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Supervisor: Doctor Cláudia Alexandra Martins Lobato da Silva

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“*I knew who I was this morning, but I've changed a few times since then.”*

- Lewis Carroll, Alice's Adventures in Wonderland

RESUMO

O potencial das células estaminais no campo da medicina regenerativa tem vindo a ganhar bastante enfâse, nomeadamente no tratamento de doenças isquémicas. Em particular, as células estaminais/estromais do mesênquima (MSCs), bem como as células progenitoras endoteliais (EPC) têm vindo a gerar bastante interesse devido às suas propriedades angiogénicas. No entanto, um dos problemas correntes neste campo é o facto de a quantidade de células que é possível obter de um organismo ser insuficiente para alcançar o objectivo clínico. Deste modo, ao longo desta tese pretendeu-se compreender como sistemas inovadores para a expansão em larga escala de MSCs se relacionam com o seu potencial angiogénico e também explorar técnicas inovadoras para não só aumentar o número de células angiogénicas progenitoras circulantes (CAPC, enriquecidas em $CD34^+$), mas também aumentar a qualidade da população gerada em relação às suas propriedades pro-angiogénicas.

Foi possível estabelecer com sucesso o *scale-up* da expansão de CAPC tanto derivadas de sangue periférico como de sangue do cordão umbilical, usando sacos de cultura celular e *spinner flasks*, respectivamente. Em ambos os casos foi possível obter não só um maior número total de células, bem como uma modulação da população final para um fenótipo mais endotelial, mostrando um maior potencial angiogénico em comparação com a população inicial.

Foi também estudado o efeito da infusão intracoronária de MSCs expandidas em diferentes plataformas de cultura (estático e dinâmico) na microcirculação de um modelo animal (porco), bem como o seu potencial terapêutico num modelo de enfarte agudo do miocárdio (também em porco). Foi verificada uma disrupção na microcirculação para ambas as células produzidas nos diferentes sistemas, mas sem diferença estatisticamente significante entre si. No entanto, foi provada a segurança da infusão destas células nas doses estudadas, embora não se tendo observado melhorias terapêuticas no tecido isquémico.

Por outro lado, outra população de bastante interesse em terapia celular são as células estaminais/progenitoras hematopoiéticas (HSPCs), que têm as mesmas limitações em termos de número mencionadas anteriormente. Assim sendo, e tendo em conta que as MSCs têm a capacidade de suportar e promover a expansão das HSPCs, estudou-se a capacidade de suporte das MSC derivadas de várias fontes, nomeadamente a medula óssea, o tecido do cordão umbilical, o tecido adiposo e fígado fetal, na expansão das HSPCs ($CD34^+$) derivadas do sangue do cordão umbilical. Foi mostrado que as MSC de diferentes fontes (com excepção do fígado fetal) são capazes de suportar a expansão das HSPCs, sem diferenças significativas. Estes resultados mostram uma vantagem em usar fontes que têm uma acessibilidade imediata e uma obtenção mais fácil em comparação com a medula óssea (BM).

Palavras-chave: Células estaminais do mesênquima (MSCs); Angiogénese; Células angiogénicas progenitoras circulantes (CAPCs); Células estaminais e progenitoras hematopoiéticas (HSPCs); expansão *ex vivo*.

ABSTRACT

Stem cell-based therapies hold a huge promise to regenerate tissues/organs based either on cell engraftment into the target tissue and/or cell's trophic activity. Several types of cells, such as Mesenchymal stem/stromal cells (MSCs) and blood derived circulating angiogenic progenitor cells (CAPCs, enriched for $CD34^{+}$) have been extensively studied for the treatment of ischemic diseases due to their angiogenic potential. However, one of the major hurdles for an effective stem cell-based therapeutic relies on the number of cells that can be retrieved. Hence, the aim of this thesis was to explore how innovative methods for the ex-vivo expansion of MSCs would relate with their angiogenic potential and to develop efficient expansion platforms for CAPCs (CD34+ cells) to obtain clinical relevant numbers with increased angiogenic potentiality.

The expansion of CAPCs derived either from peripheral blood (PB) and umbilical cord blood (UCB) was successfully scaled-up using cell culture bags and spinner flasks, respectively. In both cases, not only higher number of total cells was obtained, but a priming of the cells towards an endothelial lineage commitment, with higher angiogenic potential in comparison with the starting population.

Moreover, it was studied the effect of the intracoronary infusion of MSCs expanded under different platforms (static and dynamic) on the microcirculation of a pig model and further therapeutic effect. It was observed a disruption on microcirculation for cells produced under the different platforms, with no significant difference. However, it was proved the safety of the infusion of these cells for the doses studied although without observable improvements in the ischemic tissue.

Considering that hematopoietic stem/progenitor cells (HSPCs) are a very attractive cell-based therapy for the treatment of hematopoietic diseases, and it is known that MSCs are able to support HSPCs expansion, it was explored how the MSCs derived from different sources (adipose tissue (AT), umbilical cord matrix (UCM), bone marrow (BM), fetal liver (FL)) would

impact the expansion of these cells. It was shown that most of MSC sources (with exception for the FL) were able to support and expand HSPC ex-vivo, with no significant difference. These results show an advantage of using different MSC sources that have ready availability and are easily collected in comparison with the BM counterpart.

Key-words: Mesenchymal stem/stromal cells (MSCs); Angiogenesis; Circulating angiogenic progenitor cells (CAPCs); hematopoietic stem progenitor cells (HSPCs); *ex vivo* expansion.

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LIST OF ABBREVIATIONS

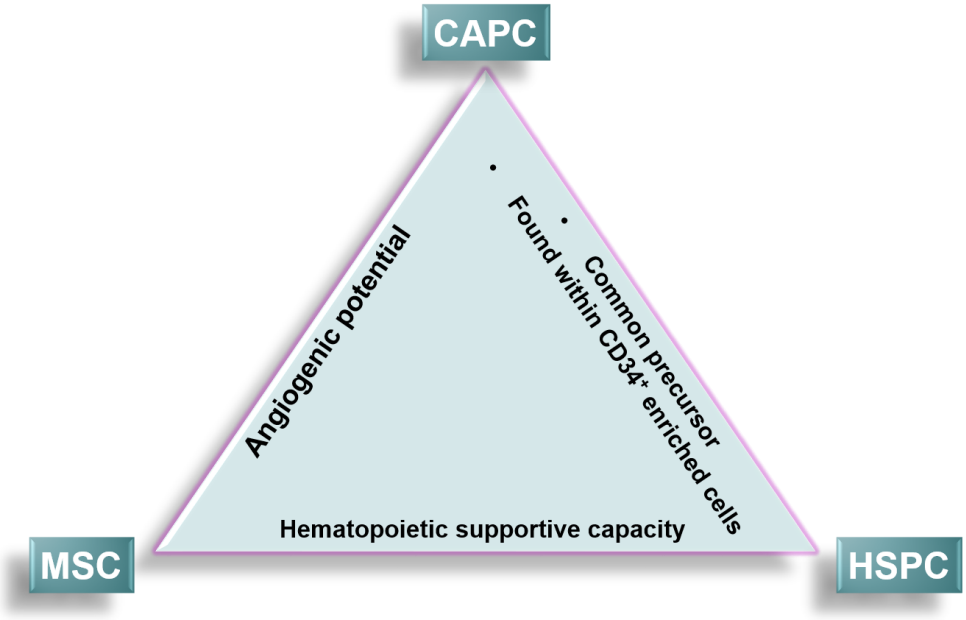
AA	Antibiotic-Antimycotic
AGM	Aortic-gonado-mesonephros
AMI	Acute myocardial infarction
Ang-1	Angiopoietin-1
ANOVA	Analyses of variance
AT	Adipose tissue
bFGF	Basic fibroblast growth factor
BFU-E	Blast-forming unit-erythrocyte
BM	Bone marrow
BOEC	Blood outgrowth endothelial cells
CAC	Circulating angiogenic cell
CAFC	Cobblestone area-forming cells
CAPC	Circulating angiogenic progenitor cells
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
CFU-F	Colony-forming units-fibroblasts
CFU-Mix	Colony-forming units-granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM	Colony-forming units-granulocyte, macrophage
CoGs	Cost of goods
Ct	Threshold cycle
CVD	Cardiovascular diseases
CXCL12	C-X-C motif chemokine 12
DMEM	Dulbecco's Modified Eagle´s Medium
DNA	Deoxyribonucleic acid
ECFC	Endothelial colony forming cells
eEPC	Early endothelial progenitor cell
EGF	Endothelial growth factor
EGM	Endothelial growth medium
EPC	Endothelial progenitor cell
EPC-CFA	Endothelial progenitor cell colony forming assay
ESC	Embryonic stem cells
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FI	Fold increase
FITC	Fluorescein isothiocyanate
FL	Fetal liver
FL1, FL2, etc.	Fluorescence parameter 1, 2, etc., on the flow cytometer
Flt-3	Fms-like tyrosine kinase 3
FSC	Forward Scatter

G-CSF	Granulocyte colony-stimulating factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GMP	Granulocyte/monocyte progenitor
GvHD	Graft-versus-host disease
h	Human
HDR	Homology-directed recombination
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HLI	Hind limb ischemia
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem/Progenitor Cells
HUVEC	Umbilical Vein Endothelial Cells
IBB	Institute for Biotechnology and Bioengineering
IC	Intracoronary
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IGF	Insulin growth factor
IL	Interleukin
IL2RG	Interleukin-2 receptor gamma
eNOS	nitric-oxide synthase
IMR	Index of microcirculatory resistance
iPSC	induced pluripotent stem cells
ISCT	International Society for Cellular Therapy
IST	Instituto Superior Técnico
LVEF	Left ventricular ejection fraction
MAC	Myeloid angiogenic cells
MACS	Magnetic Activated Cell Sorting
MI	Myocardial infraction
miRNA	Micro-RNA
MNC	Mononuclear cells
mPB	Mobilized peripheral blood
MPP	Multipotent progenitor cell
MSC	Mesenchymal stem/stromal cells
oEPC	Late outgrowth endothelial progenitor cell
PAD	Peripheral artery disease
PB	Peripheral blood
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PerCP	Peridinin chlorophyll-A protein
PFA	Paraformaldehyde
QBSF	Quality Biological serum-free medium
RNA	Ribonucleic acid
rpm	Revolution per minute

RT-PCR	Real time- polymerase chain reaction
SCF	Stem cell factor
SCID	X-linked severe combined immunodeficiency
SDF-1	Stromal-derived factor-1
SEM	Standard error of the mean
SFM	Serum free medium
SSC	Side Scatter
TGF-β	Transforming growth factor-β
TNC	Total nucleated cells
TNF-α	Tumor necrosis factor-α
TPO	Thrombopoietin
UC	Umbiical cord
UCB	Umbilical cord blood
UCM	Umbilical cord matrix
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VESC	Vascular endothelial stem cells
XF	Xeno(geneic)-free

AIMS OF STUDY AND THESIS OUTLINE

Stem cell-based therapies hold a huge promise to regenerate tissues/organs based either on cell engraftment into the target tissue and/or cell's trophic activity (i.e. delivery of soluble factors). However, one of the major hurdles for an effective stem cell-based therapeutic relies on the number of cells that can be retrieved that does not match the clinical cell doses needed. Of notice, several types of stem cells, such as circulating angiogenic progenitor cells (CAPCs, where it is also found the endothelial progenitor cells (EPCs)) and mesenchymal stem/stromal cells (MSCs) have been studied as a treatment for ischemic diseases, due to their theoretical capability of promoting the growth and/or differentiate into new blood vessels. Nevertheless, these cell-based treatments have been hindered by the quantity and quality (angiogenic potential) of the cells. Hence, the aim of this thesis was to develop efficient expansion platforms for MSCs and CAPCs (CD34+ cells) to obtain clinical relevant numbers with increased angiogenic potential. Importantly, one of the major concerns for the implementation of a cell-therapy relies on its definition and characterization. For instance, there is no concise definition of EPC. It is known that they originate from the same precursor as hematopoietic stem/progenitor cells (HSPCs), sharing a vast number of immunophenotypic markers and hence, being isolated from CD34+ cells. In addition, considering that HSPCs are a very attractive cell-based therapy for the treatment of malignant and non-malignant hematopoietic diseases, and it is known that MSCs enable the support of HSPC expansion, it was explored how the MSCs derived from different tissue sources would impact the expansion of these cells. The way the different cell types studied in this thesis are interrelated is depicted on **Scheme I-1**.



Scheme I-1 - Schematic representation of the relation present between the different cell types presented in this thesis. CAPC – circulating angiogenic progenitor cell; MSC – mesenchymal stem/stromal cell; HSPC – hematopoietic stem/progenitor cell.

The aims and main results of each chapter are outlined below:

CHAPTER III – *EX* VIVO EXPANSION OF HUMAN CIRCULATING ANGIOGENIC CD34$^+$ PROGENITOR CELLS FROM THE PERIPHERAL BLOOD.

This chapter describes the successful scale-up of the expansion process of circulating angiogenic CD34$^+$ progenitor cells from peripheral blood (PB) by using culture bags, since there is the need to move towards a dynamic and fully controlled bioreactor system in order to meet *good manufacturing practice* (GMP) standards and attain clinically meaningful cell doses in a time- and cost-effective way. The data shows effective expansion of the cultured population (7-fold), while maintaining the stem/progenitor content and increasing the endothelial population. Moreover, post-expanded cells showed higher tube formation capacity compared to pre-expanded cells. In addition, an up-regulation of the anti-inflammatory gene expression and a down-regulation of pro-inflammatory genes were observed, which suggests

that the increase in angiogenic potential is not paired with an increase in the inflammatory profile.

CHAPTER IV – EFFECT OF EXPANDED MESENCHYMAL STEM/STROMAL CELLS ON THE INTRACORONARY MICROCIRCULATION IN A PIG MODEL.

The work developed in this chapter was focused on the expansion of bone marrow mesenchymal stem/stromal cells (BM-MSC) and their immunophenotypic and morphological characterization. This was developed in a collaboration with António Fiarresga, MD, within the scope of his Ph.D. work (António José Fiarresga PhD Thesis, "*Terapia Celular Com Células Mesenquimais Por Via Intracoronária: Contributos Do Estudo Invasivo Da Microcirculação Coronária*", Tese de Doutoramento em Medicina na Especialidade de Investigação Clínica na Faculdade de Ciências Médicas, Universidade Nova de Lisboa. (April 2016)).

This work was divided into 2 phases: the first one focused the study of the intracoronary infusion (IC) of BM-MSC expanded ex-vivo in static conditions on the microcirculation in a healthy pig model; and the second focused on the comparison of the infusion of different sized cells, which were obtained through the expansion under different systems (static, with fetal bovine serum (FBS) containing medium – 'conventional'; dynamic in spinner flasks; with xenogenic-free (XF) medium – 'innovative') on the microcirculation of an induced acute myocardial infarction (AMI) pig model, as well as their therapeutic potential on the myocardial infarcted site regeneration. The microcirculation was evaluated through the index of microcirculatory resistance (IMR) measurement.

The results presented herein are focused on the expansion and characterization of the cells, which was the work developed by the PhD candidate.

MSCs were successfully expanded in static and dynamic conditions, where it was able to achieve the cell number for the animal infusion, with concomitant immunophenotypic characterization and differentiation potential within the parameters established for these cells.

The results of phase one showed that the IMR was significantly higher in MSC-injected animals and intragroup analysis showed a significant increase from baseline to 30 minutes after cell infusion, although no electrocardiographic changes or clinical deterioration were noted. Hence, it was demonstrated the feasibility of using IC delivery to administer culture-expanded human MSC without compromising hemodynamic, electrical stability and epicardial coronary flow.

