wide experiments and results in this work, it is necessary further research in this field due to the conflicting evidence and inadequate information about several cell surface markers.
Resumo

As doenças valvulares cardíacas são um problema social, econômico e de saúde grave no mundo, com a realização de aproximadamente 300 000 cirurgias para substituição de válvulas cardíacas. A Engenharia de Tecidos na investigação e desenvolvimento de células cardiacas com capacidades para reparar e substituir válvulas cardíacas, pode vir a ultrapassar as limitações das atuais próteses valvulares. Contudo uma das maiores dificuldades desta investigação, tem sido encontrar uma fonte válida de células do músculo liso. A fonte ideal de colheita de células deve resultar de uma técnica minimamente invasiva e deve disponibilizar inicialmente um número elevado de células de modo a reduzir drasticamente o tempo necessário para a proliferação. Por estas razões há muitos estudos em curso de modo a investigar se as células do músculo liso podem ser obtidas a partir de vários tipos de células mesenquimatosas adultas (MSC): células do cordão umbilical (UC-MSC), medula óssea ("BM-MSC), tecido adiposo (AD-MSC) e vilosidades coriónicas (CV-MSC), como possíveis fontes para a terapêutica de válvulas cardíacas patológicas. As MSC de diferentes fontes, foram isoladas e cultivadas a partir de diferentes dadores saudáveis. Neste estudo foi estabelecido um protocolo independente para as células CV-MSC porque nunca tinha sido realizado neste grupo de investigação. Uma vez que as células CV-MSC são raramente descritas em estudos, esta fonte de MSC foi caracterizada por citometria de fluxo, para possível comparação com outro tipo de MSC que estão bem caracterizadas na literatura. Posteriormente, as MSC foram diferenciadas, isoladas e cultivadas num meio de cultura contendo o facto de crescimento TGF-β1 e BMP4 baseado num protocolo de diferenciação de 7 dias. A diferenciação foi analisada usando quatro marcadores diferentes de músculo liso (α-SMA, SM22α, Calponin and SM-MHC) através de imunofluorescência (IF) e imunohistoquímica (IHC). Para uma caracterização completa do fenótipo antes e depois da diferenciação, foi também realizada por citometria de fluxo uma análise mais extensa usando os marcadores de músculo liso e os outros marcadores utilizados por diferentes estudos na caracterização das MSC. A IF mostra bons resultados uma vez que as células diferenciadas mostraram ser positivas para os marcadores musculares enquanto que as células não diferenciadas não mostraram evidência dos marcadores. Por outro lado, a IHC e a citometria mostraram resultados contraditórios para a diferenciação, uma vez que a expressão não foi consistente nas MSC não diferenciadas e diferenciadas. Os resultados mostram que que as MSC são facilmente acessíveis e podem ser um boa alternativa para tratamento de válvulas cardíacas e apesar de se ter realizado uma análise extensiva, é necessário mais investigação para clarificar alguns pontos poucos claros que ficaram para responder em relação aos marcadores.
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List of Abbreviations

AB – Antibody
AD-MSC – Adipose Derived Mesenchymal Stem Cells
APC – Allophycocyanin
Aqua dest. – Distilled H₂O
ASC – Adult Stem Cell
BM-MSC – Bone Marrow Mesenchymal Stem Cells
BMP4 – Bone Morphogenetic Protein-4
BSA – Bovine Serum Albumin
C₆H₈O₇ – Citric Acid Monohydrate
CD – Cluster Differentiation or Cluster of Determinate
CH – Switzerland
CVDs – Cardiovascular Diseases
CV-MSC – Chorionic Villi Mesenchymal Stem Cells
DAPI – 4′,6-diamidino-2-phenylindole
DE – Germany
DK – Denmark
DMEM – Dulbecco’s Modified Eagle’s Medium
DMSO – Dimethyl Sulfoxide
ECM – Extracellular Matrix
EDTA – Ethylenediaminetetraacetic Acid
ESC – Embryonic Stem Cell
FBS – Fetal Bovine Serum
FGF-Basic – Basic Fibroblast Growth Factor
FISH – Fluorescence In Situ Hybridization
FITC – Fluorescin
HCL – Hydrochloric Acid
HSC – Hematopoietic Stem Cells
HUASMC – Human Umbilical Artery Smooth Muscle Cells
HUVEC – Human Umbilical Vein Endothelial Cells
IF – Immunofluorescence
IHC – Immunohistochemistry
IL – Israel
iPSC – Induced Pluripotent Stem Cells
JP – Japan
M – Molar
mM – Millimolar
MSC – Mesenchymal Stem Cells
NL – Netherlands
°C – Degree Celsius
PBS – Phosphate Buffered Saline
PE – R-Phycoerythrin
PE-Cy™7 – Is a tandem fluorochrome that combines PE and a cyanine dye
Pen Strep – Penicillin Streptomycin
PerCP – Is a component of the photosynthetic apparatus
PerCP-Cy™5.5 – Is a tandem conjugate that combines PerCP with a cyanine dye
PFA – Paraformaldehyde
RT – Room temperature
SM-MHC – Monoclonal Anti-Myosin (Smooth) antibody produced in mouse
SMCs – Smooth Muscle Cells
TGF-β1 – Transforming Growth Factor-β1
UC – Umbilical Cord
UC-MSC – Umbilical Cord Mesenchymal Stem Cells
UK – United Kingdom
USA – United States of America
vWF – Von Willebrand factor
α-SMA – Monoclonal Anti-Actin, α-Smooth Muscle antibody produced in mouse
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XIX
Chapter I – General Introduction
Chapter I

General Introduction

1. Aim of Studies

This master thesis comprises a comparative study of four different sources of mesenchymal stem cells (MSC) as potential candidates for heart valve therapy. It comprehends an interdisciplinary work merging the subjects of Biomedical Engineering and Stem Cell Biology in order to overcome, through cellular therapy, one of the major problems among the cardiovascular diseases. The main objective of this project is to study and characterise MSC from different sources with regard to the establishment of a protocol for smooth muscle differentiation. The four different human mesenchymal cells used were (i) mesenchymal stem cells from umbilical cord matrix (UC-MSC), (ii) mesenchymal stem cells from bone marrow (BM-MSC), (iii) mesenchymal stem cells from adipose tissue (AD-MSC) and (iv) mesenchymal stem cells from chorionic villi of placenta (CV-MSC). The specific tasks of this work comprise three major goals:

- To establish an isolation protocol for chorionic villi derived human mesenchymal stem cells (CM-MSC). Characterization and comparison of MSC from the four different sources listed above. All MSC types were obtained from tissue (primary cells) and during the work all cells were expanded until passage 7 up to 8.

- To characterise the phenotype of the MSC used in this work by flow cytometry. All the cells were analysed with a group of markers between different passages. As the CV-MSC are rarely reported in studies, it was very important to perform a comparison between the CV-MSC and the well-characterised MSC: the UC-MSC, BM-MSC and AD-MSC.

- To establish a smooth muscle cell differentiation protocol using MSC from different sources. The success of smooth muscle cell differentiation was analysed by immunofluorescence and immunohistochemistry through a specific set of smooth muscle cell markers. The cells were also analysed using flow cytometry with the same group of markers used to characterise the phenotype of MSC plus smooth muscle markers. Flow cytometry was very important to analyze into details the difference between the undifferentiated and differentiated cells. All the cells used were between passages 4-7.

For analysis was used a positive and negative control, human umbilical artery smooth muscle cells (HUASMC) and human umbilical vein endothelial cells (HUVEC), respectively. The project was
executed at the AG S. Neuss-Stein, Pathology/IBMT-ZMG at Uniklinik RWTH Aachen University with collaboration of AG P. Mela (AME, Helmholtz Institute) at RWTH Aachen University.

2. Smooth Muscle Cells

Smooth muscle cells (SMC) reside in various locations throughout the human body (gastrointestinal, respiratory system, reproductive system, ocular, urinary tissues and heart) and they play an essential role in maintaining the structural and functional integrity of blood vessels. SMC have different specialized functions in each tissue in which they are found. Thus, SMC are multifunctional, for instance, they maintain the viability and phenotype of endothelium by providing physical support and regulation of blood pressure by contracting and relaxing in response to a variety of stimuli; they are responsible for the alterations in the volume and diameter of hollow organs, enabling them to generate pressure and move contents; they adjust the shape of the lens and the diameter of the pupil in the eye; they produce erections of the hairs in the body; they control the passage of food through the gut and excretion of waste products; and they determine the movement of sperm cells, eggs and the delivery of a fetus [1, 2].

The fully differentiated or mature SMC proliferates at an extremely low rate and is a cell almost completely geared for contraction. For example, fully differentiated SMC in mature blood vessels proliferate at extremely low rates and produce only small amounts of extracellular matrix (ECM) proteins. They express a unique repertoire of contractile proteins, ion channels, and signalling molecules that are required for their function and that when taken in aggregate clearly distinguish it from any other cell type [1-5].

The SMC retains remarkable plasticity, such that it can undergo relatively rapid and reversible changes in its phenotype in response to changes in the local environment. However, the plasticity of the SMC has confounded efforts to understand the cellular and molecular mechanisms that control its differentiation. A given SMC can acquire a broad spectrum of different phenotypes in response to different physiological or pathological stimuli. If an artery is injured, some SMC must be recruited to repair the injury, while at the same time the contractile function of the blood vessel must be maintained for normal cardiovascular homeostasis [1, 3, 6].

There is considerable interest in understanding the cellular and molecular regulation of the differentiation of vascular smooth muscle cells for the treatment of diseases. For example, cardiovascular diseases (CVD) are the number one cause of death globally, more people die annually
from CVD than from any other cause. An estimated 17.3 million people die annually from CVD, representing 30% of all global deaths. The number of people, who die from CVD, mainly from heart disease and stroke, will increase to 23.3 million by 2030. CVD are projected to remain the single leading cause of death [7-9]. Therefore CVD are considered more than just a health care problem because of their major consequences in terms of financial and economical impact for governments, businesses and individuals. These are a global epidemic that requires a combined action from not only the individual patients but also the pharmaceutical industry and the government. Thereby the treatments for SMC therapy have been the subject of intense research in the field of cellular therapeutics [9].

3. Heart Valves

Cardiovascular diseases represent a major worldwide health care issue. A considerable amount of these diseases is represented by heart valve failures. It’s a significant cause of morbidity and mortality; over 300 000 valve replacements are performed globally per year and the number of patients requiring heart valve replacement is expected to triple by the year 2050 [10].

Heart valve disease is characterised by damage to or a defect in one of the four heart valves: the mitral, aortic, tricuspid or pulmonary. Heart valve abnormalities are caused by birth defects, age-related changes, infections, or other conditions. The valves become too hardened to open fully, or are unable to close completely hindering the flow of blood. As the tissue of the heart cannot regenerate spontaneously, valve diseases generally necessitate surgical repair or replacement of the diseased tissue in order to avoid serious and potentially fatal cardiac or systemic consequences.

Currently used heart valve prostheses can be divided into two basic groups, namely mechanical and biological prostheses. Mechanical valves are durable but suffer risks of clot formation on their prosthetic surfaces, necessitating life-long anti-coagulant drug therapy. Bioprosthetic valves, on the other hand, have minimal risk for bleeding events but are much less durable and unsuitable for pediatric applications. These disadvantages can be overcome by valve substitutes that more closely mimic their native counterparts as to adequate mechanical function and durability, as well as the absence of immunogenic and inflammatory reactions [11-14].

Tissue engineering is a promising strategy to meet these requirements by in vitro fabrication of autologous living heart valve replacements. The final goal is to create or regenerate a living valve replacement that functions well hemodynamically, repairs on-going tissue damage, and has long-term
durability and growth potential similar to those of the natural heart valves. The most immediate need for heart valve tissue engineering and regeneration technology is in the pediatric and young adult population in which the results of valve replacement are not as favourable as those in older adults [15, 16]. However, an ideal cell source enabling the fabrication or regeneration of heart valve has not been identified yet. Ideally, the cells used to seed the developing tissues should be non-immunogenic, capable of expansion in vitro to yield increased number of cells, have the ability to provide specific cell functions, easy to harvest in a non-or minimally invasive way and should deliver an initial high number of cells in order to drastically reduce the time needed for cell expansion [17-20].

4. Tissue Engineering

Tissue Engineering has emerged as a rapidly expanding approach to address the organ shortage problem. It is “an interdisciplinary field that applies the principles and methods of engineering and life sciences toward the development of biological substitutes that can restore, maintain, or improve tissue function” [21]. Currently, tissue engineering focuses mainly on associating cells with scaffolds, in order to promote cell attachment and restrict their distribution in the tissue, direct cell distribution and differentiation, sustain large tissue losses while new tissue is formed and ultimately lead to new tissue formation [22, 23].

Figure 1.1 - Tissue Engineering strategies, adapted from [24].
In 1993 Langer and Vacanti described three strategies for the creation of new tissue *in vitro* [21]:

1. **Isolated cells or substitutes.** The concept of treating injured tissues with isolated cells is regarded as cell therapy.
2. **Tissue inducting substances.** Bioactive molecules induce cell proliferation, differentiation and metabolic activity.
3. **Cells placed on or within matrices.** Associated cells and substrates provide the injured tissue with continuity, and promote cell attachment and fixation.

As proposed by Langer and Vacanti, tissue engineering may be performed by several different approaches in order to obtain tissue regeneration, but the association showed on Figure I.1 (with the association of all three elements, composing bioactive constructs) is proposed to be the most promising option for tissue engineering [24, 25]. The present work is focused on the study of mesenchymal stem cells from different sources and their differentiation towards SMC.

### 5. Stem Cell Therapy

Stem cell research has evolved a lot in recent years due to its therapeutic potential in dealing with many diseases in the human body, which many are incurable by established therapies. The list of diseases and injuries cited as potential targets of stem cell therapy reveals, in large measure, why stem cells offer so much hope for revolutionary advances in medicine (Table I.1). These diseases are characterized by progressive cell loss, traumas and defects that become a serious health issue, because the cells that maintain the ability to divide and differentiate into more specialized cells of different tissue types are rare in adult. In contrast, the seemingly unlimited potential of the undifferentiated cells of the early embryo has made embryonic stem cells the focus of great scientific interest. The properties of stem cells need to be understood so stem cell therapy can help the body regenerate against some serious health problems [26-28].

#### 5.1 Stem Cells Basics

Stem cells are unspecialized cells in the human body that are capable of becoming specialized cells, each with new specialized cell functions. A stem cell is uncommitted, and remains uncommitted until it receives a signal to develop into a specialized cell. They serve as a repair system by being able to divide without limit to replenish other cells. When stem cells divide, each daughter cell has the
potential to either remain as a stem cell in the stem cell pool (niche) or become mature cell type with
new special functions, such as blood cells, brain cells, etc. Their self-renewal capacity combined with
their differentiation capacity, makes stem cells unique [27, 29].

Table I.1 – Potential US patient populations for stem cell-based therapies, adapted from [28].