The results of the second phase showed no significant differences in hemodynamic parameters, epicardial flow and electrocardiographic evaluation during intracoronary infusion for the three tested doses (10×10^6, 15×10^6 and 20×10^6). A significant increase in IMR was observed in animals that received cells, after the second and third doses. IMR was similar between the two groups receiving BM-MSC ('conventional' and 'innovative'). Four weeks after, IMR declined in all groups to values similar to baseline and with no significant differences between them. Myocardial infarction (MI) dimensions and the number of capillaries were similar among groups.

CHAPTER V – DIFFERENT SOURCES OF STROMAL FEEDER LAYERS TO SUPPORT THE EXPANSION OF HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS FROM THE UMBILICAL CORD BLOOD.

This chapter comprises a comprehensive comparison between different sources of MSC focusing on their hematopoietic supportive capacity. Thus, BM MSC (i.e. MSC isolated based on plastic adherence), umbilical cord matrix (UCM) MSC, adipose tissue (AT) MSC, Stro-1 MSC (i.e. MSC isolated based on Stro-1 expression following Magnetic Activated Cell Sorting (MACS) and fetal liver (FL) MSC (also isolated based on Stro-1 expression) were tested as possible feeder layers and a direct comparison amongst them was performed. All feeder layers were able to support HSPC expansion, with exception for the FL-MSC feeder layer, which gave similar results to the *no stroma* control. Although the AT-MSC feeder layer performed better in terms of fold increase of total nucleated cells, that was not observed for the fold increase of total CD34+ progenitor cells, or neither in the fold change of progenitors,

clonogenic forming units (CFUs) and cobblestone forming cells (CAFCs). Overall there was no significant difference between the feeder layers tested in terms of progenitor cell expansion and clonogenic potential, with exception for the FL-MSC that was similar to the *no stroma condition*. This may indicate the possible use of more readily available and accessible MSC sources for to support HSPC expansion ex vivo.

CHAPTER VI – DYNAMIC EXPANSION OF CIRCULATING ANGIOGENIC CD34$^+$ PROGENITORS FROM THE UMBILICAL CORD BLOOD.

The main aim of this chapter was to achieve a scalable system for the manufacturing of these cells while studying the effect of shear stress and oxygen tension in order to enhance their angiogenic potential.

Firstly, it was investigated the differences between two different media, SCGM (Cellgenix), that was used in Chapter III, and StemSpan (StemCell Technologies) (a medium developed by the manufacturer to promote the expansion of human hematopoietic stem and progenitor cells (HSPCs), when combined with proper cytokines) StemSpan medium showed not only to be able to expand the total number of cells at higher extent (57.5 ± 3.6 *vs* 19.8 ± 7.4 at day 7 and 116.2 ± 9.4 *vs* 54.6 ± 17.1 at day 10), but remarkably to keep a higher content of the more primitive population and maintain, or even increase the content on endothelial related markers.

Moreover, it was performed a preliminary study for the scale-up of the expansion of CD34$^+$ cells from umbilical cord blood (UCB) into spinner flasks under serum-free conditions, together with the *in vitro* characterization of the final product, that showed improvement in terms of quantity as well as angiogenic profile comparing with uncultured cells. Overall, it was proven to be able to scale-up the system from 2D culture to spinner-flasks, while maintaining expansion of total number of cells as well as the final population produced assessed by surface markers and their angiogenic potential measured *in vitro*. No significant difference was observed between cultures under atmospheric and hypoxic (5% O_2) conditions.

CHAPTER VII - ALPHA-TESTING OF A NEW PROTOTYPE MEDIUM FOR HEMATOPOIETIC STEM AND PROGENITOR CELL EXPANSION

Herein, the aim of this chapter was to test and compare a new medium formulation not commercially available, medium SP, developed by *Company X* for the expansion of UCB derived HSPC. Hence, static and dynamic conditions (spinner flasks), with and without stroma (BM-MSC) were used for the comparison between the medium and cytokine cocktail previously established in the lab (QBSF + Z9) (Andrade et al., 2010) with this novel SP formulation combined with a specific combination of cytokines. Overall, in both systems, the new medium and cytokine cocktail from *Company X* gave better results in terms of expansion of total cells as well as the progenitor populations and clonogenic potentiality. Interestingly, the combination of this new medium/cytokine cocktail with the system developed in our lab, with stromal feeder layer, presented the higher values of fold increase and clonogenic potential.

CHAPTER I - INTRODUCTION

I.1 BACKGROUND

Stem cells hold a great promise in the regenerative medicine field. This is due to their ability of self-renewal, lineage differentiation and, ultimately, regeneration of a given tissue (Lakshmipathy and Verfaillie, 2005). Concomitantly, these cells have a great potential to be applied as autologous and/or allogeneic therapies for acquired and inherited disorders. However, there is the need to develop and validate an effective isolation, characterization and *ex vivo* manipulation methodologies in order to achieve relevant clinical potential which harnesses quality, quantity and safety (Andrade et al., 2013b).

Roughly, stem cells can be characterized into two main groups regarding their differentiation and potency ability: (i) pluripotent and (ii) multipotent. Pluripotent stem cells have unlimited proliferative capacity and can give rise to any tissue of the three germ layers, endoderm, mesoderm and ectoderm (Passier, 2003). In this subcategory, we can find the Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs). ESCs can be derived from the blastocysts' inner mass, while the iPSC are somatic cells reprogramed to an embryonic like state through the introduction of specific transcriptional factors (Takahashi and Yamanaka, 2006). Although ESCs are attractive for regenerative medicine purposes due to their characteristics, their usage raises several ethical issues due to the destruction of the embryo, whereas iPSCs do not, and offer the possibility to develop patient-specific therapies with minimal probability of immune rejection (Yamanaka, 2010). However, their clinical use is limited by their innate tumorigenicity when injected *in vivo* (e.g., teratoma formation) and difficulty of differentiation control, usually leading to a nonhomogeneous cell population. Hence, further research to overcome these caveats needs to be done to allow the use of these cells in the clinic setting.

On the other hand, multipotent stem cells can be found in adult and neo-natal tissues and do not raise major ethical issues, when comparing with ESCs. These cells have more restricted differentiation potential to the lineage origin of the cell source and limited proliferative capacity

in vitro. They have a pivotal role in normal organ function by promoting homeostasis and tissue repair and they can be found in several tissues as bone marrow (BM), peripheral blood (PB), liver, pancreas, brain, skeletal muscle, skin, lung, gastrointestinal tract, adipose tissue (AT), umbilical cord (UC), and placenta (Mimeault et al., 2007). Indeed, the most common and well established stem cell allogeneic treatment in clinical practice has been with BM transplantation of HSPCS, used since the 1950s (Thomas; et al., 1957). These cells have not only been used as a source for hematopoietic cells, but as well as pro-angiogenic cells, such endothelial progenitor cells (EPCs), for the treatment of ischemic diseases (Asahara et al., 1999). Mesenchymal stem/stromal cells (MSCs) are another example of adult stem cells exploited in experimental clinical studies. However, regardless the cell type employed, the success of such therapies has been hindered by the number of cells needed for an adult patient, as well the variability of the quality of donor cells related with their characteristics (e.g. age, sex, genetic background, etc.). Hence, there is a need to develop bioengineering processes that will allow to reach clinical meaningful numbers, as well as enhancing the therapeutic potential of the cells. Of importance, sources considered as 'biological waste' (e.g. UC and AT) are gaining attention due to their ready availability and ease of collection. Additionally, cells retrieved from neonatal tissues, as the hematopoietic cells from the umbilical cord blood (UCB), have also the advantage of a less stringent HLA-matching to be used in bone marrow transplantation. Still, a full comprehensive characterization and potential of the cells obtained from the different sources is yet to be achieved

I.2 ANGIOGENIC THERAPY

Abnormal angiogenesis is the major cause of numerous diseases; therefore, angiogenesis itself can be useful for diagnostic/prognostic applications and can be manipulated for further clinical applications. In the case of ischemic diseases, which develop due to deficient angiogenesis, an angiogenesis stimulator can be used to induce therapeutic angiogenesis since this promotes blood vessel growth. In that way, the aim is to stimulate blood vessel growth in areas of poor vascularization to support tissue function and recovery.

For instance, acute myocardial infarction (AMI) is a common cardiovascular event and is one of the major death causes in both developed and developing countries with concomitant major causes of disability, loss of productivity and increased health costs worldwide (Reddy et al., 2015). After an AMI left ventricular remodeling occurs, which can develop to congestive heart failure (HF), and terminal heart disease. The number of patients with this disease continues to grow and is associated with an increase of the risk of death (Alaiti et al., 2010). Nonetheless, the only standard therapy that addresses cardiomyocyte loss is through cardiac transplantation, which is hindered by low supply of donor hearts and consequent need for immunosuppression and routinely therapeutic strategies for HF rely mainly on restoration of blood flow or reperfusion (Parsa et al., 2015). Consequently, there is a need for the development of therapies for the myocardial regeneration and prevention of its degeneration.

In this context, a variety of therapies have been explored for the damaged myocardium repair by either de novo cardiomyogenesis or by promotion of neovasculogenesis, by using combinations of cells, biomaterials, cocktails of biochemical factors, and bioengineered cardiac tissues (Chavakis et al., 2010; Perin and Silva, 2011; Yoo and Kwon, 2013).

Neovascularization involves a complex interaction on the 3-dimensional network of the skeletal muscle vasculature, which consists of capillaries and larger blood vessels comprising a variety of cell types that include endothelial cells, smooth muscle cells, pericytes and monocyte/macrophages that interact with the extracellular matrix (**Figure I-1**). Briefly, in response to a variety of angiogenic factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), angiopoietin-1 (ANG1), among others, endothelial cells (ECs) are recruited and are induced to proliferate and migrate via specific cell/receptor matrix interactions. Then, the secretion of proteolytic enzymes by ECs (such as matrix metalloproteinase (MMP) family members) leads to cell-directed degradation and remodeling. The final steps involve lumen formation that leads to a network of tubes and loops (Lawall et al., 2011; Potente et al., 2011; Yoo and Kwon, 2013). Blood vessel growth can occur by three different mechanisms (Watt et al., 2010): (I) Angiogenesis, which is the growth of new capillaries from pre-existing blood vessels; (II) Arteriogenesis, which is defined as the process of growth of collateral arteries, whereby pre-existing arterioles enlarge and remodel into large vessels that bypass the arterial occlusion; (III) Vasculogenesis, which is the *de novo* formation of blood vessels by EPCs and it is believed to occur only during development. However, the incorporation of these progenitor cells into the vessel structures has gained interest in some studies of angiogenic cell therapy (Asahara et al., 1997; Dong et al., 2013; Masuda et al., 2012).

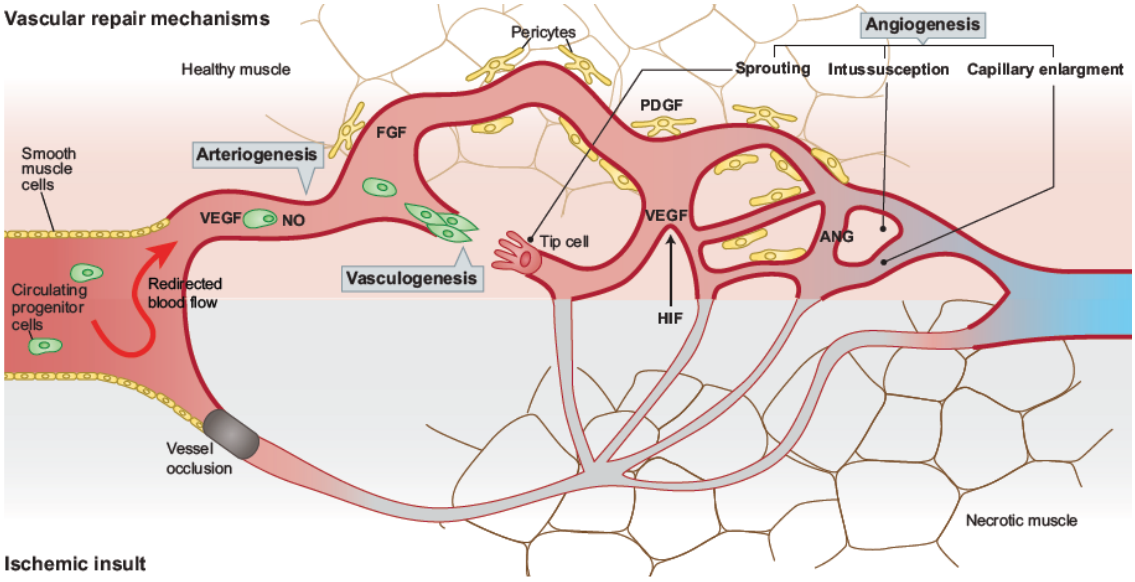


Figure I-1 - *Schematic representation of the agents and microenvironment involved in the vascular repair after ischemia (Dragneva et al., 2013).*

The potential of various cytokines such as VEGF (Kim et al., 2004), viral-based gene therapy approaches (Jazwa et al., 2013), nanoparticle-drug delivery systems (Nagahama et al., 2012) and mononuclear and stem cells (Alaiti et al., 2012; Lee et al., 2013a; Whiteley et al., 2014) have been tested as angiogenic and arteriogenic agents in pre-clinical studies. Moreover, some clinical trials that use angiogenic therapeutic agents, such as cytokines (*e.g.* VEGF) (Kim et al., 2004), and stem/progenitor cells (Assmus et al., 2002; Boyle et al., 2006; Dong et al., 2013), namely Mononuclear Cells (MNCs) (which contain stem/progenitor cells) and EPCs/CAPCs for the treatment of ischemic diseases have been occurring over the past years, but there was an inconsistency in the obtained results. There has been a great deal of speculation as to a lack of consistency in results for stem cell and gene based therapy trials (Singh and Sharma, 2014), but presently, there is no clear consensus and studies are ongoing. Thus, an effective angiogenic therapy for ischemic diseases is not available yet.

I.2.1 CYTOKINE THERAPY

A variety of growth factors that are known to have proangiogenic effects have demonstrated to induce neovascularization. These factors can be administered systematically or locally. Factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) have been used to stimulate stem cell proliferation and promote their release from the BM (Kim et al., 2011), as well as act as a proangiogenic factor by stimulating various populations of BM cells and enhancing regenerative vasculogenesis (Watt et al., 2010). Moreover, systematic administration of cytokines alone, such as VEGF, FGF and HGF, or combinations, has been conducted in clinical trials (Alaiti et al., 2010; Kim et al., 2004). Although appealing, the effect of this system may be hampered by their short half-life in circulation, low targeted uptake efficiency and some severe effects (Grochot-Przeczek et al., 2013).

I.2.2 GENE THERAPY

In order to achieve a more sustained and targeted delivery of angiogenic factors, gene therapy has emerged for the transfer of the genes of these factors directly into the ischemic tissues. Two types of vectors have been used: non-viral (plasmids) and viral (retrovirus, lentivirus) (Jazwa et al., 2013; Suzuki et al., 2015). This system can also be combined with cell therapy in order to improve the survival, proliferative, migratory and angiogenic potential (Grochot-Przeczek et al., 2013).

Pre-clinical studies have displayed optimistic results, but the translation of this system from bench to bedside is hindered by transduction efficiency, choice of route of administration, type of vector and its dose (Grochot-Przeczek et al., 2013).

I.2.3 CELL-BASED THERAPY

Stem cell/progenitor cell-based therapies for ischemic diseases have emerged, given their potential to proliferate, differentiate, and form new blood vessels or to support other cell types involved in the neovascularization process (Alaiti et al., 2010; Perin and Silva, 2011). Initial pre-clinical studies evaluated pluripotent ESC and lineage-committed multipotent stem cells as circulating progenitor cells (CAPCs), which include the endothelial progenitor cells (EPCs). Early clinical trials have suggested some potential benefits of EPC-based therapy in patients with ischemic arterial peripheral or coronary disease (Assmus et al., 2002; Dong et al., 2013; Perin and Silva, 2011).