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular Diseases</td>
<td>58 million</td>
</tr>
<tr>
<td>Autoimmune diseases</td>
<td>30 million</td>
</tr>
<tr>
<td>Diabetes</td>
<td>16 million</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>10 million</td>
</tr>
<tr>
<td>Cancer</td>
<td>8.2 million</td>
</tr>
<tr>
<td>Alzheimer’s Disease</td>
<td>5.5 million</td>
</tr>
<tr>
<td>Parkinson’s Disease</td>
<td>5.5 million</td>
</tr>
<tr>
<td>Burns (severe)</td>
<td>0.3 million</td>
</tr>
<tr>
<td>Spinal-cord Injuries</td>
<td>0.25 million</td>
</tr>
<tr>
<td>Birth Defects</td>
<td>0.15 million/year</td>
</tr>
</tbody>
</table>

Stem cells are present within most, if not all multicellular organisms and are the ultimate
drivers of growth and regeneration. They are considered to be critical biological components necessary
for proper growth and development during embryogenesis and they have also been demonstrated to
play indispensable roles in adult species, providing a source of cellular replenishment for every mature
and differentiated cell type. All stem cells originate from the fertilized egg. As a totipotent
entity, the fertilized egg has the capacity to drive the formation of all intra- and extra-embryonic tissues during
growth and development. It is during the process of embryonic maturation that determination occurs, a
variety of more specialized stem cell types are generated with different properties that allow the
development of specific tissues and organs [27, 30].

Stem cells can either be totipotent, pluripotent, multipotent or bipotent. Pluripotent stem cells
have the potential to give rise to all the cells that derive from the three embryonic germ layers, more
specifically the mesoderm, endoderm, and ectoderm. The germ layers are the embryonic source of all
cells in the body and are originated from the blastocyst, one of the earliest stages of the embryo
development. From the inner cell mass of blastocyst before it would be implanted in the uterine wall it
is possible to isolate and collect one type of pluripotent cell, the embryonic stem cell (ESC). As
pluripotent stem cells, ESC have the ability to give rise to any type of cell (except extra-embryonic tissue
such as the trophoblast / placenta) yielding an unlimited ability to self-renewal associated to high levels
of telomerase activity. ESC have a tremendous prospective for clinical application, yet their use in therapeutics is ethically controversial due to its origin and also their unmeasured proliferation can lead to \textit{in vivo} teratoma (tumour consisting of different types of tissue caused by the development of independent germ cells) formation, which make them unsafe for the therapies [29, 31-33].

The multipotent stem cell represent an undifferentiated type of cell occurring in a specialized differentiated tissue with the ability to self-renew and give rise to all types of cells from its origin tissue. Basically these stem cells have the \textit{in vitro} and \textit{in vivo} ability to give rise to a limited number of specialized cells, being capable of originating multiple cell lineages or more restricted progenitor populations that are able to produce precursors and finally mature cells. They also exhibit a self-renewal capacity and contribute to the tissue homeostasis by repairing and replacing the damaged cells after an injury. Despite their limited potential regarding differentiation compared to pluripotent stem cell, multipotent cells bring more advantages when compared to pluripotent ones in the themes of ethical concerns and safety issues. Adult stem cells (ASC) can be isolated from several sources such as bone marrow (BM), blood, cornea and retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, the lining of the gastrointestinal tract, and pancreas. Despite rare, difficult to identify, isolate and purify ASC are very promising for the therapeutic use. Hematopoietic stem cells (HSC), neural stem cells, endothelial progenitor cells, and mesenchymal stem cells are the most studied ones [27, 29, 31, 33]. Figure 1.2 graphically elucidates the classification of all types of human stem cells.
Additionally, in 2006, Takahashi and co-workers discovered a novel type of stem cell, by reprogramming an adult mouse fibroblast back to a pluripotency-like state. In 2007, the same authors were able to reproduce the same output with human fibroblast, and named them induced pluripotent stem cells (iPSC). This breakthrough of restoring pluripotency to somatic cells was awarded with the Nobel Prize and created potentially new opportunities for modelling human diseases by preventing immunosuppression and graft rejection. Alternatively, iPSC also can allow the *in vitro* simulation of each individual patient’s disease enabling the screening of potential drugs and therefore developing new therapies [34-36].

### 5.2 MSC – Definition And Sources

The MSC definition is not 100% consensual among the scientific community. Friedenstein and their colleagues reported the first evidence of these cells in 1968 through the identification of a bone marrow cell subpopulation with osteogenic potential. Later, in end of the 80s decade the same author described these cells as plastic-adherent, fibroblast-like cells that were able to develop colony-forming units (CFU-Fs) when plated into tissue culture plates, naming them stromal cells. In 1991, Caplan proposed to name mesenchymal stem cells to all the cells capable to differentiate into all mesodermal lineage cells also known as mesenchymal tissues [37-39]. The minimum criteria for their classification include: adherence to plastic, the expression of specific set of surface markers and *in vitro* multilineage differentiation (bone, cartilage, fat and muscle) as it can be seen on Figure I.3 [31, 40, 41].

MSC proliferation and senescence has been considered important by scientists seeking to use them for therapeutic purposes. *Ex vivo* expansion of MSC is required to generate a pure cell population in an amount sufficient for the clinical indication. During this expansion process, cells enter cellular senescence, which leads to a gradual reduction of their potency and significant changes in protein expression. Despite those features, MSC can be easily expanded in culture with a capacity to retain their stemness and multilineage potential over several passages; however *in vivo* demonstration of all these characteristics have not been accomplished. The limited *in vivo* knowledge of MSC is due (but not limited) to the fact that they lack a unique specific marker, besides lacking the expression of hematopoietic markers [31, 42, 43].
Figure 1.3 – The mesengenic process: cellular transitions from MSC to differentiated cells, adapted from [43].

The most *in vitro* features of MSC include the formation of CFU-Fs when plated into plastic tissue cultures in a basal medium, such as DMEM (Dulbecco’s Modified Eagle’s Medium), in the presence of animal serum, like FBS (fetal bovine serum), and have the ability of being propagated without losing their differentiation potential. MSC also have other important features: active secretion of ECM components and several cytokines, thereby having immunomodulatory and trophic properties, as well as, low immunogenicity which make them suitable for both allogeneic and autologous settings in several clinical applications. Thereby these cells have an enormous potential due to their intrinsically therapeutic features in setting-up a regenerative microenvironment releasing anti-fibrotic, anti-scaring, anti-apoptotic, mitotic and angiogenic factors [31, 42].

In adults MSC can be found in the BM compartment of long bones, iliac crest, sternum and cranium, being the major source of these cells in the human body. In those locations, a compartment of stroma-supportive cells related to the hematopoietic compartment is formed. In addition to BM, it is possible to obtain MSC population from other adult tissues such as adipose tissue, dental pulp, synovial membrane and breast milk. They also can be found in some neonatal tissues and fluids like the placenta, amniotic fluid and umbilical cord (UC): blood, perivascular cells and Wharton’s jelly. However, depending on the sources, MSC can exhibit different features namely concerning differentiation potential and other differences related with cell processing (cell yield and purity) [42, 44-46].
5.3 MSC – Niche Characterization and Homing Ability

MSC reside in their predefined niches where upon signalling and molecular activation mobilizes, migrate or home to the locus of the inflammation and tissue injury in order to modulate the immune response. Niches are structurally tailored to meet the requirements of the resident stem cells, and the resident cells in turn play an indispensable role in architectural organization of the niche. Three different niches are known to contain MSC: the BM niche, the adipose tissue and the subventricular zone. In all tissues where MSC can be collected they are in contact with a perivascular niche in close association with blood vessels, therefore these cells are often referred as pericytes (abluminal cells in perivascular locations that are in contact with the basement membrane and surrounding endothelial including the microvasculature). Pericytes play an important role in the blood vessel stabilization by secreting an amount of different factors. The course of the perivascular niche is highlighted in the housing of MSC due to the fact that alongside with a decrease of the vascular density is associated a diminishment of viable MSC. The surrounding supporting cells, ECM and signalling molecules act in coordination to activate the naive MSC, which can then proliferate, as a consequence of lost attachment with the basement membrane and endothelial cells [44, 47, 48].

Figure I.4 – Stem cells niches and their oxygen tension levels, adapted from [46].

Although MSC are located close to vascular structures, the oxygen tension within those milieus are very low, approximately 2-8%, as it can be visualized on Figure I.4. The hypoxia is thought to contribute for MSC stemness maintenance by conserving an undifferentiated state [47].

The homing properties of MSC can further enhance their therapeutic potential. The homing process is defined as the arrest of MSC within the vasculature of a tissue followed by transmigration across the endothelium. This represents a complex process involving the niche interaction and paracrine crosstalk in order to achieve a correct MSC mobilization, niche homeostasis and
consequently a better healing process. There is evidence that host MSC are able to migrate in response to inflammation via activation of mobilizing and trafficking mechanisms that still remain muddled. However, the homing of culture-expanded MSC in comparison to leukocytes and HSC, is much less efficient taking into account the lack of important chemokine and cell-adhesion receptors by MSC, as well as the difficulty in identifying, isolating and tracking these type of cells [48, 49].

5.4 MSC – Morphological and Phenotypic Characteristics

According to several studies, MSC morphological classification depends on the shape and processes (ramifications extending from the cell body) that the cell displays, usually the MSC while plastic adherent cells in culture, exhibit architecture similar to fibroblasts, thereby being classified as fibroblast like-cells in terms of their morphology. They are divided into two major subpopulations, the first one encloses MSC with a spindle-shaped morphology and at least two processes extending in different directions from an elongated cell body (generally these MSC correspond to an immature multipotent state), and the other morphological group of MSC represents large cells with a flattened, polygonal shape and a plainly visible nucleus, commonly devoid of processes or with many small processes, being termed mature MSC, which have less potential. Figure 1.5 shows the differences in MSC morphology [31, 50].

![Figure 1.5 - Morphologically distinct MSC in culture. A to C: Elongated spindle shaped cells. D to F: Flattened polygonal cells with short or no branches (adapted from [49]).](image)

Although MSC lack a specific, unique surface marker able to identify and track these cells, it is possible to identify and characterize these cells immunophenotypically because they express several surface markers. However, depending on the MSC source and their different stages during cell
proliferation and culture the expression of adult MSC surface markers can differ. Adult MSC have also been shown to have non-immunogenic surface antigens, due to weakly express major histocompatibility complex class I protein (MHC I) and no MHC II molecules. The Mesenchymal Stem Cell Committee of the International Society for Cellular Therapy proposed in 2006 minimal criteria to define these cells. Besides the plastic adherence ability and multilineage differentiation potential, human MSC have to express a certain group of markers and lack the expression for certain other markers. Nevertheless, extensive research needs to be done in this field, as not much is known about the phenotypic character and developmental origin. Also, there is not adequate information about the cell surface markers of adult mesenchymal stem cells and how they can be identified. The Table I.2 shows the most commonly used markers summarized by the scientific community to identify the MSC [31, 41, 51-53].

Table I.2 – Summary of the markers expression for MSC, adapted from [50, 52].

<table>
<thead>
<tr>
<th>Markers</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>CD13</td>
<td>CD14</td>
</tr>
<tr>
<td></td>
<td>CD29</td>
<td>CD31</td>
</tr>
<tr>
<td></td>
<td>CD44</td>
<td>CD34</td>
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<tr>
<td></td>
<td>CD73</td>
<td>CD45</td>
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<tr>
<td></td>
<td>CD90</td>
<td>CD49d</td>
</tr>
<tr>
<td></td>
<td>CD105</td>
<td>CD106</td>
</tr>
<tr>
<td></td>
<td>CD166</td>
<td>HLA-DR</td>
</tr>
<tr>
<td>Stro-1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6 MSC – Candidate Cells for Differentiation

As previously discussed on 1.2 Heart Valves the ideal cell source should be harvested in a non- or minimally invasive way and should deliver an initial high number of cells in order to drastically reduce the time needed for cell expansion. Here will be discussed the potential sources of cells that this study focused on (BM-MSC, AD-MSC, UC-MSC and CV-MSC) to evaluate which of them is most suitable for smooth muscle cell differentiation and thus for heart valve therapy.
6.1 MSC – BM-MSC Properties and Characteristics

BM is a well-organized tissue composed by a complex structure highly vascularized that shelters the basic elements from the stroma or the mesenchyma and hematopoietic systems. As it can be seen on Figure 1.6, the BM is located at the center of the large bones, where MSC compartment together with the ECM elements support the haematopoiesis process. MSC have a very important role in the BM HSC niche as a stromal supporting network by producing an array of growth factors and cytokines able to modulate immune responses and regeneration processes. Notwithstanding MSC only represent 0.001 to 0.01% of BM cell population, a very low number [29, 31, 50].

Although BM has been the main source for the isolation of multipotent MSC, the harvest of BM is a highly invasive procedure and the number, differentiation potential, and maximal life span of MSC from BM decline with increasing age. Although obtaining MSC from the bone marrow is a highly invasive procedure the BM-MSC relies on one big advantage, the patient will not have any compatibility problems. The MSC from BM are the best characterized, in many studies they compare MSC from different sources to BM to see if there’s a match [31, 54, 55].

Some analysis of proliferation capacities of BM-MSC showed that they possessed the lowers population doubling numbers and the shortest culture period. BM-MSC exhibit characteristics similar to the other cell types studied in this work (UC-MSC, CV-MSC and AD-MSC), including fibroblastoid morphology, surface proteins and differentiation potential. Cultured BM-MSC have showed differentiation potential into bone, cartilage, fat and they also been shown to regenerate cardiac and...
skeletal muscle. More recently, it has been suggested that BM-MSC can traverse lineage borders and differentiate into neural cells, epithelia of liver, lung, kidney, skin, pancreas and gastrointestinal tract. They also have been used with varying success to improve neurological, cardiovascular, blood related and musculoskeletal disorders [31, 50, 54, 55]. The most common markers used to characterize BM-MSC are listed on Table I.2.

6.2 MSC – AD-MSC Properties and Characteristics

Adipose tissue serves as an endocrine organ, functioning to maintain energy metabolism through the storage of lipids. Two types of adipose tissue exist, brown and white (there is another type described between brown and white, called beige fat; you should mention that), the white adipose yields the commonly studied AD-MSC. This perivascular niche where AD-MSC derive is highly vascularized, analogous to different mesenchymal cell populations identified in other tissues throughout the body. AD-MSC can be obtained by a less invasive method and in larger quantities than BM. It has been demonstrated that adipose tissue contains stem cells similar to BM-MSC, which are termed processed lipoaspirate cells. However, like BM-MSC it has the advantage to be collected at any time of donor’s life and has no problem with compatibility, but the differentiation potential, and maximal life span of AD-MSC decline with increasing age. These cells can be isolated from cosmetic liposuctions in large numbers and grown easily under standard tissue culture conditions [31, 54, 55].

AD-MSC are a very promising alternative source of MSC, due to their wide availability and ability to differentiate into other tissue types of mesoderm, they may serve a wide variety of applications. AD-MSC had been utilizes in studies addressing osteoarthritis, diabetes mellitus, heart disease, and soft tissue regeneration and reconstruction after mastectomy and facial repair. Also they represent potential candidates for cell-based therapies designed to treat cartilage defects/disorders, neurological disorders, autoimmune diseases, as well as organ transplantation outcomes [22, 56].

While the accessibility and quantity of ASC that can be recovered offers much appeal, the practical application and safety of using ex vivo expanded populations for clinical applications remains to be determined. However, what is clear is, the need to better understand the basic properties of culture-expanded AD-MSC in order to properly assess their growth, developmental potential, and capacity to modulate immune responses [56, 57]. Although the expression of surface markers is very similar to the BM-MSC, there slight differences exist between them; Table I.3 shows the most common markers used to characterize the AD-MSC in comparison with BM-MSC [58].
6.3 MSC – UC-MSC Properties and Characteristics

As the collection and isolation of BM-MSC and AD-MSC require invasive and often undesirable procurement procedures, investigators have begun to seek alternative sources of human MSC including the umbilical cord (UC) that was seen as biological waste product post childbirth. Human UC consists of three tissue components including the amniotic membrane, stroma (Wharton’s jelly), and blood vessels (two arteries and one vein) as can be seen on Figure 1.7. Over the last few years, UC-MSC derived from Wharton’s jelly (WJ) have been isolated to examine their stem cell nature. Although some technical and observational variations exist between laboratories, there has been a relatively common consensus regarding the immunological and functional characteristics of UC-MSC derived from WJ. WJ is a gelatinous substance that provides insulation and protection within the umbilical cord and the cells within the WJ express several stem cells genes, therefore is a potential source of ASC. These cells are believed to be more primitive than MSC derived from more mature tissues sources and to have intermediate properties between embryonic and ASC. The expression of certain embryonic and mesenchymal stem cell markers on the naïve form of these cells brings suggestive evidence as to their multipotency [59, 60].

![Diagram of UC cross section](image)

**Figure 7 – Example of a cross section, adapted from [59].**

The UC-MSC are widely reported on studies because they produce large yields of MSC and posses immunosuppressive activities, making it useful in allogeneic situations. UC-MSC cultured *in vitro* shared a similar fibroblastoid morphology like the other cell types analysed in this work, and in comparison, although they have the lowest isolation rate (approximately 61%), they are available in potentially large quantities when successfully isolated. The UC-MSC have the fastest proliferation and
expansion rate and they have significantly lower expression of senescence markers. UC-MSC normally
is the cell type capable of being cultured the longest [54, 55, 59, 60].

Successful *in vitro* and *in vivo* differentiation to several lineages makes these cells an invaluable
stem cell source, deserving further testing as a cellular therapy or other applications in tissue
engineering. Therefore, the isolation and culture of UC-MSC still need better clarification to ultimately
build an optimal standard procedure among laboratories, tissue banks, and clinics. This potential has
ultimately led many groups to initiate the evaluation of these cells in therapeutic regimens. Even though
UC-MSC still need further characterization, they have been successfully differentiated into various cell
types including adipocytes, chondrocytes, osteocytes, cardiomyocytes, skeletal myocytes, hepatocytes,
insulin-producing cells as well as neuron-like cells [54, 55, 59, 60]. The most common markers used to
classify UC-MSC are usually the same to characterize BM-MSC and are listed on Table I.3.

Table I.3 – Expected results of surface markers for BM-MSC and AD-MSC, adapted from [52, 61].

<table>
<thead>
<tr>
<th>Markers</th>
<th>BM-MSC</th>
<th>AD-MSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>CD13, CD29, CD44, CD73, CD90, CD105, CD166, HLA-ABC</td>
<td>CD13, CD29, CD34, CD44, CD73, CD90, CD105, HLA-ABC</td>
</tr>
<tr>
<td>Negative</td>
<td>CD34, CD40, CD45, HLA-DR, CD80, CD86</td>
<td>CD38, CD45, CD106, HLA-DR, CD80, CD86</td>
</tr>
</tbody>
</table>

6.4 MSC – CV-MSC Properties and Characteristics

Placenta is one of the most important organs in the uterine environment. It is short lived by
design, enabling the mammalian fetus to survive and develop within the confines of the intrauterine
environment. Placenta assumes multiple roles during gestation, including providing nutrients and
removing waste products, to secretory and immunomodulatory functions. The placenta is a vital source
for a wide range of hormones, growth factors, cytokines and transcription factors and is involved in the
protection of the fetus from various chemical, infections and immune assaults, arising from the
maternal circulation or from the cervix. More recently, however, researchers have begun to investigate
the possibility that the placenta’s utility may extend beyond fetal development to act as a source of cells
with clinically relevant properties [31, 62, 63].

The human placenta, as shown on Figure I.8 can be conceptually visualized as having two
components: a fetal part (amniotic and chorionic structures) and a maternal part originating from the
decidua. Chorionic structures are composed of chorionic mesoderm and a highly variable trophoblast
layer, forming both chorionic villi and extra villus trophoblast. Several groups have reported the isolation of CV-MSC, which display both multilineage differentiation potential and immunomodulatory properties \textit{in vitro}. Despite the phenotypic characterization of CV-MSC \textit{in vitro} with cell surface markers, there have been no studies to exploit multiple cell surface markers to identify the niche of CV-MSC in the chorionic villi of placental tissues. Furthermore, these cells have also been shown to secrete soluble factors involved in pathophysiology processes that may aid tissue repair [62-64].

\textbf{Figure I.8 – Schematic Structure of a Human Placenta, adapted from [63].}

Like the UC, the collection of placenta is non invasive, neither for the mother nor for her child. At the time of the isolation of CV-MSC it has to take in account the probability of isolating cells belong to the mother, but with the right knowledge and a selective medium this can be avoided. Due to the late onset, CV-MSC are barely reported in studies, therefore they are not well characterized. There is a lack of comparison studies with the other MSC from different sources. Nevertheless the CV-MSC are very easy to isolate, they have been reported in studies to have a fast growth, fast proliferative rate, and within all MSC studied in this work, is the source that offers the higher number of cells after harvesting [62, 63, 65].

Like the other cell types, they are very similar, CV-MSC exhibit characteristics like fibroblastoid morphology, differentiation potential and sharing the main surface markers. Cultured CV-MSC have shown differentiation into most cell types of mesodermal lineage. And some studies show the cells can acquire successfully endothelial, hepatocyte, neurogenic and osteogenic phenotype. The most commonly used markers referred on reports to characterize the MSC phenotype of the placenta cells are the same used to characterize the BM-MSC and the UC-MSC, that can be seen on table I.2 [31, 65, 66].
7. Differentiation Into Smooth Muscle Phenotype

Different signals from the extracellular microenvironment can play significant roles over the cellular performance and with a precise control over the levels and sequence of the signalling molecules within a specific location may positively regulate the differentiation process. Specific manipulation protocols of MSC have been established in order to obtain enriched and even highly purified mature cell populations. Development of in vitro models to study SMC differentiation has been hindered by some peculiarities intrinsic to these cells, namely their different embryological origins and their ability to undergo phenotypic modulation in cell culture. Although many in vitro models are available to study SMC differentiation, careful consideration should be taken so that the model chosen fits the questions being addressed. In vitro differentiation is obtained by either chemical or mechanical stimulation, and is determined by expression of smooth muscle cell markers [56, 67].

A study shows that through a chemical approach, under the stimulation of transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein 4 (BMP4), it is possible to induce smooth muscle cell differentiation in AD-MSC. This chemical approach was used on this work to see if the smooth differentiation was possible on different MSC types [40].

- **TGF-β1** – This gene encodes a member of the transforming growth factor beta (TGF-β) family of cytokines and it can act on MSC via its cognate receptor to cause cell morphology change and an increase in actin filaments. The proteins involved have a range of different functions, such as the cytoskeleton, matrix synthesis, membrane, and metabolic enzymes. Of the proteins identified by the proteomics assays, smooth muscle-actin was increased. TGF-β1 is a key regulator of cell growth, differentiation, migration, and extracellular matrix production [67].

- **BMP4** – The protein encoded by this gene is a member of the bone morphogenetic protein family, which is part of the TGF-β superfamily. BMP4 is now considered essential with varying roles during embryogenesis, skeletal formation, haematopoiesis and neurogenesis. BMP4 in adult tissue can control several cellular behaviours including differentiation, proliferation, apoptosis, and motility. This particular family member plays an important role in the onset of endochondral bone formation in humans, and a reduction in expression has been associated with a variety of bone diseases [67].
Chapter II – Materials and Methods
# Chapter II
## Materials and Methods

### 1. Materials

#### 1.1 Equipment

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<tr>
<th>Equipment</th>
<th>Manufacturer</th>
</tr>
</thead>
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</tr>
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<td>FACSCantoTM II Flow Cytometer</td>
<td>Becton Dickinson, San Diego, USA</td>
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<td>Hot Cabinet</td>
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<td>Incubator (20% O₂, 5% CO₂, 37°C)</td>
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<td>Waterbath</td>
<td>GFL, Burgwedel, DE</td>
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## 1.2 Glass Ware

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<tr>
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<th>Location</th>
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<td>Pasteur Pipettes</td>
<td>Brand, Wertheim, DE</td>
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## 1.3 Consumables

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<tr>
<td>Casyton</td>
<td>Roche, Mannheim, DE</td>
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<tr>
<td>Cell Culture Plates 24 and 48 Cavities</td>
<td>Becton Dickinson Labware, Heidelberg, DE</td>
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<td>Cryo vials 1.8ml</td>
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<td>Nitrile and Latex Gloves</td>
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<tr>
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<td>T75 Tissue Culture Flask</td>
<td>Greiner Bio-one, Frickenhausen, DE</td>
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</table>
1.4 Chemicals

- **BSA**
  - Sigma-Aldrich, Steinheim, DE
- **Citric Acid Monohydrate**
  - Merck, Darmstadt, DE
- **DAPI**
  - Sigma-Aldrich, Steinheim, DE
- **DMSO**
  - Sigma-Aldrich, Steinheim, DE
- **Hydrochloric Acid**
  - Carl Roth GmbH + Co. KG, Karlsruhe, DE
- **Methanol**
  - Carl Roth GmbH + Co. KG, Karlsruhe, DE
- **Paraformaldehyde**
  - Merck, Darmstadt, DE
- **PBS**
  - Gibco, Darmstadt, DE
- **Roti® Block 10x**
  - Carl Roth GmbH + Co. KG, Karlsruhe, DE
- **Triton-X100**
  - Carl Roth GmbH + Co. KG, Karlsruhe, DE
- **Tween® 20**
  - Carl Roth GmbH + Co. KG, Karlsruhe, DE

1.5 Solvents

- **Aqua ad Injectabilia**
  - Braun, Melsungen, DE
- **Ethanol**
  - Sigma-Aldrich, Steinheim, DE

1.6 Cell culture

- **0.05% Trypsin/0.02% EDTA**
  - Lifeline Cell Technology, California, USA
- **BIOAMF™-1 Basal Medium**
  - Biological Industries, Beit HaEmek, IL
- **BIOAMF™-1 Supplement**
  - Biological Industries, Beit HaEmek, IL
- **Collagenase A**
  - Roche Diagnostics, Indiana, USA
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<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
<th>Location</th>
</tr>
</thead>
<tbody>
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<td>Gibco, Darmstadt, DE</td>
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</tr>
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<td>DMEM (1x) Low Glucose</td>
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<tr>
<td>DMEM/F-12 (1:1) (1x)</td>
<td>Gibco, Darmstadt, DE</td>
<td></td>
</tr>
<tr>
<td>EBM®-2</td>
<td>Lonza, Cologne, DE</td>
<td></td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>PAN Biotech GmbH, Aidenbach, DE</td>
<td></td>
</tr>
<tr>
<td>Mesenpan Basal Medium</td>
<td>PAN Biotech GmbH, Aidenbach, DE</td>
<td></td>
</tr>
<tr>
<td>Mesenpan Growth-Supplement</td>
<td>PAN Biotech GmbH, Aidenbach, DE</td>
<td></td>
</tr>
<tr>
<td>Pen Strep</td>
<td>Gibco, Darmstadt, DE</td>
<td></td>
</tr>
<tr>
<td>Pen Strep Glutamin (100x)</td>
<td>Gibco, Darmstadt, DE</td>
<td></td>
</tr>
<tr>
<td>Recombinant Human BMP4</td>
<td>Peprotech, Connecticut, USA</td>
<td></td>
</tr>
<tr>
<td>Recombinant Human FGF-Basic</td>
<td>Peprotech, Connecticut, USA</td>
<td></td>
</tr>
<tr>
<td>Recombinant Human TGF-β1</td>
<td>R&amp;D Systems, Minnesota, USA</td>
<td></td>
</tr>
<tr>
<td>Trypsin/EDTA</td>
<td>Lonza, Cologne, DE</td>
<td></td>
</tr>
<tr>
<td>VascuLife® SMC Complete Medium</td>
<td>Lifeline Cell Technology, California, USA</td>
<td></td>
</tr>
</tbody>
</table>

**1.7 Kits**

Dako Kit for IHC: DAKO, Glostrup, DK
Dako Real Detection System
Dako Real Antibody Diluent
Dako Automaton Hematoxylin

**1.8 Cells**

HUASMC Obtained from Helmholtz Institute
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human AD-MSC</td>
<td>Obtained from Düsseldorf Hospital</td>
</tr>
<tr>
<td>Human BM-MSC</td>
<td>Isolation from Primary Tissue from Clinics for Orthopedics, UK Aachen</td>
</tr>
<tr>
<td>Human CV-MSC</td>
<td>Isolation from Primary Tissue from Department of Genecology and Obstetrics, UK Aachen</td>
</tr>
<tr>
<td>Human UC-MSC</td>
<td>Isolation from Primary Tissue from Department of Genecology and Obstetrics, UK Aachen</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Obtained from Helmholtz Institute</td>
</tr>
</tbody>
</table>

### 1.9 Primary antibodies for IHC and IF

- **Anti-SM22α**: Abcam, Cambridge, UK
- **Anti-Calponin**: Abcam, Cambridge, UK
- **SM-MHC**: Sigma-Aldrich, Steinheim, DE
- **α-SMA**: Sigma-Aldrich, Steinheim, DE

### 1.10 Primary antibodies for Flow cytometry

- **Calponin**: Abcam, Cambridge, UK
- **CD105 (Purified)**: BD Pharmingen, Heidelberg, DE
- **CD105-PE**: eBioscences, San Diego, USA
- **CD106-PerCP-Cy™5.5**: BD Pharmingen, Heidelberg, DE
- **CD13-APC**: BD Pharmingen, Heidelberg, DE
CD14-PerCP-Cy™5.5 eBiosciences, San Diego, USA
CD146-PE BD Phamingen, Heidelberg, DE
CD16-PE-Cy™7 BD Phamingen, Heidelberg, DE
CD166-PE BD Phamingen, Heidelberg, DE
CD19-FITC BD Phamingen, Heidelberg, DE
CD275(B7H2)-PE eBiosciences, San Diego, USA
CD29-PE BD Phamingen, Heidelberg, DE
CD31-FITC BD Phamingen, Heidelberg, DE
CD34-FITC BD Phamingen, Heidelberg, DE
CD40-PE-Cy™7 eBiosciences, San Diego, USA
CD44-PE-Cy™7 BD Phamingen, Heidelberg, DE
CD45-APC BD Phamingen, Heidelberg, DE
CD45-PerCP-Cy™5.5 BD Phamingen, Heidelberg, DE
CD49a (Purified) BD Phamingen, Heidelberg, DE
CD49d-PE BD Phamingen, Heidelberg, DE
CD56-PE-Cy™7 BD Phamingen, Heidelberg, DE
CD73-APC BD Phamingen, Heidelberg, DE
CD80-PE BD Phamingen, Heidelberg, DE
CD83-PE-Cy™7 BD Phamingen, Heidelberg, DE
CD86-V450 BD Phamingen, Heidelberg, DE
CD90-APC eBiosciences, San Diego, USA
HLA-ABC-APC BD Phamingen, Heidelberg, DE
HLA-DR-eFluor450 eBiosciences, San Diego, USA
SM-MHC Abcam, Cambridge, UK
SM22α Abcam, Cambridge, UK
Stro-1 (Purified) eBiosciences, San Diego, USA
Vimentin Abcam, Cambridge, UK
vWF-FITC Abcam, Cambridge, UK
α-SMA R&D Systems, Minnesota, USA

1.11 Secondary Antibodies

Alexa Fluor® 488 Goat Anti-Mouse Life Technologies, California, USA
Alexa Fluor® 488 Goat Anti-Rabbit Life Technologies, California, USA
FITC-Goat Anti-Mouse BD Pharmingen, Heidelberg, DE

1.12 Software

FlowJo FlowJo, LLC, Ashland, USA

2. Methods

2.1 Cell Culture Mediums

MSC-Medium (For UC-MSC and BM-MSC):
-500ml Mesenpan Basal Medium
-10ml FBS
-4ml Pen Strep Glutamin
-Mesenpan Growth-Supplement
AD-MSC Medium:
-200ml DMEM/F-12 (1:1) (1x)
-20ml FBS
-1.6ml Pen Strep
-10ng/ml Human FGF-Basic

CV-MSC Medium:
-450ml BIOAMF™-1 Basal Medium
-50ml BIOAMF™-1 Supplement
-4ml Pen Strep

HUASMC Medium:
-200ml DMEM (1x) + GlutaMax®
-20ml FBS
-1.6ml Pen Strep

Smooth Muscle Induction Medium:
-100ml DMEM (1x) Low Glucose
-1ml FBS
-0.8ml Pen Strep
-5ng/ml TGF-β1
-2.5ng/ml BMP4

2.2 Solutions Preparation

- **PBS:**
  One tablet of PBS was dissolved in 500ml in *Aqua dest.* and autoclaved at 37ºC for 20min.