MSCs have also been explored for the treatment of ischemic diseases. One of the advantages of a MSC-based therapy could lie on the immunomodulatory effects mediated by these cells, as well as the ability of MSC to promote growth, survival or differentiation of other cells in the damaged ischemic area through paracrine mechanisms (Zhang and Xu, 2014). These properties have encouraged pre-clinical and clinical research to explore the potential role of MSC as a cell-based treatment for heart disease (Wei et al., 2013).

I.3 MESENCHYMAL STEM/STROMAL CELLS

MSCs were first described by Friedenstein et al in the 1960s, where they isolated and characterized a sub-population of adherent spindle-shaped cells from the murine bone marrow (BM) able to generate colony-forming unit-fibroblasts (CFU-F) (A. J. Friedenstein et al., 1966). But only later they were nominated as mesenchymal stem cells, popularized by Caplan (Caplan, 1991), due to their progeny from mesoderm and capability to multilineage differentiation. However, due to the population heterogeneity obtained through the isolation process (i.e. based to the ability of cells to adhere to culture plastic) it was further discussed if the cells should be called as stem cells due to their biological

properties. Hence, more recently and to avoid misconception of the nature of the cells, Horwitz and colleagues have proposed to name these cells as multipotent mesenchymal stromal cells, however still maintaining the same acronym, MSC (Horwitz et al., 2005). Besides BM, these cells can be isolated from other tissues including, adipose tissue, umbilical cord matrix, synovial tissue, placenta and amniotic fluid (Hass et al., 2011b).

However, the frequency of these cells in the organism is very low, spanning between 0.001-0.01% of the total nucleated cells in the BM (Bonab et al., 2006), and physiological factors as age and sex contribute to the decrease of cell number (Caplan, 2007).

These cells are very attractive to the regenerative medicine field and, hence extensively studied. Nonetheless, a concise phenotypic characterization is yet to be achieved.

I.3.1 MESENCHYMAL STEM/STROMAL CELL CHARACTERIZATION

The expansion and isolation of MSCs can be obtained with high efficiency *in vitro* (Baksh D et al., 2004; de Soure et al., 2016) and are characterized as adherent, spindle shape cells, upon culture establishment and which can differentiate, under specific culture conditions, into mesodermal cell lineages such as osteoblasts, chondroblasts and adipocytes (**Figure I-2**) (Caplan and Correa, 2011b; Pittenger et al., 1999).

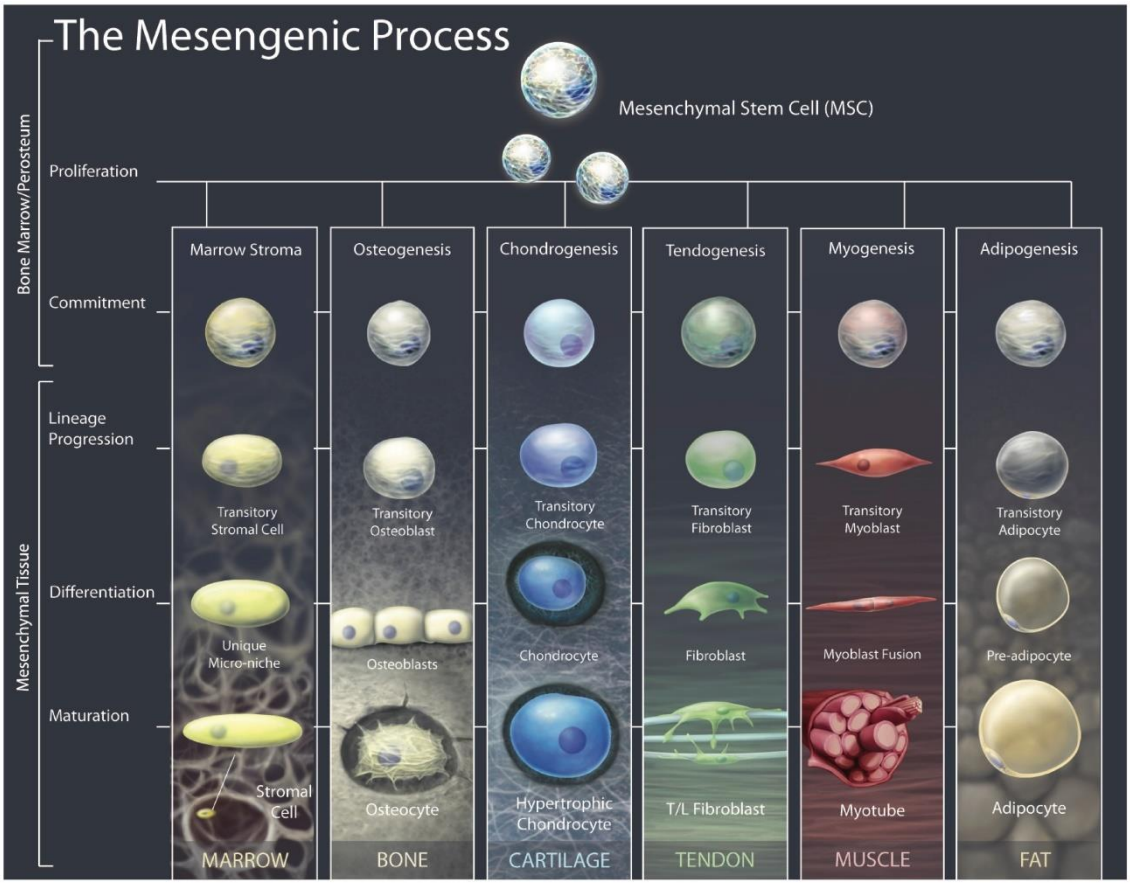


Figure I-2 – MSC differentiation into the different mesodermal lineages: bone marrow stromal cells, bone, cartilage, tendon, muscle and adipocytes. From Caplan 2011 (Caplan and Correa, 2011b).

However, the defining characteristics of these cells have not been consistent due to differences on cell isolation and expansion as well the source where they are obtained. This has been hindering the straight comparison between different works as there is no universally accepted criteria on the definition of MSC. In addition, there is a lack of specific markers for the definitions of these cells. They are characterized by a complex immunophenotypic panel, as they express a wide range of specific markers, such as CD29 (β1-integrin), CD73 (SH3 and SH4), CD105 (SH2), CD44 (HCAM1), CD90 (thy-1), Stro-1, CD271 and CD146 and lack the expression of hematopoietic related markers such as CD45, CD34, CD14 or CD11b, CD33 and CD133 (Baksh D et al., 2004; Chamberlain et al., 2007; Feng-Juan Lv et al., 2014). In order to overcome the limitation of the non-homogeneous population obtained by the isolation of these cells through

plastic adherence, several markers have been proposed as an alternative for isolation of MSCs. Between them, the ones that have received most attention and have been adopted in studies as markers to sort MSCs are Stro-1, CD271, stage-specific embryonic antigen-4 (SSEA-4), and CD146 (Bianco et al., 2013; Feng-Juan Lv et al., 2014; Psaltis et al., 2010). Nonetheless, none of these markers are specific for MSC and their biological role and therapeutic significance is yet to be understood. For instance it is known that although Stro-1 is regarded as an early mesenchymal/stromal precursor marker, namely from BM origin, (Lin et al., 2011), it is not present in all MSCs obtained from different sources (Feng-Juan Lv et al., 2014) and its expression is lost during culture (Psaltis et al., 2010).

To overcome this caveat, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell Therapy (ISCT) have proposed a set of standard criteria for MSC definition in the research field (Dominici et al., 2006): 1) adherence to plastic; 2) specific surface antigen expression assessed by flow cytometry (more than 95% of the MSC population must express CD105, CD73 and CD90 and must lack expression (less than 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class III); 3) multipotent *in vitro* differentiation potential into osteoblasts, adipocytes and chondrocytes, showed by *in vitro* staining.

Recently, Samsonraj and colleagues, proposed an extent of the phenotypic criteria to be used in combination with ISCT minimum standards, which will render an improved MSC potency assessment and potentially establish cell quality prior clinical use (Samsonraj et al., 2015)

I.3.2 MESENCHYMAL STEM/STROMAL CELLS IN REGENERATIVE MEDICINE

Upon the discovery of these cells by Friedenstein and colleagues and the demonstration of their ability of differentiate *in vitro* into several mesodermal lines, it was hypothesized that the regenerative capacity of these cells would be mainly by would migration to sites

of injury, engraft, and differentiation into functional cells, resulting in regeneration of damaged or diseased connective tissues, upon their transfusion. However, results from several studies and clinical trials from the past few decades have challenged this paradigm. Indeed, it has been shown that these cells, throughout *ex vivo* culture, secrete several paracrine factors (Meirelles et al., 2009), which can be divided in six main groups functionally: immunomodulation, anti-apoptotic, angiogenesis, support/expansion/differentiation of other stem cells, anti-scaring and chemoattraction (***Figure I-3***).

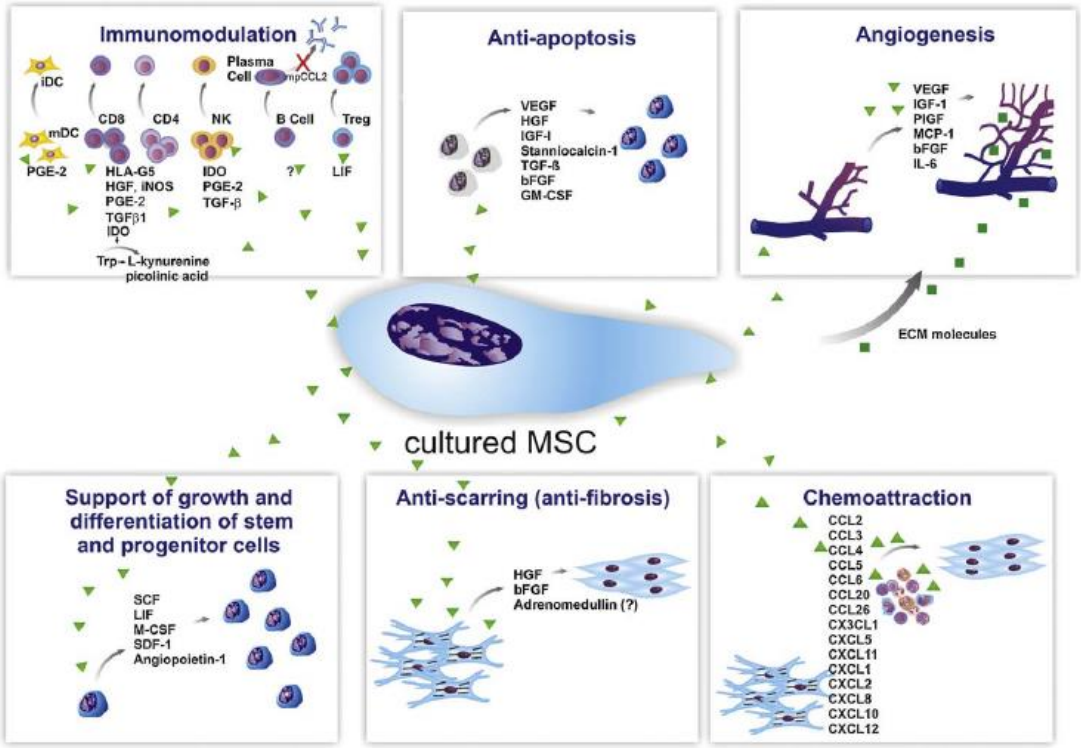


Figure I-3 - *Paracrine effects of cultured MSCs. MSC are able to secrete a broad range of bioactive molecules, which can be divided into six main categories: immunomodulation, anti-apoptosis, angiogenesis, support of the growth and differentiation of local stem and progenitor cells, anti-scarring and chemoattraction. Although the number of molecules known to mediate the paracrine action of MSCs increases every day, several factors that have been shown to be secreted by cultured MSC are depicted here for illustrative purposes. From (Meirelles et al., 2009)*

Upon injury/inflammation, MSCs are known to home to injury site, driven by expression of growth factors, chemokine and extracellular matrix receptors on MSC surface, led by initial localization by chemoattraction along a chemokine gradient, target location

adhesion/docking to vascular endothelial cells, and, ultimately, transmigration across the endothelium to the damage site (Tran and Damaser, 2015). MSCs express a set of cell surface receptors and adhesion molecules shown to be specifically involved in tissue migration and homing processes. For instance, it has been shown *in vitro* the migration stimulation, in a dose dependent fashion, by the pro-inflammatory cytokine tumor necrosis factor (TNF)- α, by increase expression of macrophage derived chemokine (MDC) CCR2, CCR3 and CCR4 receptors (Ponte et al., 2007). Furthermore, as mentioned previously, other mechanisms involved in MSC homing or docking include endothelium attachment. Endothelial cells become activated under injury conditions, being characterized by the expression of surface molecules which allow circulating cells docking. Two of these molecules, vascular cell adhesion molecule 1 (VCAM-1, aka CD106) and E-selectin (CD62E), are known to be ligands for the MSC surface molecules integrin a4/b1 (CD49d/CD29) and CD44, respectively (Ruster et al., 2006; Segers et al., 2006). Therefore, upon infusion, MSC are thought to migrate and exert their regenerative potential by stimulation of endogenous stem/progenitor cells survival and proliferation, remodeling of extracellular matrix, and neovascularization, thus prompting tissue functional recovery (Keating, 2012). This therapeutic effect is mainly due to MSC response to pro-inflammatory cytokines such as (TNF)-α, interleukin (IL)-1, interferon (IFN)-γ, toxins of infectious agents and hypoxia by secretion of a large number of biologically active molecules that exert a paracrine effect on tissue-resident cells. These molecules include epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor(TGF)-β, vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), angiopoietin 1 (Ang-1), stromal cell-derived factor 1 (SDF-1), IL-6, leukemia inhibitory factor (LIF), stem cell factor (SCF) (Meirelles et al., 2009; Segers and Lee, 2008) .

However, it is yet to be depicted if MSC tissue repair needs their persistence locally, either in naive or differentiated state. In this context, some reports have demonstrated

that MSCs disappear from circulation shortly upon intravenous infusion being mostly found in the lungs, liver and spleen, with only few engrafted in the damaged tissue (Eggenhofer et al., 2012; Lee et al., 2009; Sorrell and Caplan, 2010). In fact, recently, Caplan released a statement for the urge of MSC name change to *medicinal signaling cells*, as therapeutically recent evidence is that these cells exert the function through secretion of bioactive molecules, rather than engraftment and differentiation (Caplan, 2017).

I.3.2.1 MSC Immunomodulation

Importantly, other attractive MSC characteristic for advanced cell therapy is their immunomodulatory properties. Of notice, MSC are known to express intermediate levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I molecules and costimulatory CD40, CD80, and CD86 and negligibly low levels of HLA class II (Deans and Moseley, 2000; Dominici et al., 2006; Le Blanc et al., 2003). In addition, it has been shown the ability of allogeneic MSC of inhibiting the proliferation of MHC-mismatched lymphocytes in mixed leukocyte reactions (MLR) in vitro (Le Blanc et al., 2003). MSCs produce a variety of immunomodulatory cytokines including TGF-β1, indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF) and prostaglandin E_2 (PGE-2), which contribute to the ability of MSCs to modulate immune responses, by T cells proliferation and dendritic cell (DC) maturation suppression, B cell activation and proliferation reduction, inhibition of proliferation and cytotoxicity of NK cells, and generation promotion of regulatory T cells (Gao et al., 2016; Meirelles et al., 2009). These discoveries led to the consideration of MSC as *immunoprivileged*, and transplantation between HLA-incompatible individuals is therefore possible (Le Blanc et al., 2003). Hence, MSC have become extremely attractive as candidates for an *off-the-shelf* allogeneic therapy.