- **PBS with 0.05% Tween:**
  0.5ml of Tween was added to 999.5ml of a PBS solution.
• **PBS with 0.2% Triton-X:**
  200µl of Triton-X was added to 99.8ml of a PBS solution.

• **Rotiblock 1:10:**
  1ml of Rotiblock was added to 99ml of PBS/0.05% Tween solution.

• **Rotiblock 1:100:**
  100µl of Rotiblock was added to 99.9ml of a PBS/0.05% Tween solution.

• **4% PFA Solution:**
  To prepare 100ml of a 4% PFA solution, 100ml of PBS were heated while stirring to 60°C. When the PBS was hot 4g of PFA were added. The solution was stirred until it was clear. Then, the solution was cooled down to room temperature and storage in the fridge (4°C).

• **Reconstitute Human FGF-Basic:**
  To reconstitute 50µg of human FGF-Basic for a final concentration of 10ng/ml (in the required medium), 50µg of Human FGF-Basic were dissolved in 500µl of Aqua dest. Then, to get the desired solution, the solution was diluted with 49.5ml of Aqua dest. The solution was filtered and distributed for 50 aliquots (with an aliquot concentration of 1µg/ml) and preserved in the freezer (-20°C). Each aliquot was used for 100ml of the required medium.

• **Solution of 8mM of Citric Acid:**
  To obtain 100ml of a 8mM of Citric Acid solution 0.168g of C₆H₈O₇ was dissolved in Aqua dest. The solution was stirred until it was clear and then stored in the fridge.

• **Solution of 4mM of HCl with 0.1% BSA:**
  A solution of HCl with a concentration of 4mM was prepared in Aqua dest. For 50ml of the final solution 500mg of BSA were added to the solution. The solution was stirred until it was clear and stored in the fridge.
• **Reconstitution TGF-β1:**
  To reconstitute 2µg of TGF-β1 for a final concentration of 5ng/ml (in the required medium), 2µg of TGF-β1 were dissolved in 100µl of 4mM of HCl with 0.1% BSA. The solution was then diluted in 7.9ml of *Aqua dest*, filtrated and distributed for 8 aliquots (with a concentration of 2.5µg/ml). Each aliquot was used for 50ml of the required medium.

• **Reconstitution BMP4:**
  To reconstitute 5µg of BMP4 for a final concentration of 2.5ng/ml (in the required medium), 5µg of BMP4 was dissolved in 50µl of citric acid. The solution was then diluted in 39.950ml of *Aqua dest*, filtrated and distributed for 40 aliquots (with a concentration of 1.25µg/ml). Each aliquot was used for 50ml of the required medium.

---

### 2.3 Cell Counting With CASY-1®

The CASY-1® Electronic Cell counter (Figure II.1) is a system to determine the cell amount in a sample in terms of viability, concentration and volume of the cells. This technology was developed in Germany, and is a very useful tool in cell culture, since it gives a very accurate number of the cells. This equipment consists of an electrolyte developed specifically for cell counting and aspirating them through a precision measuring pore of defined geometry at a constant flow speed.

![Figure II.1 – CASY-1 Electronic Cell Counter.](image)

The precision measuring pore is designed as a hole within an artificial precious stone, which is cast inside the capillary body. During the measurement process, a pulsed low voltage field with 1 MHz is applied to the measuring pore via two platinum electrodes. The electrolyte-filled measuring pore...
represents a defined electrical resistance. During their passage through the measuring pore, the cells displace a quantity of electrolyte corresponding to their volume. Since intact cells can generally be considered isolators, an increased level of resistance is achieved over the measuring pore. This resistance is a dimension for the volume of the cells. By contrast, dead cells whose membrane no longer acts as an electrical barrier are recorded by the size of their cell nucleus. It is important that the cells are passing through the measuring pore individually. The amount of cells is readily determined in counts/ml. Measurement is performed by suspending 100µl the cells in 9,9ml of CASY® ton.

2.4 Isolation And Culture Of Human Mesenchymal Stem Cells

2.4.1 Isolation and Culture of Human UC-MSC

The stem cells were isolated from different and freshly provided UCs. The UCs were obtained from the department of Genecology and Obstetrics at University Hospital Aachen (UKA, Aachen, DE) after informed consent of the patients, according to the local ethics committee of the RWTH Aachen University. After obtaining the UC, it was cut into pieces to facilitate the removal of the Wharton’s Jelly. Under sterile conditions on a petri dish and with the help of a scalpel, the pieces of the UC were cut in the middle. The Wharton’s Jelly was scraped very carefully to prevent drill the artery or the vein. After scraping the Wharton’s Jelly of all the UC pieces was immersed in 10ml of collagenase and incubated overnight at 37°C. After the incubation the sample was centrifuged (at 1200xg, for 10min) and the supernatant was discarded. Then, 10ml of trypsin was added to the solution and incubated for 10min at 37°C. After the incubation time, the sample was centrifuged (at 1200xg, for 10min) and the supernatant was discarded. The sample was washed with 10ml of PBS, resuspended in 5ml of MSC-Medium and transferred to a T25 cell culture flask. The cells were expanded in an incubator (37°C, 5% CO₂ and 20% O₂ humidified atmosphere) until they reached confluence. After that, cells are washed with 5ml of PBS and fresh medium is added. Medium change occurs twice a week.

2.4.2 Isolation and Culture of Human BM-MSC

Human BM-MSC used in this experiment were isolated from spongiosa or aspirate of the BM. The clinic for Orthopaedics at University Hospital Aachen (UKA, Aachen, DE) provides femoral heads for the pathology, after informed consent of the patients, according to the local ethics committee of the RWTH Aachen University. The femoral heads are obtained by hip endoprosthesis surgery. After obtaining the femoral head the isolation should occur as fast as possible to ensure that many cells survive. For that,
the femoral head was placed into 10ml of MSC-Medium (warmed up to RT). Medium was flushed with a sterile syringe through the spongiosa. Then the medium was transferred to a 50ml tubes tube. The procedure was repeated until the spongiosa was white. The sample was centrifuged (at 1200xg, for 10min) and supernatant was discarded. Cells were resuspended in 10ml of MSC-Medium and transferred to a T75 cell culture flask. The cells were maintained in an incubator (37°C, 5% CO₂ and 20% O₂ humidified atmosphere) to expand and proliferate. After that, cells are washed with 5ml of PBS and fresh medium is added. Medium change occurs twice a week.

2.4.3 Isolation and Culture of Human CV-MSC

CV-MSC are barely reported in studies and have a huge potential to the field of tissue engineering. The cells used in this experiment were isolated from the placenta of a newborn right after his birth. It was the first time in our research group that someone tried to isolate mesenchymal stem cells from the placenta. The placenta was obtained from the department of Genecology and Obstetrics at University Hospital Aachen (UKA, Aachen, DE), and since the placenta was considered waste, it was simple obtain permission of the local committee.

Figure II.2 – Schematic overview of CV-MSC isolation. In the picture 1 is visible a placenta after processing. Picture 2 and 3 shows pieces of the processed placenta, the tissue was weight and than extensively minced. The picture 4 shows the processed placenta tissue at an incubator shaker.
The following isolation protocol was based on the method of Castrechini et al. [68] and Schmidt et al. [17]. After the birth, the placenta was collected, to proceed the isolation of the stem cells. After collection, the placenta was washed extensively with PBS and the decidual surface of the placenta was removed. An overview of CV-MSC isolation was displayed on Figure II.2. Small pieces of placental tissue (about 1g) were taken from a central cotyledon of the villous vascular bed and transferred to a petri dish (Figure II.2-1 and 2). The small pieces were maintained in medium to prevent them from drying. Each piece was extensively minced (Figure II.2-3) and immersed in 10ml of collagenase. The 50ml tubes were incubated between 1-2h at an incubator shaker (37°C) and were removed when the placenta tissue was completely dissolved (Figure II.2-4). The 50ml tubes were centrifuged (at 1200xg, for 10min), the supernatant was discarded and 10ml of trypsin/EDTA were added to each 50ml tubes. The cell solution was incubated (37°C) for 10min. After the incubation time the samples were washed, resuspended in 5ml of CV-MSC-Medium (BIOAMF™-1) and transferred to 20 T₂⁵ cell culture flasks. The cells were expanded in an incubator (37°C, 5% CO₂ and 20% O₂ humidified atmosphere) until they reached confluence. After that, cells are washed with 5ml of PBS and fresh medium is added. Medium change occurs twice a week.

2.5 Thawing And Freezing Cells

Due to the high number of cells and cell types used during this work it was very frequently used frozen cells and it was needed to froze and thawing cells. Some mesenchymal stem cells used in this experiment, like AD-MSC and HUASMC were kindly provided by the Düsseldorf Hospital and Helmholtz Institute.

2.5.1 Freezing

Cells can be stored in liquid nitrogen tanks for a long period of time. In order to store, cells are trypsinized from their respective cell culture flaks (normally the amount of trypsin is 4ml for each T₂⁵ cell culture flask) and then counted (described on 2.3 Cell Counting With CASY-1®). The number of frozen cells strongly relies on the cell type. For example, the average number of CV-MSC for freezing is 3 million per vial and the average number of UC-MSC for freezing UC-MSC is 1 million. The amount of cells desired was resuspended in 900µl of their respective medium. For every 900µl of cell suspension was added 100µl of DMSO under sterile conditions into a cryovial. The cryovials were quickly transferred to a freezer because DMSO is very harmful for the cells, beginning the process of freezing.
They were maintained at the freezer at -20°C for 60min. After that, they were incubated overnight at a temperature of -80°C. In the next day, the cells were transferred to the liquid nitrogen tanks. Long-time storing occurs in a liquid nitrogen tank (-196°C) where it is very important to store the cells in the right zone (gas phase or liquid phase). The MSC should be stored in the gas phase.

2.5.2 Thawing

The thawing is the reverse process, to recuperate the frozen cells. It should be a quick process since the DMSO is very harmful to the cells at RT. The greater the number of frozen cells, higher is the probability of more cells being alive. The cryo vials were removed of the liquid nitrogen tank and briefly thawed at 37°C in a water bath. The cell suspension from the cryo vials was transferred to a 50ml tubes with 9ml of pre-warmed of their respective medium. The sample was centrifuged (at 1200xg, for 10min), and the supernatant was discarded, followed by the addition of 10ml of the respective medium. The cells were transferred to a T75 cell culture flask and incubated (37°C, 5% CO₂ and 20% O₂ humidified atmosphere).

2.6 FISH Test

Fluorescence In Situ Hybridization (FISH) was used to detect the karyotype of the CV-MSC after their isolation. It is important to know if the isolated cells belong to the baby or the mother. FISH provides a way to visualize and map the genetic material in an individual's cells, including specific genes or portions of genes. Unlike most other techniques used to study chromosomes, FISH does not have to be performed on cells that are actively dividing. Thus, it is a very versatile procedure. The lab staff performed this procedure. Here I would explain, that the Y-chromosomes are stained so that you could be sure – when using placenta of male babies – which you do not work with the mother’s tissue.

2.7 Induction of Smooth Muscle Differentiation

To induce smooth muscle differentiation of mesenchymal stem cells, the basal medium (DMEM (1x) Low Glucose) was supplemented with TGF-β1 (5ng/ml) and BMP4 (2.5ng/ml) (more detailed on 2.1 Cell Culture Mediums). This method of differentiation was adapted from Wang et al. [40].
2.8 IF and IHC Staining

To analyse the expression of smooth muscle markers, the positive control (HUASMC) and the cell types in studied cultured in the differentiation medium and their respective standard medium, IF and IHC analysis were performed after 7 days of culture. Four smooth muscle markers were used for IHC and IF: SM22α, α-SMA, Calponin and SM-MHC.

SM22α is a major component of differentiated smooth muscle cells and is a calponin-related protein that is expressed specifically in adult smooth muscle. This gene encodes a smooth muscle cell-specific cytoskeletal protein. The encoded protein is structurally similar to calponin, an actin-binding protein. In mouse models of atherosclerosis the gene product may be involved in plaque cell and atherosclerotic lesion formation during atherogenesis [69].

Calponin binds to actin, myosin, Ca²⁺ binding proteins and tropomyosin that inhibits the actomyosin ATPase as well as the movement of actin filaments over myosin in vitro. These properties have led to the proposal that calponin may be involved in the Ca²⁺ dependent regulation of actin-myosin interaction and consequently of smooth muscle contraction. Calponin is localized in both the contractile and the cytoskeletal parts of the smooth muscle cell and may have a structural function in smooth muscle cells. Calponin may play an important role in signal transduction from the membrane receptor to the contractile proteins in smooth muscle [70].

The protein encoded by SM-MHC is a smooth muscle myosin belonging to the myosin heavy chain family. The gene product is a subunit of a hexameric protein that consists of two heavy chain subunits and two pairs of non-identical light chain subunits. It functions as a major contractile protein, converting chemical energy into mechanical energy through the hydrolysis of ATP [71].

α-SMA an isoform typical of SMC and present in high amounts in vascular SMC was localized in microfilament bundles, strengthening the assumption that it is the functional isoform in these cell types and supporting the assumption that pericytes exert contractile functions. This protein belongs to the actin family of proteins, which are highly conserved proteins that play a role in cell motility, structure and integrity [72]. A detailed plan can be visualized on Appendix A – IF and IHC Experiments Plan.