Although MSC allogeneic therapy is regarded as safe (Lalu et al., 2012), there has been recent reports that have shown MSC rejection combined with adverse clinical events in

an equine model (Joswig et al., 2017), as well as other animal models, supporting the evidence that allogeneic MSCs are rejected *in vivo* (Berglund et al., 2017). Indeed, recent evidence has regarded MSCs as *immune evasive*, rather than *immunoprivileged* as several studies have shown evidence of mismatched MSC immunogenicity (Ankrum et al., 2014). Hence, further investigation into the immune responses towards allogeneic cells and if immune responses affect the therapeutic outcome of MSC therapy are warranted. In addition, it has been proposed a serial of *in vitro* assays, such as MLR, cytotoxicity assays and ELISPOT, that could depict an immune response by the recipient and thus, adding safety to the therapy (Berglund et al., 2017).

I.3.2.2 MSCs in Angiogenesis

MSCs are also known to facilitate healing of ischemic tissues by the secretion of proangiogenic proteins. Angiogenic factors produced by MSCs include basic fibroblast growth factor (bFGF), VEGF, TGF-β, PDGF, Ang-1, placental growth factor (PGF), IL-6, and monocyte chemotactic protein-1 (MCP-1), HGF, which facilitate tissue regeneration (Meirelles et al., 2009; Tao et al., 2016). Indeed, they have been integrated in several clinical trials for the treatment of ischemic diseases, such as PAD and AMI (Lee et al., 2014; Rodrigo et al., 2013; Tao et al., 2016). As specified in **I.2**., angiogenesis is a complex process that involves an orchestra of different cell types and signaling factors. Hence, MSCs exert their angiogenic ability by promoting resident cell growth, stimulating neovascularization, decreasing apoptosis, reducing inflammation and finally, preventing fibrosis.

On the other hand, it was recently suggested that among soluble factors, extracellular vesicles (EVs) play an important role in intercellular communication and are involved in angiogenesis and that the paracrine functions of MSCs could, at least in part, be mediated by EVs. Moreover, EVs derived from MSCs have shown encouraging therapeutic effects in various animal models (Rani et al., 2015; Teng et al., 2015). This may represent an alternative to whole-cell therapy which may offer specific advantages

for patient safety such as lower propensity to trigger innate and adaptive immune responses and inability to directly form tumors (Yu et al., 2014). Nonetheless, further studies need to be pursued as the therapeutic mechanism of both MSC and MSC-EVs is not clearly understood, and direct comparison of therapeutic potential of the cells *vs* derived EVs should be conducted. Indeed, MSC-mediated immunomodulation is still a matter of debate if the regulatory effects are cell-to-cell contact-dependent, or if, as postulated by most groups, soluble factors are sufficient. The contact dependency of MSC-mediated effects has been much less investigated than the soluble factors effective in immunosuppression, thus warranting further investigation.

I.3.2.3 MSC in Acute Myocardial Infarction

Upon several years of research on cell-based therapies for cardiovascular regeneration, the best cell type as well as administration route to promote angiogenesis is yet to be established. In this context, and due to their homing to injured tissues and angiogenic capabilities by the secretion of paracrine factors as discussed above, MSCs have been widely studied in cardiovascular diseases (CVD) and implemented in several pre-clinical trials which have been shown to engraft and reduce infarct size in rodent and large animal models (Kanelidis et al., 2017; Squillaro et al., 2016; Watt et al., 2013), as well as in clinical trials (reviewed in (Majka et al., 2017)).

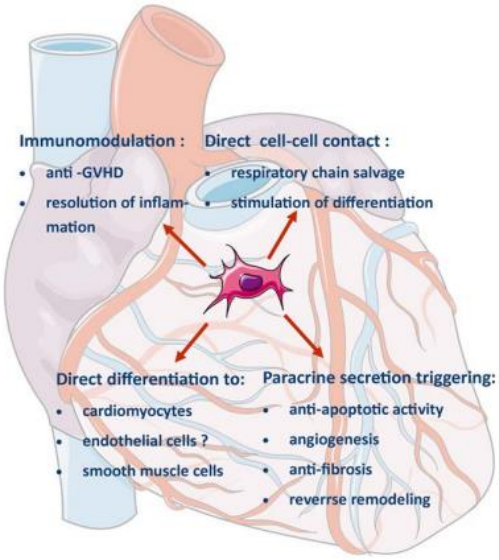


Figure I-4 - Mesenchymal stem/stromal cells mechanisms of action in cardiovascular diseases. Abbreviation: GVHD, graft-versus-host disease. From (Majka et al., 2017)

Despite of the positive results of early-phase trials, MSC-based therapy efficacy has not been uniform throughout the studies. This may be explained by the different isolation and expansion methodologies, administration route, cell dose and effect measurement. Therefore, further research on the regenerative properties of MSCs are required for the establishment of a safe and effective MSC-based therapy for AMI.

Several MSC administration routes have been explored in AMI therapy context, such as intravenous (IV), transendocardial injection (TEI) and intracoronary. (IC) Nonetheless, most of the clinical trials in heart regenerative medicine have used the IC route for cell delivery, due to the ability of delivering a high number of cells into the myocardial region (Dib et al., 2011). However, IC delivery of MSC remains a debate matter, since these cells are larger (10-20 µm) than the heart capillaries (5-10 µm), and therefore the risk of vessel obstruction is increased (Sanganalmath and Bolli, 2013).

Recently, Kanaledis *et al.* performed a meta-analysis to test hypothesis that the route of MSC delivery influences the reduction in infarct size (IS) and improvement in left ventricular ejection fraction (LVEF) in myocardial infarction (AMI) and chronic ischemic cardiomyopathy (ICM) 58 preclinical studies (mouse, rat and swine). They have concluded that MSC therapy improves cardiac function in animal models of both AMI and ICM, with route of delivery playing a role in modulating the efficacy of MSC therapy in AMI swine studies and clinical trials, suggesting the superiority of TEI due to its reduction in IS and improvement of LVEF, which can have important design implications of future studies (Kanelidis et al., 2017).

On the other hand, the mechanism through which these cells exert their regenerative potential (***Figure I-4***) is still a matter of debate. Although differentiation mechanism into myogenic cells have been reported (Pittenger and Martin, 2004), recently there has been several studies criticizing this mechanism for the MSC cardiac therapy (Tao et al., 2016), mainly due to poor survival and low engraftment in the ischemic tissue. Hence, new insights on the therapeutic effect through paracrine action in an AMI setting have been explored, with some reports showing this mechanism *in vivo* (Cai et al., 2016; Yao et al., 2015). Nonetheless, to date, there is a lack of a consensus on the existence of myocardial regeneration or a meaningful clinical benefit of cell therapy in MI context (Behbahan et al., 2015; Leri and Anversa, 2013), hence additional well designed randomized multicenter studies are urged before MSCs treatment can become a therapy of choice for CVD.

Although majority of the studies use BM derived MSCs, some efforts have been made in the use of more readily available MSCs sources. Recently with some clinical trials, showed AT and UCM-MSC administration safety and, in some cases, clinical improvement (Bartolucci et al., 2017; Gao et al., 2015; Musialek et al., 2015; Perin et al., 2014). Nonetheless, further research in order to depict MSCs derived from alternative sources in the regenerative potential in an AMI setting should be chased.

In a mutual relationship to clinical trials, key issues that need to be addressed at the pre-clinical and early clinical stage of MSCs applications involve (i) cell source, (ii) cell dose, (iii) administration route and timing, (iv) expansion methodology (v) rejection reduction or elimination, (vi) development of improved delivery techniques to enhance myocardial retention and engraftment, and (vii) cell engineering and /or preconditioning to enhance regenerative capacities and enhance survival.

I.3.3 ISOLATION AND SOURCES OF MESENCHYMAL STEM/STROMAL CELLS

BM is the first and widest studied and characterized MSC source, which hinder them as the comparison *standard* against other sources. Furthermore, these cells have been successfully isolated from other adult sources, such as AT (Kern et al., 2006), dental pulp (Poltavtseva et al., 2014), and synovial membrane (SM) (Sugita et al., 2016), as well as from perinatal sources such as UCM (Simões et al., 2013), UCB (Sibov et al., 2012), amniotic fluid (Bai et al., 2012) and the placenta (Vellasamy et al., 2012) and fetal tissue as fetal blood, BM and liver (FL) (Campagnoli et al., 2001). Indeed, MSCs can be isolated from almost all postnatal organs including brain, spleen, liver, kidney, lung, thymus and pancreas, suggesting that MSCs are diversely distributed *in vivo* and mainly related to perivascular niches (Klingemann et al., 2008). The focus of this thesis will be on the most studied sources, namely BM, AT and UCM-derived MSC.

Normally, BM samples are firstly subjected to a density gradient centrifugation step using a polymeric solution (e.g. Ficoll-PaquePLUS (1.078 g/mL), Percoll (1.130 g/mL)) to separate MNC fraction from other blood constituents such as erythrocytes and plasma (Gottipamula et al., 2014). This MNC fraction contains, among other cell populations, a small percentage (0.01%) of MSCs which can be further isolated in two different approaches: (i) adherence to plastic (polystyrene) tissue culture flasks or (ii) immunobased cell-sorting methods. The latter method has the advantage of allowing the collection of a more homogeneous cell population, and different markers have been used for such effect, such as Stro-1, CD271, CD105 between others (Feng-Juan Lv et al.,

2014). This later leads to a hypothetical more homogeneous population, but it also presents technical constrains such as is scalability deprived and has a high associated process cost. Hence most of BM-MSC isolation protocols is based on plastic adherence with high yields (100%) (Kern et al., 2006). Thus, MNC fraction is plated onto tissue culture flasks, where morphologically fibroblast-like cells (which are enriched for MSC) adhere to the plastic surface, while other cell contaminants (hematopoietic for e.g.) are washed out through medium change and enzymatic passaging (Chamberlain et al., 2007). However, this source presents several constraints as it is obtained through an invasive procedure which can cause complications to the donor (Baksh et al., 2007), such as infections and chronic pain. Hence, an alternative source such as AT and UCM present several advantages when envisioning clinical MSC therapy.

AT represent an attractive alternative source as can be obtained through liposuction products in large quantities and in a less invasive way (Hass et al., 2011a; Kern et al., 2006). AT-MSCs are obtained by enzymatic treatment of the adipose tissue, usually with collagenase, yielding an initial stromal vascular fraction (SVF) in which MSC isolation is further processed by plating and adherence to plastic (Gimble et al., 2007). Similarly to BM isolation, MSC enriched population adhere to the plastic and other contaminants are washed out by media change and enzymatic passaging. AT MSCs can be isolated in larger numbers than BM (as it is at least 100-fold more frequent that in BM) with high yields (100%) and exhibit stable growth kinetics in culture (Kern et al., 2006).

Finally, UCM is also rated as an attractive MSC source since is widely available, can be harvested with no ethically restrictions (considered as biological waste) and is painless for the donor (Simões et al., 2013). The isolation process of UCM-MSC normally consists on blood vessels removal and dissection of the cord segment into very small pieces (1–3mm^3), followed by enzymatic treatment using collagenase, hyaluronidase, or trypsin dissociation agents (De Bruyn et al., 2011). To present, enzymatic treatment with collagenase is the most broadly used. Alternatively, instead of enzymatic treatment,

isolation can be performed by explants, i. e. by simply growth of the dissected pieces as explants on plastic (Bongso and Fong, 2013; De Bruyn et al., 2011). Importantly, the harvesting yield of MSCs in UCM was found to be significantly higher than BM ($4x10^5$ cells per sample, $10x10^{3–5}$ cells per centimeter of UC) (Can and Karahuseyinoglu, 2007; Troyer and Weiss, 2008).

However, these isolation methods have the drawback that they are usually performed with xenogeinic elements such as use of fetal bovine serum (FBS) containing medium or animal derived dissociation agents, such as collagenase, thus hindering the future clinical application. This concern has been brought to attention and efforts on development of a GMP compliant and scalable method have been pursued (Rojewski et al., 2013; Simões et al., 2013).

Moreover, comparative studies have shown no significant difference between BM, AT and UCM regarding isolation efficiency, morphology and immunophenotyping, which was within the minimal criteria established by ISCT (Kern et al., 2006; Motaln et al., 2010). Nonetheless, when comparing to adult MSCs, UCM-MSCs have shown to have a higher proliferative capacity (shorter doubling times), frequency of colony forming units fibroblasts (CFU-F), and immunosuppressive properties (Baksh et al., 2007; Kern et al., 2006; Simões et al., 2013). However, it has been reported different differentiation abilities for the different sources. BM-MSCs were capable of more robust osteogenic and chondrogenic differentiations, whereas AT-MSCs were described as more prone to differentiate into the myogenic and cardiomyogenic lineages (Bourin et al., 2013). Furthermore, UCM has shown delayed osteogenic and adipogenic differentiation abilities (Kern et al., 2006; Motaln et al., 2010). Moreover, immunophenotypic differences have also been described between the different MSC sources (Chamberlain et al., 2007; Hass et al., 2011a).

Taking these differences into account, it is important to depict if the cells derived from a certain source will have the same clinical benefit. Hence, systematic and comprehensive studies regarding a specific applied therapeutic outcome should be pursued and further generalization of studies to all MSC sources needs to be critically viewed.

I.3.4 EX-VIVO EXPANSION OF MESENCHYMAL STEM/STROMAL CELL

Considering the limited MSCs numbers that can be obtained in most tissue sources with the usual cell doses that have been used in several clinical trials (1-5x10^6 cells per kg body weigh), the efficient *ex vivo* expansion in order to attain, in a cost effective way, clinically meaningful cell numbers with therapeutic properties is critical for the implementation of MSC-based therapies (Hoch and Leach, 2014). Hence, several critical culture parameters such as, plating cell density, cell passage, type of medium and serum used, oxygen tension and cell culture platforms in order to attain maximum yield while maintaining/enhancing cell intrinsic therapeutic potential must be considered (de Soure et al., 2016). However, concomitant with the isolation procedure, most studies have focused on the static 2D MSC expansion in polystyrene flasks, usually using medium formulations containing xenogeneic components, as FBS. FBS, specifically, has been critically rated by regulation agencies due to batch-to-batch variation, be a potential source of harmful contaminants (e.g. virus and prions), and to possibly elicit immune reactions (Jung et al., 2012) Indeed, most of the clinical trials that use expanded MSCs are within these parameters. Importantly, in order to establish a clinical grade cell product, manufacture needs to be GMP compliant.

Considering these factors, it has been an effort in the development of GMP medium formulations (human derived, such as human platelet lysate (hPL) or serum/xeno(geneic)-free) together with innovative 3D expansion methods (microcarrier-based) within fully controlled systems (bioreactors). Indeed, it has been shown successful isolation and MSC culture with the use of HPL as an FBS substitute. In addition, it has been reported a higher proliferative profile upon using the humanized

medium. Nonetheless, although some reports state no morphological, viability, differentiation ability and immunophenotype when compared with traditional FBS containing medium (Hemeda et al., 2014; Juhl et al., 2016), recent studies have demonstrated that hPL-expanded MSC presented diminished immunomodulatory potential (Abdelrazik et al., 2011; Oikonomopoulos et al., 2015). These contradictory results must be due to batch-to-batch variability, which is one of the drawbacks on the use of this supplement.

Furthermore, the ideal candidate for GMP production would be a completely defined serum-free (SF), xeno-free (XF) culture medium formulation. In this context, several companies have developed medium formulations within these characteristics. As an example, our group has previously reported the isolation and expansion of MSC from different sources under static and dynamic systems using Life Technologies™ StemPro® MSC SFM XenoFree culture, without compromising multilineage differentiation potential (Carmelo et al., 2015; Dos Santos et al., 2014; Santos et al., 2011; Simões et al., 2013). Of notice, the MSC isolation using this medium formulation has been somewhat challenging (Chase et al., 2012) and it needs to be taken into consideration that optimal cell culture conditions, including medium used, may vary with cell source and culture system.

In addition, oxygen tension is also another important parameter in cell culture efficiency and cell quality. Indeed, In BM, oxygen concentration seems to be around 0 to 4%, which is a hypoxic environment (Mohyeldin et al., 2010). Local hypoxia can also occur in certain pathological conditions, like ischemia, infection or tissue injury, in which MSCs are believed to have a regenerative impact through their homing capacity and trophic activity (Meirelles et al., 2009). As an example, our group has reported a comparison in proliferative and clonogenic potential of BM-MSCs when cultured at 2% and 21% O_2, which shown significantly higher proliferative and clonogenic potentials for cells under hypoxic conditions (Dos Santos et al., 2010).

I.3.4.1 Scalable *ex vivo* expansion of human MSCs using microcarrier-based systems

As mentioned previously, the establishment of a scalable, cost-effective, and fully controlled manufacturing system is vital for MSC-based therapies. Hence, several types of bioreactor systems have been explored for the scalable expansion of MSCs, including multilayered cell factories, roller bottles, microcarrier-based stirred bioreactors, hollow-fiber bioreactors, and fixed-bed bioreactors (reviewed in (Godara et al., 2008; Rodrigues et al., 2011)).

Beyond the performance of bioreactors for supporting effective growth and maintenance of the cells properties, specific features of different bioreactor types should also be considered from a practical standpoint, namely: simplicity of operation, attainable cell density, disposability, ability to incorporate online monitoring and control, automation, ease of harvest and effectiveness in terms of cost and time.

Specifically, microcarriers increase surface area in comparison with 2D planar systems, providing a very effective and attractive option for the immobilization and cultivation of adherent cells in dynamic culture systems such as stirred bioreactors. Moreover, in addition to a greater scalability, by simply increasing vessel volume, microcarrier-based manufacturing of MSCs offers advantages by avoiding consecutive passaging (Carmelo et al., 2015; Dos Santos et al., 2014).

Importantly, stirred culture systems, such as spinner flasks and stirred bioreactors, promote uniform mixing conditions, avoiding the generation of concentration gradients generally found in the classical planar culture systems (Rodrigues et al., 2011). The mechanical agitation is provided by an internal impeller either controlled by a magnetic stirrer plate in the case of spinner flasks, or by a mechanical shaft in the case of stirred bioreactors. Furthermore, stirred bioreactors comprise *on line* monitoring and control of several culture parameters, such as pH, temperature, dissolved oxygen (DO) and carbon

dioxide levels which require a minimal need for human intervention, representing a critical improvement in what concerns process robustness and cell product standardization. In addition, different feeding regimes (batch, fed-batch, perfusion) can be applied to meet nutrient and metabolic needs, which are highly variable, depending on MSC source and other factors such as culture medium formulation. Importantly, spinner flasks are an important system for the lab-scale expansion development, where several parameters can be studied and optimized before moving towards bioreactor culture, which is usually accompanied by the need to use larger volumes. However, novel fully controlled disposable systems that enable high throughput needed for process development have become recently available, such as the Eppendorf DASBox® and Sartorius Ambr15®.

There are several commercially available microcarriers that have been applied for MSC expansion, both in spinner flasks (reviewed in (de Soure et al., 2016)) and in bioreactors, that present distinctive physical properties, such as stiffness and nanotopography, and can be produced from different of materials such as polystyrene, gelatin, alginate and dextran, and designed to be either non-porous (e.g. SoloHill® Plastic microcarriers), microporous (e.g. Cytodex®-1 (dextran-based)) or macroporous (e.g. CultiSpher®-S (gelatin)). Macroporous microcarriers can have the advantage of potentially providing a higher surface-area for cell growth, while enabling some hydrodynamic shear protection from stirred cultures, although oxygen limitation within the microcarrier may occur (Rodrigues et al., 2011).

To allow functional attachment and growth support, microcarrier surfaces can be functionalized with positively charged groups, gelatin, collagen, other extracellular matrix (ECM) proteins and peptides (*e.g.* RGD peptide). Furthermore, cell attachment can be also promoted by microcarrier coating with attachment factors (for instance, the ones that come along with commercially available XF culture medium formulations) or even by incubation with culture medium prior to cell inoculation (Carmelo et al., 2015; Rafiq et

al., 2016). Hence, microcarrier choice and performance is highly dependent on culture medium chosen, as well as the MSC source, since it has been demonstrated differentially surface marker expression (Hass et al., 2011a).

Interestingly, Rafiq and colleagues have recently reported a comprehensive comparison of several microcarriers (Collagen, Cultispher-G®, Cytodex-1 and 3TM, FACT III, SphereCol®, ProNectin® F, Cytopore 1 and 2^{TM}, Enhanced Attachment, Glass, Hillex®, MicroHexTM, Plastic, Plastic Plus, PVA and Synthemax II®) in the expansion capability of BM-MSC cultured under XF conditions, harvest efficiency, final immunophenotype and differentiation potential. Based on the combination of the aforementioned factors, SoloHill Plastic microcarriers were selected as the optimal microcarriers for BM-MSC expansion capacity (Rafiq et al., 2016).

Furthermore, another important factor to have into consideration in microcarrier-based culture is the harvest efficiency. In a therapeutic setting, it is important not only to achieve the demanded cell numbers, as well as to be able to retrieve the cells without microcarrier contaminants, in the most efficient way, with retention of their properties. Usually this step is done using an enzymatic agent. However, it has been shown that to enzymatic action compromise surface marker expression of cells, which can potentially alter cell function and ultimately viability. In addition, usually this consist in 3 open step processes: dissociation of cells from microcarriers, filtration and volume reduction by centrifugation. Nonetheless, the current process does not offer scalability and might have severe implications in the manufacture of a MSC-based therapy. Hence, some efforts have been made in that direction, by reduction of the exposure to the enzymatic agent in a scalable process (Nienow et al., 2014), where dissociation inside the spinner flask was aided by agitation rate increase, and by applying tangential flow filtration for MSC concentration (Cunha et al., 2015).

- *Shear Stress*

It is important to consider fluid mechanics in stirred systems, that generate shear stress and can affect cell behavior and even induce differentiation (Stolberg and McCloskey, 2009). Agitation leads to transfer of energy from the impeller to the liquid contents of the bioreactor. This energy causes the formation of small areas of intense turbulence throughout the medium, termed eddies, that dissipate the energy. Particle damage under stirred conditions is mostly related to particle-impeller and particle–particle collisions and particle interaction with microscale turbulent eddies. In these cases, damage can proportionally increase with particle size, particle concentration and agitation rate. In addition, shear depends on the geometric characteristics of the vessel and the impeller, the presence of baffles, and physical properties of the medium. Furthermore, direct gas sparging, which is required for adequate oxygenation in large-volume/high cell density may add to shear stress cell damage (Cherry and Papoutsakis, 1988; Nienow, 2006).

Cell damage in microcarrier-based system has been associated to the relation between the size of the microcarriers and the size of Kolmogorov-scale turbulent eddies. It has been reported for fibroblasts, that cell damage would only be significant when eddies' size reached two-thirds (or less) of the microcarriers' size (Croughan et al., 1987). Additionally, this criteria can be translated for MSCs cultures, as reported by Hewitt and colleagues (Hewitt et al., 2011). The smallest eddies, Kolmogorov eddies, generate localized shear on particles suspended in the bioreactor, including the microcarriers surfaces. This damage usually occurs only when the eddies' size becomes smaller than the suspended particles. A turbulent eddy larger than the microcarrier beads can surround and rapidly accelerate a single bead to the eddy's velocity or move groups of beads without creating a large relative velocity between them. Having eddies smaller than the beads, the smallest eddies would not be able to readily accelerate the beads, dissipating their kinetic energy against the cells on the beads surfaces (Nienow, 2006).

Overall, further efforts need to be made to bring this technology to a reality for the robust and reproducible production of MSC. Designing an optimal culture system for a specific cell type depends on understanding the characteristics of the cell and the growth requirements. It is important to note that microcarrier-based dynamic cultures are quite complex systems influenced by a number of important engineering and biological factors most of which are not fully understood.

To achieve a successful, consistent large-scale production of MSC, each of the parameters critical for the microcarrier-based stirred cultures should be identified, optimized, and standardized: (i) cell source; (ii) medium composition; (iii) microcarrier type; (iv) bioreactor geometry; (v) operational parameters (such as DO, pH, temperature, agitation speed); (vi) agitation strategy (continuous/intermittent); (vii) microcarrier and inoculum cell density, (viii) feeding strategy (batch, fed-batch, perfusion), (ix) process intensification (passage, bead-to-bead transfer), (x) downstream processing (dissociation, separation and concentration).

I.4 ENDOTHELIAL PROGENITOR CELLS

Endothelial Progenitor Cells were first isolated from adult peripheral blood (PB) in 1997 (Asahara et al., 1997) and were shown to be able to incorporate into *foci* of physiological or pathological neovascularization (Asahara et al., 1999). These cells can be found within the BM, mobilized peripheral blood (PB) and UCB (Asahara et al., 1997; Ingram et al., 2004; Shi et al., 1998). It has been recognized that the measurement of circulating EPCs levels can provide clinical information on the atherosclerotic burden and even on the future cardiovascular risk (Alaiti et al., 2010).

I.4.1 CELL CHARACTERIZATION

A consensus definition of EPCs is not yet established in the field. EPCs can be characterized into two subsets: hematopoietic (which includes early EPCs (eEPCs)) and non-hematopoietic (which includes late outgrowth EPCs (oEPCs)) (Asahara et al., 2011).

eEPCs are angiogenic in a sense that these can extend vasculature from pre-existing blood vessels, acting through the paracrine signaling. These cells arise from hematopoietic cells that may include monocytes, myeloid progenitor cells, and T lymphocytes and are also characterized by their limited *ex vivo* proliferative capacity. On the other hand, human oEPCs are truly vasculogenic with studies demonstrating that these can form *de novo* human-murine chimeric blood vessels when transplanted into immunodeficient mice (Yoder et al., 2007). Nevertheless, it is believed that both hematopoietic and endothelial lineages come from a common embryological ancestor, the hemangioblast; in adulthood, a common precursor for both cell lineages can potentially persist (Ingram et al., 2004). The exact development of EPCs from the hemangioblast or a precursor to the hemangioblast has not been proved yet. In fact, the exact EPC maturation cycle is still to be revealed.

The qualification of these cells is primarily defined by the expression of cell surface antigens such as CD34 (Hristov et al., 2003; Schmidt-Lucke et al., 2010). Still, due to the controversy in the field regarding the definition of these cells, a variety of surface antigen expression panels have emerged, which usually includes the following: CD34, CD45, CD31, CD146, CD133, KDR. Currently, the phenotypes $CD133^{+}CD34^{+}KDR^{+}$, $CD34^{+}KDR^{+}$, or $CD14^{+}CD34^{low}$, are widely used to define or select cells that express properties attributed to EPC (Hristov et al., 2012; Mund et al., 2012). Moreover, Dimmeler and colleagues have developed, by the study of a group of patients with coronary artery disease (CAD) *vs.* healthy donors, a novel protocol adapted from the standardized so-called ISHAGE protocol for enumeration of hematopoietic stem cells to enable comparison of clinical and laboratory data, which comprises $CD34^{+}KDR^{+}CD45^{dim}$ population as true EPC (Schmidt-Lucke et al., 2010).

In addition, EPCs have also been described as adhesive cells and they have also been identified by clonogenic assays. Similarly to the case of the surface markers, there is a variety of assays available. Hence, a standardization of this type of qualification has also

been pursued by a variety of groups. EPCs can be quantitated by a commercially available kit that identifies ‘’endothelial cell colony-forming units’’ (CFU-ECs), which formed the basis for use of these cells as a predictive biomarker of vascular disease and as a cell source for angiogenic therapies (Hill et al., 2003). However, this method does not have in account the hierarchical discrimination and proper characterization of contaminating cell populations, consisting mainly of hematopoietic cells (Hur et al., 2007; Rohde et al., 2007).

Endothelial colony-forming cells (ECFCs), which are also referred as blood outgrowth endothelial cells (BOECs), have been also identified, which are organized in a hierarchy of progenitor stages that vary in proliferative potential and can be identified in clonal plating conditions (Ingram et al., 2004). Later, Yoder and colleagues compared CFU-ECs and ECFCs and have shown that the later can actually differentiate into ECs and function as EPCs whereas CFU-ECs differentiate into phagocytic macrophages and not ECs (Yoder et al., 2007). Thus, CFU-ECs derive from a hematopoietic progeny committed to the myeloid lineage. This assay demonstrated adhesive endothelial potential at different stages, high- and low-proliferative. However, despite its high reproducibility, it is likely that this does not correlate to a basic EPC phenotype. In this regard, Masuda and colleagues have developed a novel clonogenic assay (EPC-CFA) that enables the identification of primary EPCs of the endothelial lineage in non-adhesive stages derived from HSPCS as ‘blood EPC’. Namely, two types of colonies can be identified: small EPCs, which are a more primitive and proliferative cell population; and large EPCs, which in turn are a more differentiated and mature cell population (Masuda et al., 2011). A schematic representation of the assays is represented in **Figure I-5**.

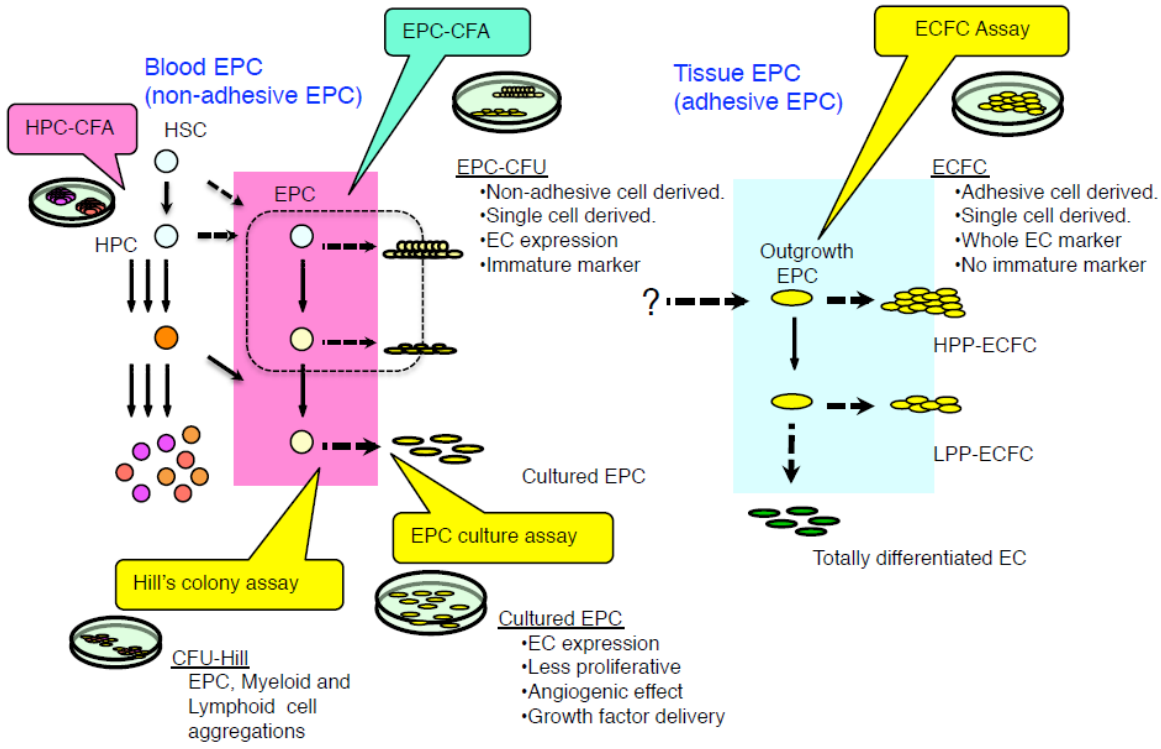


Figure I-5 *– Schematic representation of the developed assays to identify and characterize EPCs. EPC-CFA has the ability to assess EPC differentiation hierarchy in non-adhesive stages derived from HSC as 'blood EPC', in opposite from the other assays, as conventional Hill's colony assay or EPC culture assay. Quantitative evaluation based on the hierarchical proliferative potential cultured adhesive cells of tissue derived EPCs ('tissue EPC'), can be assessed by ECFC assay. Nevertheless, the relationship between 'blood EPC' and 'tissue EPC' remains unknown. From Masuda et al, 2011.*

Apart from the circulating blood-derived EPCs, a variety of tissues have been shown to contain resident stem cells able to differentiate into the endothelial lineage. This was first observed by Aicher and colleagues (Aicher et al., 2007) with mobilization of tissue resident c-Kit^{+}/CD45^{-} progenitor cells from liver and small intestine. Furthermore, other studies have revealed the presence of EPCs in the wall of human adult blood vessels (David A. Ingram and Yoder, 2005; Klein et al., 2010). Interestingly, a recent study by Wakabayashi *et. al.*, it was identified CD157+ as a marker for tissue-resident vascular endothelial stem cells (VESC) in mice with angiogenic potential *in vivo,* which respond to local injury by proliferation and regeneration of entire vasculature, thus supporting the

evidence of existence of an endothelial hierarchy within the blood vessels (Wakabayashi et al., 2018). Furthermore, high proliferative potential ECFCs are enriched in the CD157+CD200+ EC fraction of vascular endothelium and can be prospectively isolated using this marker. This work has significant impact on the controversial field of the existence of tissue derived EPCs.