2.8.1 Immunofluorescence:

The IF staining is one of the techniques used to evaluate the smooth muscle differentiation after 7 days. The following IF protocol was based on Wang et al. [40] and Vazão et al. [6].
The MSC from different sources cells were differentiated (1.8 Cells) in well plates with 48 cavities during 7 days. Then, the IF protocol was performed to evaluate the smooth muscle differentiation with the use of four different smooth muscle markers (1.9 Primary antibodies for IHC and IF). The medium was removed and the cells were fixed with PFA solution during 10min or Methanol in the case of α-SMA during 30 min at RT. The cells were washed with PBS/0.05% Tween three times and then was added 0.2% Triton-X during 10min at RT for cell membrane permeabilization. The cells were washed again and blocked with Rotiblock 1:10 in PBS/0.05% Tween during 30 min at RT (to block nonspecific binding sites). After that the cells were stained with anti-human primary antibodies (diluted in Rotiblock 1:100 in PBS/0.05% Tween) specific for α-SMA (1:1000), SM22α (1:1000), Calponin (1:500) and SM-MHC (1:50). The cells were then incubated overnight using humidified dark chambers at 4ºC. After incubation, the cells were washed and stained with secondary antibodies (1.11 Secondary Antibodies) (diluted in Rotiblock 1:100 in PBS/0.05% Tween) using a dilution of 1:200 and were incubated during 1h at RT in humidified dark chambers. The cells were washed and DAPI was added for 5 min for counter-nuclear staining. PBS was added to prevent the cells from drying until observed under the microscope.

2.8.2 Immunohistochemistry

The IHC is another technique used to evaluate the smooth muscle differentiation. The following protocol was based on the kit from Dako Autostainer and Rodriguez et al. [5].

The IHC follows the same initial protocol of IF until the addition of the primary antibodies. After blocking, the cells were stained with anti-human primary antibodies (diluted in AB-dilution from DAKO kit) specific for α-SMA (1:1000), SM22α (1:1000), Calponin (1:500) and SM-MHC (1:100) during 1h at RT. The cells were washed and then added the secondary AB (from DAKO kit) for 15 min at RT. The washing step is needed for each new step. Streptavidin-HRP (containing stabilizing protein and antimicrobial agents) (from DAKO kit) was added for 15min at RT. It was added DAB-chromogen (from DAKO kit) for 10min at RT that followed by the addition of Haematoxylin (from DAKO kit) for counter-nuclear staining. PBS was added to prevent the cells from drying until observation under the microscope.
2.9 Preparation of Cells for Flow Cytometry

For analysis of the expression of the different markers (Table II.1), either for characterization of the MSC or to evaluate the smooth muscle differentiation, the cells were harvested after 7 days of culture. The experiment was performed on well plates with 48 cavities to share the same conditions as for IF and IHC. The cells were trypsinized from the well plate obtaining the cells suspension that was distributed 1.5ml tubes. From this step until the end of the cell preparation the cells had to be preserved on ice. The experiment plan for flow cytometry can be visualized on Appendix B – Flow Cytometry Experiments Plan.

Table II.1 – Interpretation of the Markers used for flow cytometry.

<table>
<thead>
<tr>
<th>CD13</th>
<th>This antibody binds to granulocytic and monocytic cells, mast cells, and GM-progenitor cells but not to lymphocytes, platelets or erythrocytes [73].</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>Cell surface glycoprotein that is preferentially expressed on monocytes and macrophages. Additionally, it reacts with interfollicular macrophages, reticular dendritic cells, and some Langerhans cells [74].</td>
</tr>
<tr>
<td>CD16</td>
<td>CD16 is expressed on NK cells as well as macrophages and granulocytes. Reports indicate that CD16 plays a role in signal transduction and NK cell activation [75].</td>
</tr>
<tr>
<td>CD19</td>
<td>Reacts with the type I transmembrane glycoprotein expressed during all stages of B-cell differentiation and maturation, except on plasma cells. CD19 is also present on follicular dendritic cells. CD19 plays a role in regulation of B-cell proliferation [76].</td>
</tr>
<tr>
<td>CD29</td>
<td>Reacts with the integrin β1 subunit that is expressed as a heterodimeric complex with one of six distinct α subunits, comprising the very late activation antigen subfamily of adhesion receptors. The β1 subunit has a broad tissue distribution; it is expressed on lymphocytes, monocytes and weakly on granulocytes, but not on erythrocytes. These receptors are involved in a variety of cell-cell and cell-matrix interactions [77].</td>
</tr>
<tr>
<td>CD31</td>
<td>CD31 has wide tissue distribution and is expressed on platelets, monocytes, granulocytes, and in high amounts on endothelial cells. This molecule has been implicated in a number of cellular phenomena, including vascular wound healing and angiogenesis, and transendothelial migration of leukocytes in inflammatory responses [78].</td>
</tr>
<tr>
<td>CD34</td>
<td>Reacts with a transmembrane glycoprotein expressed on hematopoietic progenitor cells, vascular endothelium and some tissue fibroblasts. The intracellular chain of the CD34 antigen is a target for phosphorylation by activated protein kinase C suggesting CD34 may play a role in signal transduction. CD34 may also play a role in adhesion of specific antigens to endothelium [79].</td>
</tr>
<tr>
<td>CD40</td>
<td>CD40 is expressed on B-lymphocytes, but is not expressed on terminally differentiated B-cells.</td>
</tr>
<tr>
<td><strong>CD40</strong></td>
<td>Endothelial cells, basal epithelial cells and some epithelial cell carcinomas, follicular dendritic cells, macrophages, fibroblasts, keratinocytes also express CD40. This antibody is useful for studying the roles played by CD40 in B-cell growth, proliferation, and differentiation including immunoglobulin isotype switching [80].</td>
</tr>
<tr>
<td><strong>CD44</strong></td>
<td>CD44 is the receptor for hyaluronic acid. CD44 is expressed on leucocytes, erythrocytes, epithelial cells and weakly on platelets. CD44 is also called extracellular matrix receptor type III and has functional roles in cell migration, lymphocyte homing and adhesion during haematopoiesis and lymphocyte activation [81].</td>
</tr>
<tr>
<td><strong>CD45</strong></td>
<td>Present on all human cells leucocytes, including lymphocytes, monocytes, granulocytes, eosinophils and basophils, Pan-leucocytes marker. Is a protein tyrosine phosphatase that regulates Src kinases required for T and B-cell receptor signal transduction [82].</td>
</tr>
<tr>
<td><strong>CD49a</strong></td>
<td>CD49a is known as a very late antigen 1α subunit (VLA-1) of the integrin family of cell adhesion molecules. It is expressed on activated T cells, monocytes, neuronal cells and smooth muscle cells. CD49a has been reported to play a role in cell attachment during the development of both the central and peripheral nervous systems [83].</td>
</tr>
<tr>
<td><strong>CD49d</strong></td>
<td>CD49d is a integrin α subunit and it is present on lymphocytes, monocytes, thymocytes, NK cells, and several B and T-cell lines [84].</td>
</tr>
<tr>
<td><strong>CD56</strong></td>
<td>CD56 is a heavily glycosylated protein that is present on a subpopulation of peripheral blood large granular lymphocytes which demonstrate natural killer activity. This antigen is a pan-NK-cell marker [85].</td>
</tr>
<tr>
<td><strong>CD73</strong></td>
<td>CD73 is expressed on subsets of T and B-lymphocytes, follicular dendritic cells, epithelial cells, endothelial cells and mesenchymal stem cells. CD73 has enzymatic activity and catalyses the dephosphorylation of adenosine monophosphate (AMP) converting it to adenosine [86].</td>
</tr>
<tr>
<td><strong>CD80</strong></td>
<td>CD80 is a member of the immunoglobulin supergene family, is expressed on activated B-cells, macrophages, and dendritic cells. CD80 is also expressed on activated CD4+ and CD8+ T-cells [87].</td>
</tr>
<tr>
<td><strong>CD83</strong></td>
<td>CD83 is composed of a single V-type immunoglobulin extracellular domain with a C-terminal cytoplasmic tail. Mainly follicular dendritic cells, circulating dendritic cells and interdigitating dendritic cells in lymphoid tissues express CD83. CD83 is also expressed on some germinal center B-cells and some lymphoblastoid cell lines [88].</td>
</tr>
<tr>
<td><strong>CD86</strong></td>
<td>CD86 is expressed primarily on monocytes and activated B-cells. CD86 may play an important role in co-stimulation of T-cells in primary immune response [89].</td>
</tr>
<tr>
<td><strong>CD90</strong></td>
<td>CD90 is expressed on cord blood cells, BM cells and on 1-4% of human fetal liver cells. Anti-CD90 is useful for enriching high proliferative potential colony-forming cells, which are primitive progenitor cells [90].</td>
</tr>
<tr>
<td><strong>CD105</strong></td>
<td>CD105 presents an integral membrane homodimer protein with subunits found on vascular endothelial cells and syncytiotrophoblasts of placenta. CD105 is expressed on activated macrophages and mesenchymal stem cells. CD105 is increased on activated endothelium in tissues undergoing angiogenesis, such as in tumours, or in cases of wound healing or dermal inflammation [91].</td>
</tr>
<tr>
<td><strong>CD106</strong></td>
<td>CD106 is a type I transmembrane sialoglycoprotein. CD106 is expressed at high levels on the surface of cytokine-stimulated endothelium, and at minimal levels on unstimulated endothelium [92].</td>
</tr>
<tr>
<td><strong>CD146</strong></td>
<td>CD146 is a member of the immunoglobulin superfamily and is expressed on endothelial cells, melanoma cells, smooth muscle and intermediate trophoblasts. It has been reported as being present on a subpopulation of activated T-cells [93].</td>
</tr>
<tr>
<td><strong>CD166</strong></td>
<td>CD166 is composed of an immunoglobulin-like extracellular domain, a transmembrane region and a short cytoplasmic tail. It is expressed on neurons, activated T-cells, activated monocytes, epithelial cells and fibroblasts [94].</td>
</tr>
<tr>
<td><strong>CD275</strong></td>
<td>CD275 co-stimulates the proliferation and cytokine production of human T-cells. It is weakly expressed on peripheral blood mononuclear cells, but it is expressed on monocyte-derived dendritic cells [95].</td>
</tr>
<tr>
<td><strong>HLA-DR</strong></td>
<td>HLA-DR reacts with the major histocompatibility Class II (MHC II). This clone also cross-reacts with a subset of peripheral blood lymphocytes and monocytes [96].</td>
</tr>
<tr>
<td><strong>HLA-ABC</strong></td>
<td>HLA-ABC specifically binds to a monomorphic epitope on the alpha chain of major histocompatibility Class I (MHC I). MHC I antigens are widely distributed on human nucleated cells [97].</td>
</tr>
<tr>
<td><strong>HSTRO-1</strong></td>
<td>STRO-1 is a BM (immature) mesenchymal cell surface protein. Multipotent stromal cells can give rise to several lineages: osteoblasts and chondrocytes, which form bone, adipocytes or hematopoietic cells [98].</td>
</tr>
<tr>
<td><strong>vWF</strong></td>
<td>vWF is a multimeric plasma glycoprotein synthesized by endothelial cells and megakaryocytes. In plasma it functions as a carrier for Factor VIII and is a mediator of initial platelet adhesion to subendothelium and platelet-platelet interaction [99].</td>
</tr>
<tr>
<td><strong>Vimentin</strong></td>
<td>Intermediate filaments are a subset of cytoskeletal proteins which function to give overall structural integrity to the plasma membrane as well to organize cells into specific tissues. Vimentin belongs to the type III category of intermediate filaments proteins which are expressed in leukocytes, blood vessel endothelial cells, some epithelial cells, and mesenchymal cells [100].</td>
</tr>
</tbody>
</table>

Each cell type was split on the amount of 1.5ml tube necessaries for the use of the correct AB (for example the BM-MSC undifferentiated was split in eleven 1.5ml tubes, the AD-MSC differentiated in nine 1.5ml tubes, etc). A detailed plan is shown on Appendix C – Flow Cytometry Plan. PBS was added
to the samples and then centrifuged (at 8000xg, for 4min) since the smooth muscle differentiation was also been evaluated through flow cytometry, there was a fundamentally step for the smooth muscle markers. For the 1.5ml tubes containing the smooth muscle markers it was performed the permeabilization of the cell membrane. The cells were fixed with 4%PFA for 30 min and washed with PBS. In the end, PBS/0.05% Tween was added for 7 min to complete the cell permeabilization. All the samples were washed with PBS and the supernatant was discarded. The cells was resuspended in PBS/0.1% FBS or with PBS/0.05% Tween for the samples with smooth muscle markers. Primary AB was added to the solution. All the samples were incubated for 30 min to 1h in the dark and on ice. After the incubation time the samples were washed, the supernatant was discarded and the cells were resuspended in PBS/0.1% FBS. The secondary AB was added. The samples were incubated for 30 min in the dark on ice. After the incubation time the cells were washed followed by the addition of PBS/0.1% FBS. The samples were transferred to round bottom tubes for flow cytometry. Immediately after the staining the cells were measured at the flow cytometer.

2.10 Flow Cytometry

The cytometer detects the characteristics of each member of a population by carrying individual cells in a flow of liquid past an illumination source. With a flow cytometer it is possible to identify different cells and it can sort out cells of a particular type of interest from a mixture. The illuminating light interacts with the cell and photoactive labels attached to the cell. If an antibody specific to a certain cell-surface molecule is linked to a fluorescent dye, any cell bearing this molecule will bind the antibody and will be identified amongst other cells when it fluoresces in the flow cytometer. A group of sensors measure the light scattered by the cell, and the fluorescence emission of the cell. For measurement, the cell suspension was mixed with PBS/0.1% FBS (the sheath fluid). The suspension was forced through a nozzle, which forms tiny droplets containing at most, one single cell, which passes through a laser light beam. The light scattered by each cell can be measured at the same time as the fluorescence. From measurements of the scattered light, the size and shape of the cell can be determined. From measurements of the fluorescence, wanted characteristics like antibody binding to surface molecules can be determined. In this way, cells that have desired properties can be identified. A computer stores the signals from the sensors, making a set of data points that correspond to the measured characteristic for each member of the population [101, 102]. An overview of a flow cytometer is depicted on Figure II.3.
Figure III.3 – Schematic overview of how works Flow Cytometry, adapted from [38].
Chapter III – Results and Discussion
Chapter III

Results and Discussion

1. Isolation and Characterization of CV-MSC

The isolation of CV-MSC was never been performed in the research group, so before adapting the smooth muscle cell differentiation protocol, it was necessary to establish the isolation and then evaluate and characterize the phenotype of the CV-MSC. The removal of the cells from the chorionic villi could be complicated due to the fact that the placenta is a reservoir of cells both from the mother and the baby. During the isolation of the CV-MSC there must be special attention just to isolate the cells derived from the trophoblast of the former blastocyst. So to be sure that no parental cells were isolated, a selective medium was used for this type of cells, and further a FISH analysis was performed to identify the karyotype, in particular the Y chromosome of the isolated cells. In order to obtain an accurate analysis, CV-MSC were isolated from placenta of newborn baby boys in order easily exclude mother cells as donor cells or the presence of a mixed cell population (mother/newborn) in culture. For further characterization of the CV-MSC, flow cytometry was performed to compare with other mesenchymal stem cells well reported on the scientific community, the UC-MSC, the BM-MSC and the AD-MSC.