EPC identification and characterization has been challenging and controversial. The nomenclature for these cells has become increasingly complex, which fail to align with the latest scientific evidence and led to a wide variety of cell types being named as EPC. This has hampered the comparability between published studies and hence advances in the field and clinical translation. An effort to standardize the nomenclature and EPC characterization is being made amongst the scientific community (Fadini et al., 2012). Indeed, Medina and colleagues have recently published a review where they propose precise terminology based on defined cellular phenotype and function (Medina et al., 2017). Here, it is recommended to avoid using the term ‘’hematopoietic EPC’’ and considering adult hematopoietic cells as endothelial progenitors, since they are based in unproved assumptions, as the existence of an ‘’adult hemangioblast’’. Also, regarding the circulating angiogenic cells (CACs) used to define eEPCs, there is no sufficient proof to confirm the ‘’circulating’’ status *in vivo* and, in fact, it has been suggested to be generated *in vitro* through specific culture conditions (Shoji et al., 2004). Therefore, there is the recommendation to define these cells as ‘’myeloid angiogenic cells’’ (MAC), as they not give rise to endothelial cells, remaining true to their hematopoietic nature and having their angiogenic potency through the secretion of paracrine factors (Medina et al., 2011). Finally, regarding the so called BOEC, also obtained *in vitro*, the recommendation is to use the term ECFC, and not EPC due to its ambiguity. A schematic representation of the nomenclature used divided into the two groups, hematopoietic and endothelial, is represented in **Figure I-6**, as a guidance.

Hematopoietic Lineage

- Myeloid angiogenic cells (MAC)
- Circulting angiogenic cells (CAC)
- Collony forming unit-Hill EPC (CFU-Hill)
- Early EPC
- Early outgrowth EPC
- Hematopoietic EPC
- Small EPC
- Myeloid EPC

Endothelial Lineage

- Endothelial colony forming cells (ECFC)
- Outgrowth endothelial cells (OEC)
- Blood outgrowth endothelial cells (BOEC)
- Late EPC
- Late outgrowth EPC
- Non-hematopoietic EPC
- Large EPC

MAC	ECFC
CD45+CD31+CD14+	CD31+CD105+CD146+
CD146-CD34-	CD45-CD14-
Conditioned medium enhances endothelial network formation *in vitro* and *in vivo.*	Intrinsic tube formation capacity *in vitro* and *in vivo.*
Paracrine factors act as angiogenic stimulants	Building blocks for new blood vessel formation or vascular repair.

Figure I-6 *- Cell types studied for their pro-angiogenic properties. Complex nomenclature can be divided into two distinct groups according to phenotypic lineage: hematopoietic and endothelial. Table provides minimal criteria to define MACs and ECFCs based on immunophenotype, including negative markers for purity; and function, assessed as a potency assay. Adapted from (Medina et al., 2017).*

There are two main mechanisms in which EPCs can exert their effect in neovascularization: through direct contribution, where there is *de novo* formation of blood vessels via *in situ* migration, proliferation differentiation and/or incorporation of BM-derived EPCs into regenerating vasculature; and through indirect contribution, by the secretion of trophic factors, promoting proliferation and migration of existing ECs and thus activating angiogenesis (**Figure I-7**) (Asahara et al., 2011). These processes are briefly described in section I.2. However, due to controversial definition and existence for instance of tissue derived EPCs, the underlying mechanism is not yet completely understood (Piatkowski et al., 2013).

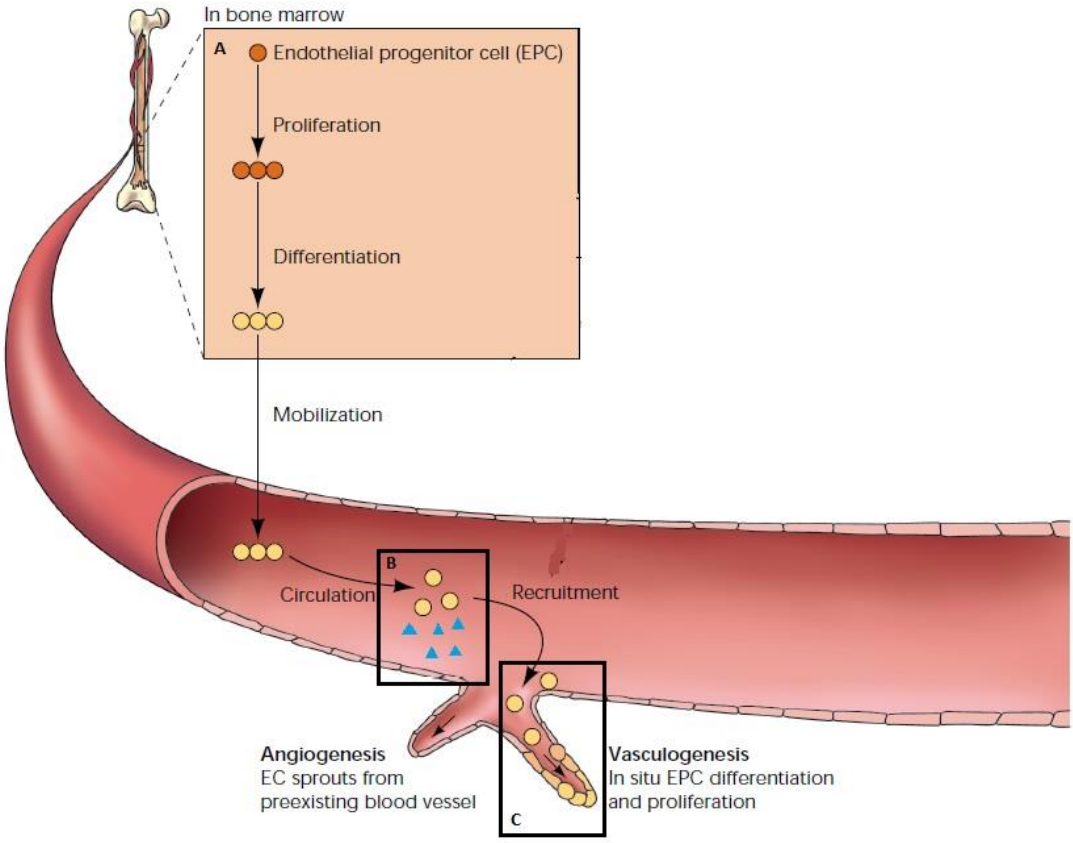


Figure I-7 *- Mechanism of EPC action in neovascularization. A – Circulating EPCs derived from Bone Marrow (BM) are mobilized into peripheral blood to the ischemic site. Participation of EPC in neovascularization can be either through secretion of proangiogenic factors (B) or through integration and differentiation into mature EC (C). Adapted from Musarawa et al., 2004.*

I.4.2 CELL EXPANSION *EX-VIVO*

EPCs can be isolated through $CD34^+$ enrichment (considered as EPC containing cell fraction) using magnetic-activated cell sorting (MACS) (Schmidt-Lucke et al., 2010). However its frequency in peripheral blood is very low (less than 0.1%) limiting the obtained cell numbers (Scheubel et al., 2003). In addition, other aspects as increasing age and disease (such as diabetes and classical cardiovascular risk factors), can affect the angiogenic potential of the cells.(Fadini et al., 2012; Scheubel et al., 2003; Tepper et al., 2010) (Numaguchi et al., 2006). Furthermore, the collection and isolation procedure can represent a physical burden to the patients. To overcome this problem, the use of an allogeneic therapy by retrieving cells from UCB and priming them *ex vivo* may be of an added value, as its collection is non-invasive and the availability is immediate.

In this context, an effective therapeutic product based on $CD34^+$ progenitor cells demands their *ex-vivo* expansion, not only to achieve clinically meaningful cell numbers, but mostly due to the superior quality of cultured cells when compared to freshly isolated $CD34^+$ cells, which has been reported to result in an higher extent of neovascularization after transplantation (Asahara et al., 2011; Kalka et al., 2000). To this end, different EPC-enriched culture methods have been developed.

I.4.2.1 Adherent culture

Adherent culture is done by plating whole MNCs or purified progenitors ($CD34^+$ or $CD133^+$) on either collagen or fibronectin coated plates (Colombo et al., 2013), with an endothelial cell culture medium (*e.g.* endothelial basal medium-2 and endothelial growth medium-2), which contains a variety of combinations of angiogenic growth factors and serum. This method has some associated hurdles such as time, since it takes several weeks to isolate the cells, as well as the animal derived products content (such as Fetal Bovine Serum (FBS)). In that way, several groups have developed some work in order to overcome the use of animal products in the expansion culture. For instance, it has been shown that human platelet lysate (hPL) is a suitable alternative to substitute animal serum (Andreas Reinisch, 2009; Hofbauer et al., 2014). Moreover, Moon and colleagues have also developed an humanized xeno-free autologous culture system for EPCs derived from UCB, with isolation of extracts (UCE) and collagen (UC-collagen) from umbilical tissue to replace the animal-derived counterparts (Moon et al., 2013).

Although these systems have presented a high vasculogenesis performance *in* vivo, several weeks are needed to isolate the cells, and hence, this process is time consuming (Hur et al., 2004) and can further enhance cellular senescence. In addition, cells have to be incubated with dissociation agents (trypsin/EDTA) in order to harvest the cells as the final product, which contributes to the increase of the process time. Thus, adherent culture systems still present challenges when translating to the clinical setting, due to the amount of labor and associated costs.

I.4.2.2 Suspension culture

Alternative suspension culture methods have been developed, where a higher quantity and quality of the final cell product has been claimed (Bouchentouf et al., 2011; Masuda et al., 2012; Masuda et al., 2014; O et al., 2011). In this setting, either isolated progenitor cells ($CD34^+$ or $CD133^+$ cells) or whole MNCs are cultured in suspension in medium containing a variety of cytokines known to promote cell expansion as well the cell priming towards a more angiogenic potential (***Table I-1***). For instance, Masuda and colleagues have developed a novel serum free expansion method with optimal cytokine and growth factors (stem cell factor (SCF), thrombopoietin (TPO), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and flt-3 ligand) of a purified UCB $CD133^+$ population to enhance the therapeutic EPC properties in a quantitative and qualitative manner as after seven days the cell number significantly increased and the cell product showed a differentiation potential towards the endothelial lineage with higher angiogenic and anti-inflammatory properties (Masuda et al., 2012). This system has also been shown similar effects and improved wound healing when using cells from diabetic mice, which may indicate that this system is robust and may facilitate cell-based therapies for diabetic patients in an autologous setting (Tanaka et al., 2013). Moreover, considering the clinical setting and concomitant good manufacturing processes (GMP) for the production of these cells, this system has also shown positive and similar results when culture was performed in disposable cell culture bags (Mata et al., 2015).

Of note, there are some reports demonstrating the benefit of co-culture systems (such as EPC-MSC, CD34+/CD45+, CD34 hybrid cells), as similar to the crosstalk between different cell types that occurs in the niche, that showed a positive effect on cell survival and angiogenic potential (Duttenhoefer et al., 2013; Kwon et al., 2014; Lee et al., 2013a; Whiteley et al., 2014).

***Table I-1**- Methods of EPC expansion culture under suspension conditions to enhance its properties*.

Authors	Cells	Cocktail	Outcome	Reference
Masuda et al.	UCB $CD133^+$	TPO, VEGF, SCF, IL-6, Flt-3 ligand	Preservation of LV function post myocardial infarction	(Masuda et al., 2012)
Masuda et al.	PBMNCs	TPO, VEGF, SCF, IL-6, Flt-3 ligand	Blood perfusion recovery in ischemic hindlimbs	(Masuda et al., 2014)
Eunju O et al.	UCB $CD34^+$	SCF, Flt-3 ligand, IL-3, bFGF	Blood flow and neovascularization recovery in hind-limb ischemia	(O et al., 2011)
Bouchentouf et al.	PBMNCs	IGF-1, VEGF, FGF, EGF	Preserved cardiac function after myocardial infarction	(Bouchentouf et al., 2011)
Janic et al.	UCB $CD133^+$	Flt-3 ligand, TPO	EPC characteristics were preserved in long term culture	(Janic et al., 2010)
Ott et al.	UCB $CD34^+$	SCF, VEGF, SCGFβ	LV improvement after myocardial infarction	(Ott et al., 2005)
Lippross et al.	BM $CD34^+$ and $CD133^+$	bFGF and PRGF	PRGF boost the long term expansion of EPCs	(Lippross et al., 2011)
Schlechta et al.	UCB $CD34^+/CD133^+$	SCF,TPO, Flt-3 ligand, IL-6	Improvement of ejection fraction after myocardial infarction	(Schlechta et al., 2010)
Whiteley et al.	UCB $CD34^+/CD45^+$	FGF4, SCF, Flt-3 ligand	Improvement in blood flow and reduction of foot necrosis in hind-limb ischemia model	(Whiteley et al., 2014)
Senegaglia et al.	UCB $CD133^+$	bFGF, ILF-1, VEGF	Improvement in LV ejection fraction after myocardial infarction	(Senegaglia et al., 2010)

I.4.2.3 Parameters influencing expansion

Resembling the natural niche of EPCs, several studies have been made where the influence of parameters such as oxygen tension and shear stress were explored in the proliferation, differentiation and angiogenic potential of these cells.

Oxygen tension

The original reservoir of EPCs is the adult BM, which means that the physiological microenvironment is under hypoxia (around 1% to 7% of oxygen, compared to atmospheric air -21%). Based on this fact, several studies have studied the impact of this factor on the expansion and final product quality of EPCs (Ceradini et al., 2004; Hung et al., 2007; Lee et al., 2013b) where it has been shown that hypoxia can prevent proliferative senescence, increase the proliferative potential of cultured cells, and their lifespan while maintaining the stem cell properties through the HIF-1α induced TWIST expression. Moreover, a secretion of a variety of proangiogenic factors (such as VEGF, bFGF and placental growth factor) by EPCs has also been shown after hypoxia preconditioning (Akita et al., 2003). Importantly, there is a specific survival ability of EPCs under hypoxia without leading to any aberrant genetic or biological changes (Lee et al., 2013b).

Shear stress

Mechanical forces, such as shear stress and stretch tension, exert an effect over endothelial cells in the vascular system. In particular, shear stress generated by blood and tissue flow plays an important role in angiogenesis and vascular remodeling. Indeed, there is a growing body of evidence that the fate and renewal of EPCs is regulated by shear stress through means of mechanosensitive proteins, as for instance their differentiation into endothelial cells (ECs) and upregulation of endothelial-related markers (Obi et al., 2012; Obi et al., 2009; Yamamoto et al., 2003). The process of shear

stress sensing by ECs is complex, and multiple transduction pathways are activated through membrane molecules and cellular microdomains, ultimately changing shape, function and gene expression (Ando and Yamamoto, 2009; Jaalouk and Lammerding, 2009). Moreover, it has been shown that the shear stress differentiation effect on circulating angiogenic progenitor cells (i.e. in suspension culture) is induced through the PI3K/Akt/mTOR pathway, which regulates several genes known to be related with endothelial cell's response to shear stress (Obi et al., 2012).

Based on this background, several studies have been made where the impact of fluid shear stress forces are investigated in EPC culture. In fact, it has been demonstrated that shear stress increases proliferation, differentiation, adhesion, migration, antipoptosis and vasculogenic potential of both adherent (Abaci et al., 2012; Yamamoto et al., 2003) and circulating phenotype (Obi et al., 2012).

I.4.3 CLINICAL APPLICATIONS OF ENDOTHELIAL PROGENITOR CELLS

A variety of clinical trials are ongoing and trying to clarify the EPC therapeutic effects seen in several pre-clinical studies in animal models of ischemic diseases, where cell populations believed to be EPC enriched are used (Alaiti et al., 2010). Such trials includes ischemic diseases as acute myocardial infarction (AMI) (Assmus et al., 2002; Taljaard et al., 2010), critical limb ischemia (CLI) (Burt et al., 2010; Losordo et al., 2012) and pulmonary arterial hypertension (PAH) (Wang et al., 2007). However, the outcomes of these clinical trials are beyond the expected, considering the optimistic results retrieved from the animal models. Such results can be explained by: 1) lack of standardization of the 'EPC' cells and a variety of surface markers, isolation processes and cell populations are used; 2) a set of different animal models for the same condition is available and usually such models do not take into account the co-morbidities; 3) quality of the cells used when performing an autologous therapy, as these cells decrease their numbers and biological activity with age and disease; 4) cell-dose; 5) route and timing of administration (Alaiti et al., 2010; Asahara et al., 2011; Fadini et al., 2012).

Thus, more controlled and randomized studies need to be performed in order to assess these factors.

Besides their pathophysiological and therapeutic implications, EPCs have also been extensively studied as a novel prototype of cardiovascular risk biomarkers, where it is shown a decrease in the circulation and function of these cells with cumulative cardiovascular risk (Hill et al., 2003).

I.5 HEMATOPOIETIC STEM CELLS

Hematopoietic Stem Cells (HSCs) are the most extensively studied cells in stem cell biology and medicine. These cells are a rare population found in BM, which are able to give rise to all blood cell types throughout adult lifespan, through a process denominated as hematopoiesis (Grove et al., 2004; Wagers and Weissman, 2004). This process of blood-cell production occurs through the orchestrated proliferation, self-renewal and differentiation of HSCs in the BM, followed by circulating blood incoming of mature progeny. Within the bone marrow exists a tightly controlled local microenvironment, or niche, that regulates the quiescence, proliferation and differentiation of HSCs, in order to minimize exhaustion through uncontrolled cell proliferation, thus maintaining the stem cell pool (Mikkola and Orkin, 2006; Orkin and Zon, 2008; Zhang et al., 2003). In fact, it has been shown that in the BM niche, the majority of HSCs are kept in a quiescent state (G_0), but also capable of rapidly respond to stress situations such as infection (Mikkola and Orkin, 2006). Therefore, these cells render an optimal candidate for the treatment of malignant and non-malignant hematopoietic disorders.

The original HSC compartment in humans arises during embryogenesis in a complex process that encompasses multiple anatomical locations during ontogeny (Arora et al., 2014). Although the first blood cells emerge within yolk sac before circulation is initiated,

the definitive long-term engraftment HSCs arise later in the aorta-gonad-mesonephros (AGM) region. At this stage hematopoietic activity can also be detected in the placenta, vitelline and umbilical arteries (Pietras et al., 2011). Furthermore in the development progress, the fetal liver (FL) becomes the main organ harboring hematopoiesis until soon before birth, when HSCs then migrate to the final lifelong primary adult hematopoiesis location, the BM (Mikkola and Orkin, 2006).

Furthermore, accordingly to HSC's self-renewal ability and consequent *in vivo* blood formation supportive capacity, current knowledge reveals that these can be divided into two types: long-term self-renewing HSC (LT-HSC) and short-term self-renewing HSC (ST-HSC). LT-HSC give rise to cells of both lymphoid and myeloid lineages throughout the whole lifespan of the individual, whereas ST-HSC is only able to provide hematopoietic reconstitution for a limited period of time (Doulatov et al., 2012)

I.5.1 HEMATOPOIESIS AND THE BONE MARROW NICHE

Multipotent HSCs differentiate into oligolineage progenitor cells which lose their self-renewal capacity giving rise to mature blood cells (Doulatov et al., 2012) (***Figure I-8***). During the initial step of differentiation, HSCs gradually lose their self-renewing capacity producing a downstream population that still retains the multilineage differentiation potential, the multipotent progenitor cell (MPP) population. MPPs, in turn, become further restricted into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). Myeloid progenitors then generate red blood cells, platelets, and cells responsible for cellular immunity such as macrophages and granulocytes; whereas lymphoid progenitors differentiate into cells that are primarily involved in humoral immunity, including B cells, T cells, natural killer (NK) cells, and dendritic cell lineages. The generation of mature blood cells from multipotent HSC (hematopoiesis) involves a highly regulated progression through successive stages as commitment to a specific cell lineage, terminal differentiation of lineage-restricted progenitor cells, growth arrest and apoptosis (Doulatov et al., 2012). The expression of different receptors on the surface of

hematopoietic progenitors permits the interaction with various regulatory elements present in their environment, which includes stromal cells, extracellular matrix molecules and soluble regulatory cytokines as growth and differentiation factors (Zhang et al., 2003)

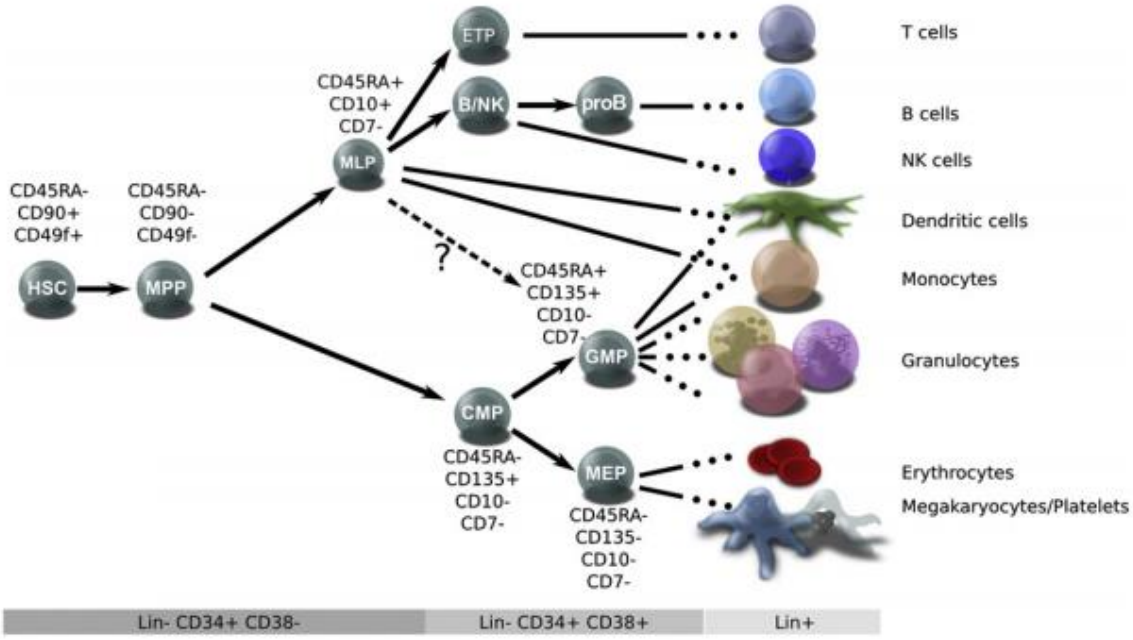


Figure I-8 - *Representation of the human hematopoietic hierarchy lineage determination. Surface markers associated with human HSC and their downstream progeny are presented. HSC: Hematopoietic Stem Cell; MPP: Multipotent Progenitor; MLP: Multi-Lymphoid Progenitor; CMP: Common Myeloid Progenitor; GMP: Granulocyte-Monocyte Progenitor; MEP: Megakaryocyte-Erythroid Progenitor. From (Doulatov et al., 2012)*

The localized presence of the hematopoietic tissue within the protected confines of the bones provides clues to the regulatory interdependence of bone and marrow beyond the obvious advantage to the marrow: a well-shielded location from which it can produce an estimated $500X10^9$ cells per day (Hans-Georg Kopp, 2005). The bone marrow can be subdivided into a hematopoietic cell compartment and the stroma, which is mainly composed of fibroblasts, adipocytes, nerves, and the bone marrow's vascular system.

A large body of evidence suggests that HSCs and hematopoietic progenitor cells (HPCs) are not randomly distributed in the bone marrow but rather are localized close to the endosteum of the bone (endosteal niche) and around blood vessels (perivascular niche)

(***Figure I-9***). Some studies also shown that the spatial distribution of HSCs and HPCs is distinct, depending on their respective level of lineage commitment, which supported the fact of the presence of HSC niches within the endosteal region of the BM as reviewed by B. S. Lam (B. S. Lam, 2010). Nonetheless, differences between both niches in hematopoiesis' regulation remains unknown (Oh and Kwon, 2010)

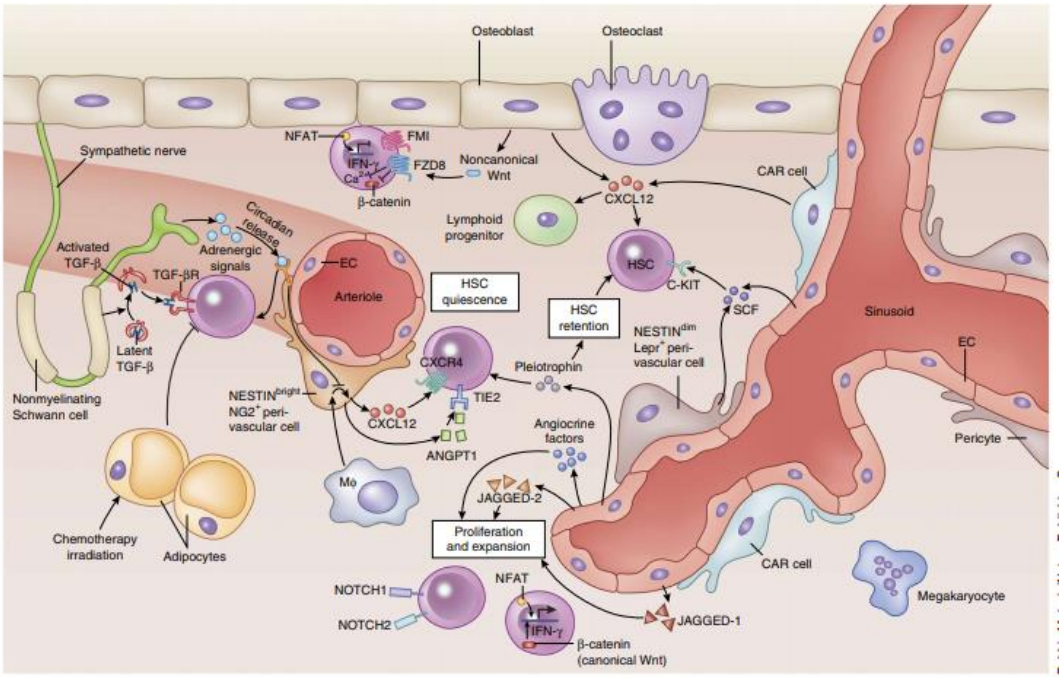


Figure I-9 - *Model illustrating the HSC niche. HSC are preferably located in close proximity with blood vessels under the direct influence of distinct cell types. Endothelial cells and several perivascular cells, including MSC, and CAR cells promote HSC maintenance by producing stem cell factor (SCF) and CXCL12 molecules among others. It is also likely that sympathetic nerves, non-myelinating Schwann cells, macrophages, osteoclasts and hematopoietic progenitors also contribute to HSC maintenance and regulation. From (Mendelson and Frenette, 2014)*

The endosteal region consists on the inner surface of the bone at the interface of bone and BM, and hence, is covered by bone-lining cells that include bone-forming osteoblasts (OB) and bone-resorbing osteoclasts (Kiel and Morrison, 2008). Although still controversial, recent studies have suggested that OB do not directly maintain HSC in the bone marrow (Ding and Morrison, 2013). In the perivascular niche, which is located near sinusoidal endothelial cell-lining blood vessels and adjacent perivascular cell population, it has been suggested that a population of perivascular cells expressing melanoma-

associated cell adhesion molecule (MCAM, also called CD146) are stromal progenitors within the BM (Persichini et al., 2017), Indeed, a subset of these population express specific bioactive molecules, such as platelet-derived growth factor receptor-α (PDGFR-α), CD51 (also called ITGAV) and the intermediate filament protein nestin, which have been later identified as defined as MSCs, giving CFU-F formation ability. In addition to being localized near both HSCs and adrenergic nerve fibers, these cells express high levels of genes mediating HSC maintenance, retention, and homing including those encoding the cytokines chemokine (C-X-C motif) ligand 12 (CXCL12) and SCF, as described previously (I.1). Furthermore, other cell populations can be found in the perivascular niche, such as (CXCL12)-abundant reticular (CAR) cells, leptin-receptor $(LEPR)^{+}$ cells, as well as neuronal and glial cells (Mendelson and Frenette, 2014). In addition to CXCL12 and SCF, further studies have also identified perivascular cells as central components of the HSC niche regulating HSCs through the expression of other bioactive molecules such as fms-related tyrosine kinase 3-ligand (Flt-3) and ANG-1, known to be implicated in HSC quiescence maintenance; and vascular cell adhesion molecule-1 (VCAM-1), which mediates HSC adhesion (Ding and Morrison, 2013; Frenette et al., 2013). In addition, glial fibrillary acidic protein (GFAP)-positive non-myelinating Schwann cells within the sympathetic nerve fibers located around the blood vessels have also been found to promote the TGF-β activation, a factor implicated in the control of HSC dormancy (Riether et al., 2015). Importantly, transplantation studies reported that HSCs preferentially home to the trabecular bone region. However, successful engraftment and subsequent hematopoietic recovery is dependent on the presence of intact vasculature (Morrison and Scadden, 2014). Collectively, these studies suggest that HSC may indeed reside in the perivascular regions, but with a preference for the trabecular region of BM, and perivascular cells are most likely the main regulators of HSCs, whilst the factors near bone surfaces have a more indirect role in HSC regulation (Mendelson and Frenette, 2014).

In addition, important pathways as Notch and Wnt signalling have been recently studied and have shown to an important role in HSC regulation and maintenance. However, the full specific mechanism and how it affects HSC regulation is still yet to be discovered, with some reports showing contradictory results (Mendelson and Frenette, 2014).

Moreover, it has been proposed that HSCs in the BM niche are distributed along an O_2 gradient (***Figure I-10***), with stem cells residing in the most hypoxic areas (0–3% O_2) and proliferating progenitors in higher O_2-content areas (Mohyeldin et al., 2010).