1.1 CV-MSC Morphology

The isolated placental cells exhibited an elongated spindle-shaped (fibroblast-like) morphology and large nuclei as depicted on Figure III.1. The Figure III.1 shows the morphology of different passages from CV-MSC. From A to C is the passage 0 isolated directly from primary tissue. Picture A was taken after 5 days of the isolation and it was observed that the cells grow in small colonies right after the isolation. Picture B is from the same donor but after 12 days of culture and picture C shows the cells after 18 days of culture (the cells were nearly confluent and right before passaging). Picture E to F are the same donor but within different passages. Picture D corresponds to passage 2, picture E to passage 4 and picture F to passage 6. The change in the morphology was not evident between the passages but in the passage 6 the differences start to appear, they look more flat, they grown slower and they occupy more space.
Figure III.1 – Pictures of a donor of CV-MSC in different passages, with a resolution of 50. From A to C represents the passage 0 of the CV-MSC during 18 days. Picture A was taken after 5 days of isolation, picture B was taken after 12 days and picture C after 18 days. The cells on picture D are on passage 2, on picture E are on passage 4 and on picture F are on passage 6.
1.2 FISH Analysis

To determine the presence of fetal cells in our CV-MSC cultures, we analysed the karyotype from two placenta of twin babies. It was known the sex of the babies, one twin was a boy (Twin 1) and the other was a girl (Twin 2). So after the isolation and expansion of the cells the test was performed and the results obtained are shown on Figure III.2.

![Fluorescence Images of FISH Analysis of CV-MSC from Twin Babies. Twin 1 Is the Baby Boy and the Twin 2 is the Baby Girl.](image)

The DAPI staining visualizes cell nuclei, the X and Y are the single gonosomes and the MIX is the overlay of both pictures. As is visible, the Twin 1 shows positive results for the X and Y chromosomes and in the MIX is visible the two chromosomes, so as it was expected the twin 1 was a
boy. The twin 2 only shows positive results for the X chromosome and in the MIX is visible two X chromosomes, so as it was expected it was a baby girl. These results indicate that CV-MSC lines derived by the isolation protocol contain predominantly fetal cells in Twin 1, although, the presence of maternal cells could not be completely ruled out. Relative to the Twin 2 it was impossible to know if the cells belong to the mother or the baby.

1.3 Phenotype Analysis

The CV-MSC expresses several cell surface and intracellular markers typical of stem/progenitor cells. In accordance with the minimal criteria to define MSC, as set out by the International Society for Cellular Therapy, they should be positive for the markers CD73, CD90 and CD105 and should not express CD45, CD34, CD14 and HLA class II.

![Figure III.3](https://Example.com/figureIII3.png)

*Figure III.3 – Some results of flow cytometry analysis of the expression of surface markers for a CV-MSC donor.*

The CV-MSC are positive for a group of markers that are also commonly reported positive on other MSC, like CD73, CD44, CD83, CD105 and CD166, and they are negative for a group of markers also commonly reported as negative on other MSC, like CD45, CD86, HLA-DR, CD14 and CD106 [51, 54, 66]. The Figure III.3 illustrates the most commonly reported positive and negative markers of a representative flow cytometry experiment, from a CV-MSC donor. CD90 is normally highly expressed in MSC, but for this CV-MSC donor, had a moderate expression and not as high as expected. The remaining results of this CV-MSC donor are presented on Appendix C – Flow Cytometry Plan.
further analysis of the CV-MSC phenotype Table III.1 illustrates the results of flow cytometry analysis from three different sources of MSC: UC-MSC, BM-MSC and CV-MSC. A specific group of markers for was prepared for analysis of these three different sources MSC. Although some results vary a little within the different sources, there are a group of markers, either positive, either negative that had the same result for the three MSC sources. For comparison a table is presented on Appendix E – Surface Markers Categories.

**Table III.1 – Results of flow cytometry analysis of three different donors of each MSC source (n=3).**

<table>
<thead>
<tr>
<th>Markers</th>
<th>UC-MSC (%expression)</th>
<th>BM-MSC (%expression)</th>
<th>CV-MSC (%expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>0.14±0.69</td>
<td>0.87±1.03</td>
<td>0.29±0.47</td>
</tr>
<tr>
<td>CD19</td>
<td>6.03±9.18</td>
<td>2.8±4.22</td>
<td>12.95±17.81</td>
</tr>
<tr>
<td>CD34</td>
<td>19.58±33.33</td>
<td>15.56±24.42</td>
<td>37.63±33.04</td>
</tr>
<tr>
<td>CD40</td>
<td>41.10±24.64</td>
<td>39.23±17.8</td>
<td>54.70±31.81</td>
</tr>
<tr>
<td>CD44</td>
<td>99.53±0.38</td>
<td>93.20±10.48</td>
<td>99.80±0.26</td>
</tr>
<tr>
<td>CD45</td>
<td>0.72±1.05</td>
<td>0.62±0.24</td>
<td>0.14±0.18</td>
</tr>
<tr>
<td>CD49a</td>
<td>33.20±14.19</td>
<td>40.07±18.00</td>
<td>84.23±13.74</td>
</tr>
<tr>
<td>CD56</td>
<td>33.72±41.21</td>
<td>42.97±36.65</td>
<td>52.78±41.05</td>
</tr>
<tr>
<td>CD73</td>
<td>88.53±13.04</td>
<td>70.23±21.95</td>
<td>96.30±5.37</td>
</tr>
<tr>
<td>CD80</td>
<td>29.74±45.38</td>
<td>16.01±20.60</td>
<td>45.45±44.82</td>
</tr>
<tr>
<td>CD83</td>
<td>66.20±18.74</td>
<td>65.93±11.32</td>
<td>86.37±10.98</td>
</tr>
<tr>
<td>CD86</td>
<td>0.78±1.07</td>
<td>0.31±0.23</td>
<td>0.78±1.05</td>
</tr>
<tr>
<td>CD90</td>
<td>75.67±29.03</td>
<td>75.30±10.44</td>
<td>54.43±42.71</td>
</tr>
<tr>
<td>CD105</td>
<td>91.03±10.03</td>
<td>74.73±17.77</td>
<td>96.63±2.32</td>
</tr>
<tr>
<td>CD106</td>
<td>0.3±0.46</td>
<td>0.24±0.06</td>
<td>0.06±0.10</td>
</tr>
<tr>
<td>CD146</td>
<td>13.38±18.25</td>
<td>20.58±25.97</td>
<td>48.05±36.33</td>
</tr>
<tr>
<td>CD166</td>
<td>59.60±32.74</td>
<td>50.67±10.16</td>
<td>84.73±12.67</td>
</tr>
<tr>
<td>CD275</td>
<td>1.37±1.24</td>
<td>0.84±0.70</td>
<td>4.95±5.71</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>81.07±22.35</td>
<td>56.83±43.17</td>
<td>92.63±10.58</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0.35±0.52</td>
<td>0.29±0.04</td>
<td>0.23±0.38</td>
</tr>
<tr>
<td>Stro-1</td>
<td>24.42±33.14</td>
<td>23.23±21.45</td>
<td>44.30±23.47</td>
</tr>
<tr>
<td>vWF</td>
<td>54.57±39.48</td>
<td>54.57±29.88</td>
<td>92.03±13.63</td>
</tr>
<tr>
<td>Vimentin</td>
<td>75.17±4.97</td>
<td>90.87±11.89</td>
<td>97.40±2.71</td>
</tr>
</tbody>
</table>
The group of positive markers equal within the three different sources were CD44, CD73, CD105, HLA-ABC and Vimentin. On the other hand, the group of markers that was equal negative for the three sources were CD14, CD19, CD45, CD86, CD106, HLA-DR and CD275.

CD90 and CD166 had a moderately expression and usually have a high expression on MSC [51]. Stro-1 was expected to be highly expressed on BM-MSC and had lack of expression on CV-MSC and UC-MSC [51, 53]; usually this marker is report to be use to exclude the BM-MSC from the other MSC sources [103, 104]. However, the results showed that Stro-1 had a low expression level in the three MSC donors of different sources. On the other hand CD40, CD83 and vWF should not be present in all MSC [66, 68]. In this case the results also show the opposite, the three markers show a moderately positive expression. CD83 is predominantly expressed on mature human dendritic cell [105], vWF is a marker that should be expressed after endothelial differentiation [106] and CD40 is commonly expressed by hematopoietic cells [107]. The expression of these markers may have been influenced by mycoplasma infection. Mycoplasma infection is very serious and it can infect cells for long periods of time without being detected and it can interfere with the cell metabolism including proliferation and signalling, thus compromising experiments and misleading results. The infection could be directly related to the expression of CD40, since there are studies referring the expression of this marker in presence of infection/inflammation [108, 109], the MSC in the presence of inflammatory cytokines induced expression of CD40 [109]. In relation to vWF, it has already been reported in recent studies to detect cells with MSC properties [68]. Regarding the other marker, there are no studies reporting the expression of this marker on MSC, probably the mycoplasma infection mislead the results.

The markers CD56 and CD146 have been controversially reported. In several studies these markers are described to be positive or negative on MSC [68, 110], and the moderately expression of this markers could be related with the results observed in the previous studies. Lastly, the CD49a is been used to identify CV-MSC [111] and this was verified in the results, since the expression is highly positive for CV-MSC but only moderately positive for UC-MSC and BM-MSC.

The surface marker panels used allowed for clear characterization of the different cell sources expressing the typical MSC markers CD90⁺, CD73⁺, CD105⁺, CD44⁺, CD34, CD45⁺ and CD106 while CV-MSC additionally expressed CD83⁺, CD166⁺ and CD49a⁺, as reported in the literature [66, 111]. The CV-MSC also proved their similar morphology to typical MSC and is lacking analysis of their differentiation potential to prove all the MSC characteristics.
2 Smooth Muscle Differentiation

The induction of smooth muscle differentiation on the different MSC sources was achieved through a chemical way by applying a combination of TGF-β1 and BMP4 [40]. This combination of growth factors was added to the differentiation medium (that had lower levels of glucose compared to basal medium of the MSC) every time the cells were fed. After 7 days in differentiation medium the success of differentiation was analysed.

Figure III.4 – Expression of SMC differentiation markers in MSC.

To determine if the MSC were able to acquire an SMC phenotype when treated with TGF-β1 and BMP4, four smooth muscle cell specific proteins (1.9 Primary antibodies for IHC and IF) were analysed by immunofluorescence (IF) and immunohistochemistry (IHC). During the differentiation also occurs a maturation of markers (Figure III.4). There are markers that are developed early, when the cell is acquiring smooth muscle cell phenotype, and markers that have a late development, when the cell become a smooth muscle cell. The markers can be denominated early (α-SMA and SM22α) and late (Calponin and SM-MHC) markers [1, 5, 40].

The differentiation process was also analysed by flow cytometry to analyse all the surface markers of MSC before and after differentiation, including the smooth muscle cell specific proteins. The three analysis methods were performed in triplicates with different donors for each MSC source. The three analysis methods were performed in triplicates with different donors for each MSC source using a total of two donor repeats.
2.1 Immunohistochemistry Analysis

After 7 days of induction, the MSC were analysed by IHC with smooth muscle cell markers to see if they acquire the smooth muscle cell phenotype. In parallel, expression of these markers in HUASMC was also examined to serve as a positive control. One representative result of each MSC source is depicted on Figure III.5 (BM-MSC) and in Appendix F – Immunohistochemistry MSC Pictures (AD-MSC, CV-MSC and UC-MSC).

![Figure III.5 - SMC-specific proteins. IHC staining of a BM-MSC donor under differentiation conditions during 7 days (n=3).](image)

As shown on Figure III.5 and Appendix F – Immunohistochemistry MSC Pictures, α-SMA and SM22α do not show any significant difference between the undifferentiated and differentiated state on all MSC sources. While α-SMA was already reported to be expressed in MSC, because it confers
plasticity to the cell [1, 112], SM22α was never identified in MSC and is a marker almost exclusively to SMC [1]. Calponin is the best result in BM-MSC and AD-MSC. It shows a significant increase in calponin expression in the differentiated state. Concerning the CV-MSC and UC-MSC (see Figure in Appendix F – Immunohistochemistry MSC Pictures) results are not clear. IHC expression seems positive for every marker for both states. Nevertheless CV-MSC show visible changes of morphology - with cells becoming more flat and larger - among differentiated and undifferentiated state which can be a possible indicator for a change of phenotype.”

The SM-MHC is the most important marker to define the smooth muscle cell because is an essential component of the contractile system [1]. However, the experiments did not get any significant results even on the positive control. That indicates that HAUSMC used as positive control for the experiments are phenotypically not valid. These results are confirmed by the flow cytometry data.

For clarification of the results, the experiments are advised to be repeated.

2.2 Immunofluorescence Analysis

After 7 days in induction medium, MSC were analysed by IF to visualize smooth muscle cell markers to check whether they acquired the smooth muscle cell phenotype. In parallel, expression of these markers in HUASMC was also examined to serve as a positive control One representative result of each MSC source is presented on Figure III.6 (BM-MSC) and in Appendix G – Immunofluorescence MSC Pictures (AD-MSC, CV-MSC and UC-MSC). The IF results for the SM-MHC were not overall conclusive therefore presented in Appendix G – Immunofluorescence MSC Pictures.

As visible on Figure III.6 and Appendix G – Immunofluorescence MSC Pictures, the undifferentiated BM-MSC and AD-MSC did not show expression for the smooth muscle cell markers. On the other and the differentiated BM-MSC and AD-MSC presented expression of the smooth muscle markers, being the SM22α the marker with higher expression in the differentiated cells. In comparison of these results with the positive control, SM22α had a similar expression. For the markers Calponin and α-SMA the expression in differentiated BM-MSC and AD-MSC was not high as the observed for the positive control. Despite these markers did not present an expression similar to the positive control, it is possible to observe an expression that did not occurred on undifferentiated BM-MSC and AD-MSC.

MSC and UC-MSC show comparable results to the presented ones for BM-MSC that can be seen on Appendix G – Immunofluorescence MSC Pictures. Although the UC-MSC and CV-MSC showed a basal expression of SM22α in the undifferentiated state, when exposed to the differentiation medium,
the expression of SM22α was enhanced. Calponin and α-SMA showed very strong results, while the undifferentiated CV-MSC and UC-MSC did not show expression, the differentiated state of the MSC showed an expression for these markers. SM22α had a similar expression all over the conditions and on the positive control. For the Calponin and α-SMA the results were similar to the previous results seen on the BM-MSC. Their expression was not high as the observed for the positive control but it was possible observed an expression that did not occurred on undifferentiated CV-MSC and UC-MSC.

![Figure III.6 – SMC-specific proteins. IF staining of a BM-MSC donor under differentiation conditions during 7 days (n=3).](image_url)

This results show that MSC acquired SMC phenotype as evidenced by their expression of specific structural proteins during synergistic induction with TGF-β1 and BMP4. It was also observed a basal expression of SM22α in the expanded cultures of undifferentiated UC-MSC and CV-MSC whereas the same basal expression was not detected on the undifferentiated AD-MSC and BM-MSC. However there was an increase of level expression by stimulation with BMP4 and TGF-β1. As an early marker of SMC differentiation, SM22α is often adopted to characterize an early SMC phenotype [113], however this expression itself does not provide a definitive evidence for an SM lineage [40]. Although, SM22α is expressed specifically in adult smooth muscle, the above phenomena had also been observed on [5, 40] as they detected basal expression of SM22α in MSC.
Calponin has been widely accepted as a late marker of SMC differentiation with more SMC correlativity; it has been postulated to function as a regulator of SMC contraction \[1\]; and is believed to be only expressed in SMC but not in any other cell types \[114\]. All these results suggest that with stimulation of TGF-\(\beta\)1 and BMP4 its possible to initiate SMC differentiation of MSC. However, in order to clarify this hypothesis more studies have to be done, including the use of SM-MHC, since it is a very important marker to characterize a contractile smooth muscle phenotype.

### 2.3 Flow Cytometry Analysis

After 7 days of differentiation cells of all conditions (HUVEC, HUASMC, BM-MSC, CV-MSC, UC-MSC) were analysed by flow cytometry. For the MSC characterization, a MSC marker panel was included (see Appendix E – Surface Markers Categories). Additionally, a CV-MSC marker panel was included, according to state of the art knowledge of the CV-MSC phenotype \[63, 111\] in order to characterize this cell source/isolation protocol in our hands. Further, an AD-MSC marker panel was included to identify the phenotype of a cell type, which is widely known \[51\] to express markers normally absent in the traditional sources of MSC. At last, a SMC differentiation panel, consisting of all markers previously used for IHC and IF analysis was included in order to evaluate efficiency of the differentiation protocol applied. The complete panels of markers analysed is seen at Figure III.7

Due to the mycoplasma infection it was not possible to perform the last experiment for analysis of the smooth muscle markers. Results might be compromised due to mycoplasma infection but that is something is being tested at the moment, since testing of frozen stocks is still ongoing. These results are presented in two figures by a heat map depicted on Figure III.7 that includes the results of MSC characterization. The Figure III.8 presents the results of smooth muscle cell characterization. A heat map is a graphical representation of data where the individual values contained in a matrix are represented as colours. The expressions of the markers vary within a range from 0 to 100%, represented by a colour gradient.

A detailed quantification of flow cytometry analysis can be seen on Appendix H – Flow Cytometry Analysis of Different MSC sources. The appendix will serve to a better overview of the discussion of the results presented.
2.3.1 Human Umbilical Vein Endothelial Cells

HUVEC are cells derived from the endothelium of veins from the umbilical cord and were used as a negative control in this experiment, since they should not express smooth muscle markers. There is lack of literature concerning the expression of the mesenchymal markers on the HUVEC. The same group of markers used for the characterization for the UC-MSC, BM-MSC and CV-MSC was applied on the HUVEC to exploit their differences. The HUVEC, which are thought to be differentiated endothelial cells, should be stained positive for the vascular markers, such as vWF, CD106 and CD146 [115] and some MSC markers, such as CD44, CD73 and CD105 [115, 116]. As is visible on Figure III.7, HUVEC had a high expression for CD44, CD73, CD105, CD146 and vWF (>80%). vWF should have been only expressed on HUVEC and not been expressed at all on MSC. However, this was not verified, all MSC sources were stained positive for vWF.

Visualizing the figure III.8 it is possible observe CD146 had a higher expression (>90%) when compared to other MSC types (<40%) and since it is a marker to identify endothelial cells [33] it was possible to conclude that. Studies show that CD106 should be positive, and that is not what the results show (0%). The remaining results are similar to the other MSC, unless the CD49a and CD90 marker. The CD49a is a mesenchymal marker, and is reported variable on MSC [104], so the lack of expression is accepted (<10%). CD90 is a marker commonly used to characterize the MSC but had lack of expression on HUVEC (<1%). This marker was found to be the best marker to separate the endothelial cells from the MSC [116] and that can be verified on Figure III.7.

2.3.2 Undifferentiated and Differentiated UC-MSC

As a usual standard the MSC should express mesenchymal markers and should be negative for hematopoietic markers, however as previous studies had shown, this is not always verified [51, 54, 55, 116]. In search of markers able to characterise the cell population during differentiation, the undifferentiated UC-MSC had a positive expression of the common well-defined MSC markers: CD44, CD90, CD73 and CD105 (>80%) and also for CD166, CD83, HLA-ABC, vWF and Vimentin (>60%).

In the case of the markers CD34, CD40, CD49a, CD56, CD80 and stro-1, despite of has not shown a significantly expression, it was possible to observe some expression (±30%), as can be observed on Figure III.7. The markers vWF, CD40, CD80 and Stro-1 were expected to be negative, the positive results obtained could be associated with the cell passage state.
On the other hand, all the markers that presented no expression for the undifferentiated UC-MSC: CD14, CD19, CD45, CD86, CD106, CD146, CD275 and HLA-DR (<8%) are in concordance with the results obtained in previous studies (Appendix E – Surface Markers Categories).

Comparing the undifferentiated and differentiated UC-MSC, they showed almost identical results, except for the CD34, CD40, CD49a, CD56 and CD80. On the differentiated UC-MSC, the CD34 has not shown almost any expression (decreased from 20% to 5%), this lack of expression was also observed in previous studies (Appendix E – Surface Markers Categories). The expression of CD80 decreased (±20%) and the expression of CD40, CD49a and CD56 increased (±20%) compared to
undifferentiated stated of the UC-MSC. Curiously these four last markers are shown in literature to have an irregular expression within MSC.

### 2.3.3 Undifferentiated and Differentiated BM-MSC

The undifferentiated BM-MSC had the same results as the undifferentiated UC-MSC, as can be observed on Figure III.7. The undifferentiated BM-MSC had a positive expression of the common well-defined MSC markers: CD44, CD90, CD73 and CD105 (>75%) and also for the markers CD166, CD83, HLA-ABC, vWF and Vimentin (>60%). In the case of the markers Stro-1, CD40, CD49a, CD56 and CD80, despite of had not showed a significantly expression it was possible to detect a weak expression (<40%). For all the markers that showed lack of expression: CD14, CD19, CD45, CD86, CD106, CD146, CD275, HLA-DR and CD34 (<8%). The results were as expected except for the Stro-1 (±25%), that should had been a higher expression, since it’s one of the most commonly markers used to identify BM-MSC from the other MSC [104]. Stro-1 was another molecule described to be highly specific for BM CFU-F. However, Stro-1 expression is downregulated during prolonged culture [117]. The function of Stro-1 on MSCs remains largely unknown; in one study, Stro-1 expanded MSCs were reported to have a better homing capacity, compared to expanded Stro-1 MSCs, suggesting its potential role in MSC migration and attachment to extracellular matrix [118].

The antibody Stro-1 was not conjugated with a fluorochrome and for flow cytometry analysis was needed to conjugate with a secondary antibody, FITC goat anti-mouse (Appendix C – Flow Cytometry Plan). Since the secondary antibody was also conjugated with another primary antibodies, and was observed for that antibodies the expression were positive (CD105 and CD49a), it was excluded that something was wrong with the secondary antibody. Or the conjugation with the secondary and primary antibody had some problem, or perhaps the primary antibody did not work properly. The results are discussed on 1.3 Phenotype Analysis.

Comparing the undifferentiated and differentiated BM-MSC, they showed almost identical results, unless for the CD34, CD40, CD45, CD73 and CD86. On the differentiated BM-MSC the CD34, as was expected (Appendix E – Surface Markers Categories), did not show almost any expression (±10%). The expression of the CD73 decreased (±20%), what may indicate that the BM-MSC was starting to lose the MSC phenotype towards SM differentiation. The expression of CD40, CD45 and CD86 increased (±25%) in comparison with the undifferentiated stated of the BM-MSC.
2.3.4 Undifferentiated and Differentiated CV-MSC

As previously discussed on 3.1.2 the undifferentiated CV-MSC showed very similar results when compared with the undifferentiated BM-MSC and UC-MSC. Almost all the markers had similar expression within the three MSC sources (results can be seen on Appendix H – Flow Cytometry Analysis of Different MSC sources), except for CD49a, CD90 and CD146. CD49a and D146 had a higher expression, more ±50% and ±35%, respectively. The CD90, one of the most commonly markers used to identify MSC phenotype, had a smaller expression, with a decreased expression of ±25% when compared with the undifferentiated BM-MSC and UC-MSC.

Comparing the undifferentiated and differentiated CV-MSC, the level of expression is not very significantly but there are some differences in the expression among the markers. CD34, CD40, CD49a, CD73, CD146 and CD166 had a decrease of expression level. Since the CD73, CD146 and CD166 (had a decrease of ±20%) are commonly markers used to characterize MSC phenotype, may mean the cells were losing the MSC phenotype towards SM differentiation. CD34 and CD40 also showed a decrease of expression (±25%) on UC-MSC and BM-MSC when comparing the undifferentiated and differentiated state. On the other hand CD80, CD90 and Stro-1 showed an increase on the level of expression. CD90 should have had a higher expression on the undifferentiated CV-MSC, but showed an increase of 35%. CD80 and Stro-1, had an increase of 10% expression when compared with the undifferentiated CV-MSC, but should have had no expression.

2.3.5 Undifferentiated and Differentiated AD-MSC

Within the AD-MSC marker panel (Figure III.7), some markers were used to compare AD-MSC phenotype to the typical sources of MSC, some to identify origin of the AD-MSC population used since processed lipoaspirate were obtained from a cooperation group. Some markers were the same used to characterize the MSC phenotype of BM-MSC, UC-MSC and CV-MSC. The undifferentiated AD-MSC showed lack of expression for CD14, CD34, CD45 and CD106 (<5%). The markers: CD44, CD90, CD166 and Vimentin (>75%) had a positive expression, the same result observed for the undifferentiated state of the others MSC. In the case of the undifferentiated AD-MSC, CD56 and CD73 were moderately expressed (±40%) and CD105 had lack of expression. Literature addresses the CD56 as consistently not expressed on AD-MSC [61], but for the other MSC, this marker is variably reported [68, 110]. And due to the moderately expression of this marker among the MSC, this result seems to be coherent. For the case of the results obtained for CD73, AD-MSC are heterogeneous cell populations with pluripotent capacity to differentiate into different types of cells, and can be divided in two
subpopulations, CD73+ and CD73− [119]. There are no differences in morphology between them and they both have the ability to differentiate in adipocytes and osteoblasts in vitro. However, CD73+ AD-MSC possesses a higher potential to differentiate into cardiomyocytes than the CD73− AD-MSC [119]. In the case of the CD105, there are some studies that showed that CD105 present no expression, when related to AD-MSC [104].

Regarding the markers only used on AD-MSC, CD13 is the only marker that presented a high expression (>95%). This result is in concordance with the literature since CD13 is usually one of the markers constantly expressed on AD-MSC and up regulated with culture [61]. The undifferentiated AD-MSC had a moderately expression for CD49d and CD16 (±40%). Although CD49d is reported variable in literature [103] CD16 is commonly not expressed on AD-MSC, since it’s a hematopoietic marker [61, 103], this was not observed in the results. For the CD31 and CD29 the AD-MSC had a lack of expression (<3%). CD31 is an endothelial marker, so it’s usually negative [61, 120] but the CD29 is commonly expressed on MSC and consequently AD-MSC [51, 61]. Since CD29 is constantly up regulated in culture [61], perhaps at the time the cells were analysed they were in a passage with lack of expression for this marker.

Comparing the undifferentiated and differentiated AD-MSC, they show almost identical results, unless for the CD73 and CD16. The CD73 had a decrease on level expression (±25%) and CD16 had an increase of level expression (±15%). As discussed above probably the AD-MSC used were CD73 negative. However, the increase of expression for CD16 was not understood.

### 2.3.6 MSC Smooth Muscle Phenotype Characterization

The normal time of differentiation in other cell lineage is usually around 21 days. In this experiment, the smooth muscle cell differentiation followed a published 7-day differentiation protocol [40]. The differentiation was already analysed through IHC and IF, showing inconsistent results on IHC but clear differentiation on IF. Flow cytometry was performed to quantify the percentage of SM markers on undifferentiated and differentiated MSC. Results are depicted on Figure III.8.

As discussed previously, α-SMA is an important marker that confers plasticity to the cells [1] and is mainly positive for SMC. The SMC, used as positive control, show a high expression of α-SMA (±95%) as it was expected, but the remaining cells, either undifferentiated or differentiated MSC and HUVEC, also showed expression for this marker (>50%). This marker was already identified on MSC [1, 112] but the results are irregular. Once the cells start to acquire SMC phenotype, the expression of α-
SMA should be more positive [1], and that’s not what is verified on the results depicted on Figure III.8. The results are more detailed on Appendix H – Flow Cytometry Analysis of Different MSC sources.

SM22α is often adopted to characterize an early SMC phenotype [113]. The SM22α is expressed specifically in adult smooth muscle, and this was not observed in the results. There was expression of this marker on all MSC, either on undifferentiated, either on differentiated MSC (>90%). Although the expected result was pointed out to be negative on undifferentiated MSC and HUVEC and positive on differentiated MSC, some studies show an expression of this marker on MSC [5, 40]. These values showed similar results with the IHC since in all experiments, an expression of SM22α was observed in undifferentiated and differentiated MSC.

<table>
<thead>
<tr>
<th></th>
<th>ADMSC</th>
<th>UCMSC</th>
<th>BMSSC</th>
<th>CVMSC</th>
<th>HUVEC</th>
<th>HUASMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>U</td>
<td>D</td>
<td>U</td>
<td>D</td>
<td>HUVEC</td>
<td>HUASMC</td>
</tr>
<tr>
<td>SM22α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calponin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM-MHC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Calponin has been widely accepted as a late marker for SMC differentiation and has been postulated to function as a regulator of SMC contraction [1]. Moreover, Calponin should be negative for undifferentiated MSC and HUVEC and positive for the differentiated MSC and the positive control. As visible on Figure III.8 the positive control (±95%) had a higher expression of Calponin compared to MSC Appendix H – Flow Cytometry Analysis of Different MSC sources. The only MSC source that had an increase of Calponin during differentiation was BM-MSC (±15%). The remaining MSC had a moderate expression of Calponin (±30%), except for the undifferentiated and differentiated CV-MSC, that had an expression superior a 55%. Calponin expression was sometimes irregular as differentiated MSC expressed a few times a lower level of calponin than undifferentiated MSC. Although the positive control had a higher expression then the other MSC, the Calponin expression was irregular.

SM-MHC is the most important marker for SM characterization, because it’s an essential component of the contractile system [1]. During the IHC and the IF experiments, SM-MHC always got
compromising results and didn’t allowed to take any conclusions. As visible on Figure III.8 the results for SM-MHC are similar to Calponin. SM-MHC had a high expression on the HUASMC (±95%) and a lack of expression on HUVEC. The SM-MHC should presented lack of expression for the undifferentiated MSC be positive for the differentiated MSC. Despite of the positive control showed a positive expression and the and negative control showed negative expression, the different results of expression obtained among the undifferentiated and differentiated MSC, contradicted the results expected and didn’t allowed any type of conclusion Appendix H – Flow Cytometry Analysis of Different MSC sources. The AD-MSC and BM-MSC were the MSC sources that showed an increasing of SM-MHC expression from the undifferentiated to differentiated state (±15%). On the other hand the UC-MSC and CV-MSC showed a moderate expression and when compared to differentiated state the expression of the marker decreased ±20%. The results for SM-MHC expression are also inconclusive at this point.

Interestingly, results obtained for SMC phenotype on flow cytometry and IHC coincide. Some possible explanations for the conflicting results among the MSC, besides the mycoplasma infection, could be due to the donor heterogeneity and MSC source (as previously discussed on 1.6, there are some significant relevance among the MSC sources). The isolation protocol used may also influence the levels and pattern of marker expression [64], and such expression patterns may also vary with different proliferative stage of the cells in culture [121]. The relevance of the observed differences of marker expression during a differentiation has not been properly investigated yet. However, studies suggested the differentiation of the MSC in adipocytes, chondrocytes and osteocytes downregulated the expression of MSC markers, suggesting that the MSC markers that my indeed be markers of the most potent/undifferentiated MSC [122].

There was a slightly pattern when comparing undifferentiated and differentiated MSC. Some commonly expressed MSC markers had a decrease of expression (±25%) from the undifferentiated to the differentiated state. This could indicate the MSC start losing their MSC phenotype towards smooth muscle differentiation. Given the relatively short period applied for differentiation it is clear from the results that longer differentiation periods are advisable in order to test validity and reproducibility of the TGF-β1/BMP4-based protocol used.
Chapter IV – Conclusions and Future Remarks
Chapter IV

Conclusions and Future Remarks

The need to investigate new therapeutic strategies using MSC from different sources has increased in the recent years. Since the MSC from different sources have different therapeutic potential, their surface markers characterization had to be investigated to better understand how the MSC behave. Different studies have identified positive and negative surface markers for MSC with conflicting information about some of the cell surface markers. As the results presented the surface markers vary among the different MSC sources. Although some markers well established by literature, was used to identify the MSC. Cells from the chorionic villi placenta had been successfully isolated and characterized by flow cytometry. In addition FISH analysis demonstrates that the cells isolated from placenta were not of maternal origin. The CV-MSC showed many benefits as an alternative MSC source. Besides the placenta possesses a large reservoir of stem cells, the CV-MSC can be easily obtained by an inexpensive and non-invasive way. Moreover, the controversy associated with the use of CV-MSC can be easily avoided since the ethical and legal considerations are minimal.

Due to the poor replicative capacity of the SMCs, the MSC potential for smooth muscle cell differentiation was explored in this work for using in heart valves therapy. To achieve this goal the MSC were submitted to a combination of growth factors, TGF-β1 and BMP4. The differentiation was analysed through immunohistochemistry, immunofluorescence and flow cytometry. The IHC showed different results within the MSC sources. Any of the smooth muscle markers was consistent and didn’t allow for any conclusion referred to IHC. In contrast, the IF showed very good results. The smooth muscle markers showed lack of expression and positive expression for undifferentiated and differentiated MSC, respectively. CV-MSC and UC-MSC showed a basal expression of SM22α. This phenomenon was already reported in literature, allowing coherent results. The MSC start to acquire smooth muscle cell phenotype when treated with TGF-β1 and BMP4 as shown by immunofluorescence.

The flow cytometry had served to a systematic evaluation of surface markers and quantification of smooth muscle markers of the undifferentiated and differentiated MSC. As the results showed, there was a pattern among the MSC phenotype. All the MSC had a positive expression for the common well-defined MSC markers: CD44, CD90, CD73 and CD105 (>70%) and had a lack of expression for the markers CD14, CD45 and CD106 (<5%). Some differentiated MSC showed a slight variation of the surface markers expression when compared with their undifferentiated state. The variations of expression of the surface markers were not significant and didn’t allow finding any significant pattern
between the MSC. Moreover, the flow cytometry results also didn’t allow any conclusive results about evolution of surface markers during the differentiation. Concerning the quantification of the smooth muscle cell markers the flow cytometry results showed odd results. Although the smooth muscle cell markers showed positive expression for the positive control, the results were not consistent with the undifferentiated and differentiated MSC. The results showed positive expression for almost all conditions. It was expected the differentiated MSC had an increasing of expression for the smooth muscle markers after 7 days, when compared with the undifferentiated MSC, but that was not verified. Although the expression obtained in flow cytometry was similar to the results obtained on IHC, the mycoplasma infection seems to have been a major impact in the markers expression. Despite of the wide experiments and results in this work, it is necessary further research in this field due to the conflicting evidence and inadequate information about several cell surface markers.

To overcome these issues it is necessary to repeat the flow cytometry and IHC experiments. Although the results were not coherent, they show very promising outcomes. The cells should be tested in a late passage without any infection to overcome the advantages presented in the results.

Regarding the CV-MSC, they are barely reported in literature, so more studies have to be done to have a better characterization of this MSC type. The interesting in the CV-MSC should rely on surface markers characterization and their differentiation multilineage potential, because they show to be a very promising alternative source for UC-MSC and BM-MSC.
Chapter V – References
Chapter V
References


Chapter VI – Appendixes
Chapter VI
Appendixes

Appendix A – IF and IHC Experiments Plan
As identified on the pictures, there are well plate 1, 2 and 3. The well plate 1 it has the four MSC from different sources and contains the undifferentiated cells, this means it has the MSC with their basal medium. The well plate 2 has also the four types of different MSC, but contains the differentiated cells. This means in this well plate the cells were submitted to the differentiation protocol, instead of their basal medium, it contains a differentiation medium supplemented with growth factors. The well plate 3 contains the HUASMC, the positive control with their basal medium. All the cells were seeded with a concentration of 25000 cells/well.

Three things to have in attention, first that each column used on the experiment was used to evaluate one different smooth muscle marker, column A with SM-MHC, column B with SM22alpha, column C with Calponin and column D with α-SMA; second, the row highlighted with red was used to background control, to see if the antibody was working properly, It was not added the secondary antibody to that row to see if the primary antibody was specific; third, this three well plates were used for only one staining method, so three well plates for IF and other three for IHC.
Appenidix B – Flow Cytometry Experiments Plan

Like on IF and IHC there were 3 well plates, so the same could be replied on the different methods. The same description for IF and IHC can be applied on cells used for flow cytometry. The well plate 1 had the undifferentiated cells, the different MSC from the different sources and their respective basal medium. The well plate 2 had the differentiated cells, the same cells but they were submitted to the differentiation protocol, instead of their basal medium, it contained a differentiation medium.
supplemented with growth factors. And the well plate 3 had the positive and negative control, HUASMC and HUVEC respectively. The cells were also seeded on a concentration of 25000 cells/well. The red line is only to get a better understand the picture separating the different MSC sources.
## Appendix C – Flow Cytometry Plan

Table C.1 – Flow cytometry plan for undifferentiated and differentiated AD-MSC.

<table>
<thead>
<tr>
<th>Primary AB-(fluorochrome) (amount used)</th>
<th>Secondary AB (amount used)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unstained Cells</strong></td>
<td></td>
</tr>
<tr>
<td>CD34-FITC (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD90-APC (5 µl)</td>
<td></td>
</tr>
<tr>
<td>CD49d-PE (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD56-PE-Cy™7 (5 µl)</td>
<td></td>
</tr>
<tr>
<td>CD14-PerCP-Cy™5.5 (5 µl)</td>
<td></td>
</tr>
<tr>
<td>CD31-FITC (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD73-APC (5 µl)</td>
<td></td>
</tr>
<tr>
<td>CD29-PE (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD44-PE-Cy™7 (5 µl)</td>
<td></td>
</tr>
<tr>
<td>CD45-PerCP-Cy™5.5 (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD105 (Purified) (5 µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>CD13-APC (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD166-PE (20 µl)</td>
<td></td>
</tr>
<tr>
<td>CD16-PE-Cy™7 (5 µl)</td>
<td></td>
</tr>
<tr>
<td>CD106-PerCP-Cy™5.5 (5 µl)</td>
<td></td>
</tr>
<tr>
<td>SM22α (5 µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>Calponin (5 µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>Vimentin (5 µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>α-SMA (5 µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>SM-MHC (5 µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>Primary AB (fluorochrome) (amount used)</td>
<td>Secondary AB (amount used)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Unstained Cells</td>
<td></td>
</tr>
<tr>
<td>CD49a (Purified) (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>CD73-APC (5μl)</td>
<td></td>
</tr>
<tr>
<td>CD166-PE (20μl)</td>
<td></td>
</tr>
<tr>
<td>CD40-PE-Cy™7 (5μl)</td>
<td></td>
</tr>
<tr>
<td>HLA-DR-eFluor450 (5μl)</td>
<td></td>
</tr>
<tr>
<td>Stro-1 (Purified) (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>CD45-APC (20μl)</td>
<td></td>
</tr>
<tr>
<td>CD146-PE (20μl)</td>
<td></td>
</tr>
<tr>
<td>CD106-PerCP-Cy™5.5 (5μl)</td>
<td></td>
</tr>
<tr>
<td>CD83-PE-Cy™7 (5μl)</td>
<td></td>
</tr>
<tr>
<td>CD19-FITC (20μl)</td>
<td></td>
</tr>
<tr>
<td>HLA-ABC-APC (20μl)</td>
<td></td>
</tr>
<tr>
<td>CD275-PE (5μl)</td>
<td></td>
</tr>
<tr>
<td>CD14-PerCP-Cy™5.5 (5μl)</td>
<td></td>
</tr>
<tr>
<td>CD86-V450 (5μl)</td>
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<tr>
<td>CD105 (Purified) (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
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<tr>
<td>CD90-APC (5μl)</td>
<td></td>
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<tr>
<td>CD34-PerCP-Cy™5.5 (20μl)</td>
<td></td>
</tr>
<tr>
<td>CD44-PE-Cy™7 (5μl)</td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
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</tr>
<tr>
<td>vWF-FITC (5μl)</td>
<td></td>
</tr>
<tr>
<td>CD80-PE (20μl)</td>
<td></td>
</tr>
<tr>
<td>CD56-PE-Cy™7 (5μl)</td>
<td></td>
</tr>
<tr>
<td>SM22α (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>Calponin (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>Vimentin (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>α-SMA (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>SM-MHC (5μl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
</tbody>
</table>
Table C.3 – Flow cytometry plan for HUASMC.

<table>
<thead>
<tr>
<th>Primary AB-(fluorochrome) (amount used)</th>
<th>Secondary AB (amount used)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained Cells</td>
<td></td>
</tr>
<tr>
<td>SM22α(5µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>Calponin(5µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>α-SMA(5µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
<tr>
<td>SM-MHC(5µl)</td>
<td>FITC Goat Anti-Mouse</td>
</tr>
</tbody>
</table>
Appendix D – Flow Cytometry Results for a CV-MSC donor

Figure D.1 – Remaining flow cytometry results of the expression of surface markers for a CV-MSC donor (n=3).
### Appendix E – Surface Markers Categories

**Table E.1** – Commonly expressed markers on MSC. The markers in red are variably reported on literature, from being expressed to not being expressed, adapted from [51-54, 66].

<table>
<thead>
<tr>
<th>Category</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesenchymal Markers</strong></td>
<td>CD13, CD29, CD44, CD49a, CD56, CD73, CD90, CD105, CD166, Stro-1</td>
<td>CD40, CD49a, CD56, Stro-1</td>
</tr>
<tr>
<td><strong>Hematopoietic Markers</strong></td>
<td>CD34</td>
<td>CD14, CD16, CD19, CD34, CD45, CD49d,</td>
</tr>
<tr>
<td><strong>Immune Response Markers</strong></td>
<td>HLA-ABC, CD83</td>
<td>CD80, CD86, CD275, HLA-DR</td>
</tr>
<tr>
<td><strong>Endothelial Markers</strong></td>
<td>CD146</td>
<td>vWF, CD31, CD106, CD146</td>
</tr>
</tbody>
</table>
Figure F.1 – SMC-specific proteins. IHC staining of an AD-MSC donor under differentiation conditions during 7 days (n=3).
Figure F.2 – SMC-specific proteins. IHC staining of a CV-MSC donor under differentiation conditions during 7 days (n=3).

Figure F.3 – SMC-specific proteins. IHC staining of an UC-MSC donor under differentiation conditions during 7 days (n=3).
Appendix G – Immunofluorescence MSC Pictures

Figure G.1 – SMC-specific proteins. IF staining of an AD-MSC donor under differentiation conditions during 7 days (n=3).
Figure G.2 – SMC-specific proteins. IF staining of a CV-MSC donor under differentiation conditions during 7 days (n=3).
Figure G.3 – SMC-specific proteins. IF staining of an UC-MSC donor under differentiation conditions during 7 days (n=3). Appendix H – Flow Cytometry Analysis of Different MSC sources.
## Appendix H – Flow Cytometry Analysis of Different MSC sources

Table H.1 – Results of flow cytometry analysis for MSS phenotype characterization of the undifferentiated MSC (n=3) and HUVEC (n=2).

<table>
<thead>
<tr>
<th>AB</th>
<th>HUVEC</th>
<th>Undifferentiated</th>
<th>Undifferentiated</th>
<th>Undifferentiated</th>
<th>Undifferentiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UC-MSC (%expression)</td>
<td>BM-MSC (%expression)</td>
<td>CV-MSC (%expression)</td>
<td>AD-MSC (%expression)</td>
</tr>
<tr>
<td>CD13</td>
<td></td>
<td>99.57±0.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>0.33±0.38</td>
<td>0.14±0.69</td>
<td>0.87±1.03</td>
<td>0.29±0.47</td>
<td>0.95±0.86</td>
</tr>
<tr>
<td>CD16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>31.12±37.49</td>
</tr>
<tr>
<td>CD19</td>
<td>2.91±3.95</td>
<td>6.03±9.18</td>
<td>2.8±4.22</td>
<td>12.95±17.81</td>
<td></td>
</tr>
<tr>
<td>CD29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.21±0.17</td>
</tr>
<tr>
<td>CD31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.26±3.70</td>
</tr>
<tr>
<td>CD34</td>
<td>14.42±20.34</td>
<td>19.58±33.33</td>
<td>15.56±24.42</td>
<td>37.63±33.04</td>
<td>10.84±18.58</td>
</tr>
<tr>
<td>CD40</td>
<td>44.00±13.58</td>
<td>41.10±24.64</td>
<td>39.23±17.8</td>
<td>54.70±31.81</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>98.10±1.27</td>
<td>99.53±0.38</td>
<td>93.20±10.48</td>
<td>99.80±0.26</td>
<td>65.60±47.27</td>
</tr>
<tr>
<td>CD45</td>
<td>0.10±0.13</td>
<td>0.72±1.05</td>
<td>0.62±0.24</td>
<td>0.14±0.18</td>
<td>0.11±0.11</td>
</tr>
<tr>
<td>CD49a</td>
<td>9.39±0.87</td>
<td>33.20±14.19</td>
<td>40.07±18.00</td>
<td>84.23±13.74</td>
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</tr>
<tr>
<td>CD49d</td>
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<td></td>
<td></td>
<td></td>
<td>56.15±49.48</td>
</tr>
<tr>
<td>CD56</td>
<td>42.44±49.72</td>
<td>33.72±41.21</td>
<td>42.97±36.65</td>
<td>52.78±41.05</td>
<td>40.36±52.01</td>
</tr>
<tr>
<td>CD73</td>
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<td>88.53±13.04</td>
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Table H.2 – Results of flow cytometry analysis for MSS phenotype characterization of the differentiating MSC (n=3).

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Table H.3 - Results of flow cytometry analysis for the smooth muscle markers of the undifferentiated and differentiated MSC, HUVEC and HUASMC (n=2).