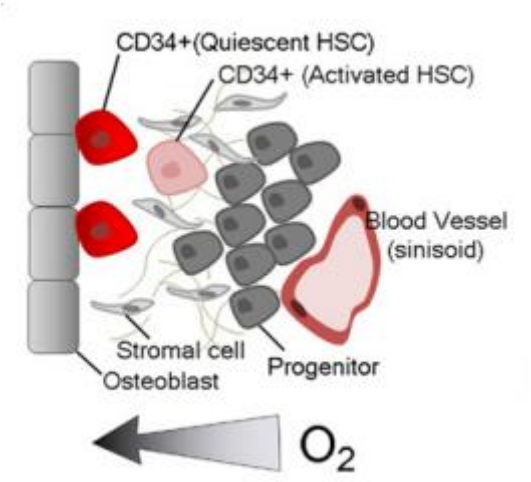


Figure I-10 – *Oxygen gradient within BM hematopoietic niche schematic representation. Quiescent cells are believed to reside in more hypoxic areas whereas proliferating cells reside in higher oxygen-content areas. From (Mohyeldin et al., 2010)*

It is now widely accepted that within the BM are gradients of oxygen from below 1% in hypoxic niches to 6% in the sinusoidal cavity (Eliasson and Jonsson, 2010). These different oxygen tension regulate HSC fate and maintenance/proliferation by an orchestra of several factors and activated pathways. For instance, HIF-1α has emerged as a likely candidate of this regulatory mechanism, as several groups have demonstrated that HIF can mediate cell-cycle arrest in several cell lines. In addition, HSCs present in the hypoxic niche express higher levels of Notch-1, telomerase, and the cell-cycle inhibitor p21 than cells closer to the vasculature (Zhang and Sadek, 2014).

As a result, *ex vivo* expansion/maintenance of hematopoietic stem progenitor cells (HSPCs) under low oxygen tensions (usually called hypoxia or 'in situ normoxia') have been explored, reporting a strong relationship between cycle/quiescence balance of more primitive cells, including long-term repopulating cells, and physiological O2 tensions (0.1–5%) in their environment, both in vitro and in vivo. Indeed, several studies have shown higher expansion yields, with concomitant progenitor maintenance (CD34+, CD34+/CD90+) and higher engraftment capability (Roy et al., 2012; Tiwari et al., 2016). Interestingly, our group has previously reported, using a MSC stromal feeder layer co-culture system, that a 10% O_2 environment was revealed to be superior in UCB CD34+CD90+ cell expansion, mostly due to increased proliferative potential of stem/ progenitor cells, rather than less pronounced differentiation (Andrade et al., 2013a). This is in agreement with the O_2 tensions measured in physiological environments of UCB cells, shown to be 3–5% O2 in the UCB vein (Lackman et al., 2001) and 8–10% O_2 in the full-term placenta (Jauniaux et al., 2000).

I.5.2 HEMATOPOIETIC STEM CELL CHARACTERIZATION

It is common consensus that, in order to successfully expand HSPCs *ex vivo*, these cells have to be previously enriched. Thus, the majority of studies for HSPC expansion/maintenance or differentiation have focus on CD34+ cell enrichment. This is a well-accepted marker, as cells isolated are able to engraftment and to reconstitute the hematopoietic system (Dorrell et al., 2000; Seita and Weissman, 2010). However, by isolating for CD34+, the product obtained is highly heterogeneous comprising of more primitive HSPCs together with more committed progenitors (Notta et al., 2011). Thus, to define and discriminate the most primitive HSPC population, further phenotypic characterization is defined (***Figure I-8***). Hence, primitive hematopoietic cell populations were primarily identified as presenting low expression of the CD90 (Thy1) marker and to not express hematopoietic lineage markers (Lin), CD38 and CD45RA (Doulatov et al., 2012; Sanchez-Dominguez et al., 2012; Seita and Weissman, 2010). More recently,

studies identified CD49f as an additional marker for further purification of primitive HSPC ($CD34^{+}$ Lin^{-} $CD90^{+low}$ $CD38^{-}$ $CD45RA^{-}$ $CD49f^{+}$) (Notta et al., 2011). By contrast, MPP can be isolated by the loss of CD90 and CD49f markers. Moreover, upon hematopoietic differentiation, CD38 and CD45RA expression is gradually acquired. (Doulatov et al., 2012; Sanchez-Dominguez et al., 2012; Seita and Weissman, 2010). In addition, CD133 (formerly known as AC133) is also a widely used marker for primitive HSPC population, especially conjugated with CD34. In fact, CD34+ CD133+ cells are described as early progenitors with a high nonobese diabetic/severe combined immunodeficiency (NOD/SCID) engraftment potential (Li et al., 2005).

Interestingly, the frequency of CD34+CD38– among CD34+ cells is higher in UCB (4%) than in BM (1%), giving further certain that UCB contains a higher proportion of hematopoietic progenitors than BM (Almici et al., 1997). However, this primitive phenotype was shown to be modulated upon culture, thus leading to an erroneous quantification of the primitive cells able to engraft (da Silva et al., 2009a).

However, some studies have revealed the uncertainty as to whether all HSPCsexpress CD34 and whether CD34 expression by human stem cells is a reversible process. Indeed, recent studies shown a high engraftment ability of CD34- cells, and hence CD34+ HSCs are suggested to originate from CD34- HSCs (Takahashi et al., 2014).

I.5.3 HEMATOPOIETIC STEM CELL SOURCES

HSPC properties have rendered them as an attractive source for a wide range of stem cell-based therapies. Indeed, HSPC transplantation has been routinely clinically explored for several years for the treatment of malignant and non-malignant hematological diseases(Dahlberg et al., 2011b; Hofmeister et al., 2007). Sources of HSPCs include BM, mobilized PB, and UCB. Originally, these cells were retrieved from BM. However, granulocyte colony-stimulating factor (G-CSF) mobilized PB appeared as

an alternative to BM collection, which is an invasive procedure. Nonetheless, it has been shown clinically that although PB infusion provide a faster recovery of neutrophils and platelets in comparison to BM, it is also associated with higher risk for of graft-versus-host disease (GvHD) (McAdams et al., 1996).

However, since the first successful UCB transplantation conducted in the late 1980s to treat a patient with Fanconi´s anemia, UCB has emerged as a valuable and attractive alternative (Gluckman, 2009; Passweg et al., 2014). In fact, UCB present several advantages in comparison with the two other sources, namely its ease of access (non-invasive, considered as biological waste), less restringing HLA matching, reduced risk of disease transmission and to contain more primitive cells. (Dahlberg et al., 2011b; Hofmeister et al., 2007).

Nonetheless, considering the therapeutic numbers usually used (a minimal cell dose >$3x10^7$nucleated cells/kg or $2x10^5$ $CD34^+$ cells/kg is recommended), UCB transplantation is usually associated with graft failure due to the low numbers (typically 2-$3x10^8$ total nucleated cells) and primitiveness of cells. (Gluckman, 2009). Therefore, the primary application of UCB-based therapies is has been limited to pediatric recipients (Chou et al., 2010; Passweg et al., 2014). Nevertheless, many efforts have been performed in order to overcome this limitation and increase the number of cells available for transplantation, strategies as double-unit transplantation and intraosseous injection have been explored (Gluckman, 2009). Nevertheless, double cord transplantation might be associated with increased GVHD and might not significantly reduce neutrophil and platelet engraftment time. Hence, interest in expanding HSPCs *ex vivo* for transplantation have urged significant interest (Flores-Guzman et al., 2013).

I.5.4 UMBILICAL CORD DERIVED – HSPC *EX VIVO* EXPANSION

The therapeutic potential of an UCB unit can be enhanced by *ex vivo* expansion, accomplishing by not only increased cell numbers with hematopoietic reconstitution potential (LT-HSC), but also providing a selective expansion of short-term engrafting HSPCs (ST-HSC). Indeed, combination of an expanded graft with 'unmanipulated' fractions of the same cell source unit, would provide more rapid initial hematopoietic reconstitution (expanded cells) and long-term sustainable hematopoiesis (unmanipulated cells) (Shpall et al., 2001). More recently, to mitigate number limitation and in order to improve long and short-term engraftment, a combination of two unrelated UCB units, which one is expanded and the other remains unmanipulated, have shown positive results (Horwitz et al., 2014; Wagner et al., 2016). In addition, this strategy has been applied in several clinical trials as it improves safety (i. e. as a back-up in case expansion fails or interferes with engraftment) and offers the possibility to track both fractions over time and measure of engraftment contribution due to the genetic differences between both units. However, due to the reports of GVHD cases, the relies on producing a product containing sufficient cell numbers while being able to promote long and short-term engraftment (Wagner et al., 2016).

Many procedures have been attempted to expand the HSPC pool, by a rational combination of many factors such as initial stem/progenitor cell enrichments (Andrade et al., 2011), oxygen tension (Andrade et al., 2013a), culture duration (Douay, 2001b), cytokine cocktails (Andrade et al., 2013b; Kumar and Geiger, 2017), blocking in vitro differentiation of early progenitor cells (small molecules) (Zhang and Gao, 2016), and co-culture with stromal feeder layers (Andrade et al., 2010; da Silva et al., 2010a; Jing et al., 2010b).

The most traditional and widely used culture method is by use of a combination of cytokines known to regulate HSPC expansion and maintenance. Throughout the literature it can be found a wide variety of cytokine/supplement combinations (***Table I-2***),

featuring not only the hematopoietic early-acting cytokines SCF and Flt-3L (present in almost all combinations) but also TPO and members of the interleukin (IL) family (reviewed in (Andrade et al., 2013b; Kumar and Geiger, 2017)). In addition, one of the Notch ligands, δ -1, has shown promising expansion of CD34+ cells (phase I clinical trial) (Delaney et al., 2010), as well as culture in the presence of copper-chelators (tetraethylenepentamine, TEPA) (de Lima et al., 2008). Furthermore, A summary of some results regarding UCB derived CD34+ cell expansion upon exposure to different cytokines with and without MSC derived feeder layer is presented on ***Table I-3***

Table I-2 – *Summary of cytokines and other molecules as supplement for ex vivo HSC expansion. Adapted from (Andrade et al., 2013b)*

Cytokines/growth factors	Reported function
Fms-like tyrosine kinase-3 ligand (Flt-3l)	Potentiates the effects of other cytokines; promotes HSC survival
Granulocyte CSF (G-CSF)	Mobilization of HSC to peripheral blood
Interleukin 3 (IL-3)	Together with IL-6, promotes proliferation of HSC
Interleukin 6 (IL-6)	Together with IL-3, promotes proliferation of HSC
Interleukin 10 (IL-10)	Helps proliferation of HSC
Interleukin 11 (IL-11)	Shortens the G0 period of the cell cycle of HSC
Jagged-1	Regulates HSC self-renewal / Notch Ligand
Platelet-derived growth factor (PDGF)	Mitogen for connective tissue cells
Stem cell factor (SCF)	Growth factor for HSC progenitor cells
Thrombopoietin (TPO)	Stimulator of megakaryocytopoiesis, but also of primitive cells
Tetraethylenepentamine (TEPA)	Copper (Cu) chelator; Cu is essential to the proliferation and differentiation of HSCs
δ -1	Enhance self-renewal through Notch system
Stem regenin (SR1)	Antagonist of aryl hydrocarbon receptor; prevents differentiation of HSCs
Angiopoietin 5 (Angp-5)	Unknown; cytokine produced by mouse fetal liver cells, which support the ex vivo expansion of mouse HSCs
Trichostatin A (TSA)	Chromatin-modifying agent promoting HSCs maintenance
5-aza-2' -deoxycytidine (5-azaD)	Chromatin-modifying agent promoting HSCs maintenance
Insulin growth factor binding protein 2	Supports survival and cycling of HSC

Furthermore, as mentioned previously, medium formulation is vital for the lab-to-bedside translation, as it should attain the higher production yield as possible, while maintaining

the desired therapeutic properties, and should be GMP compliant, preferably a chemical defined xeno-free medium and be cost-effective, since this component has heavy influence on the *cost-of-goods* (CoGs). In that sense, it is important to optimize the used cytokine cocktail in a specific system, which can be performed by *Design of Experiments* (DoE) approach, rendering in that way higher productivity with maintained cell properties (Andrade et al., 2010).

I.5.4.1 HSPC co-culture with stromal feeder layers

Stromal cells play an important role in the self-renewal and the maintenance of the multipotency characteristics of HSPC *in vivo* by the secretion of several paracrine factors, as mentioned previously. It has been widely reported the use of BM-MSC as support for HSPC culture (***Table I-3***) (Andrade et al., 2010; da Silva et al., 2010a; Jing et al., 2010b). However, other MSC sources may present attractive advantages as higher availability and ease and non-invasive collection, such as AT-MSCs and UCM-MSCs. Some studies have reported the use of these alternative sources as stromal feeder layers, as AT-MSCs (Corre et al., 2006; Nishiwaki et al., 2012), UCM-MSCs (Bakhshi et al., 2008; Friedman et al., 2007; Tipnis and Viswanathan, 2010), Stro-1 MSCs (Dennis et al., 2002; Goncalves et al., 2006). Nonetheless, results have been in some cases contradictory, which may be due to the different isolation methods and culture formulations used in the studies. For instance, Nishiwaki and colleagues have shown that AT-MSC were able to attain higher fold expansion in total and hematopoietic progenitors derived from peripheral blood than BM-MSC and to facilitate hematopoiesis more effectively in mice (Nishiwaki et al., 2012). On the other hand, Corre and co-workers compared the supportive capacity of BM-MSC and AT-MSC for the maintenance and expansion of UCB derived HSPCs and showed that the latter does not support HSPC self-renewal, but differentiation towards lymphoid and myeloid lineages (Corre et al., 2006). The same was observed by de Toni and colleagues (De Toni et al., 2011). In addition, contradiction is also observed for studies using UCM-MSC derived layer, where

some demonstrate higher potential on HSPC expansion/maintenance than BM (Tipnis and Viswanathan, 2010) and others show moderate efficiency, same or less as BM (Magin et al., 2009).

Table I-3 *- Summary of the main results of umbilical cord blood ex vivo expansion under static conditions, either in liquid suspension cultures or in the presence of mesenchymal stem/stromal-derived feeder layers. Adapted from (Andrade et al., 2013b)*

	Cytokine Cocktail	Serum	Days of Culture	CD34+ fold expansion	Author
Liquid suspension					
	SCF, TPO, G-CSF	No	10	4	(Shpall et al., 2002)
	SCF; Flt-3L; IL-6; TPO; TEPA	10% FCS	21	6	(de Lima et al., 2008)
	Notch ligand d-1; SCF; Flt-3L, IL-6; TPO; IL-3	No	16	160	(Delaney et al., 2010)
	IL-6, TPO, SCF, SR-1, Flt-3L	No	7	24	(Boitano et al., 2010)
	SCF, Flt-3L, TPO, Angpt5	No	10	n.a.	(Zhang et al., 2008)
	SCF, Flt-3L, TPO, IL-3	No	10	12	(Zhang et al., 2006)
	SCF, TPO, Flt-3L, IL-3, G-CSF, GM-CSF and IL-6	No	7	66	(Yao et al., 2006)
	SCF, Flt-3L, MGDF, IL-3	30% FBS	9	2.5	(Araki et al., 2006)
	SCF, Flt-3L, MGDF, IL-3, 5-AzaD, TSA	30% FBS	9	5	(Araki et al., 2006)
	IL-6, TPO, SCF, Flt-3L	10% FBS	21	9.5	(Gunetti et al., 2008)
	SCF, TPO, Garcinol	No	7	7.4	(Nishino et al., 2011)
	SCF, TPO, Flt-3L	No	14	8.9	(Du et al., 2012)
	SCF, Flt-3L, TPO, G-CSF	No	14	80	(Duchez et al., 2012)
	SCF, Flt-3L, TPO, IL-6 (10% O2)	No	8	20	(Tursky et al., 2012)
Stromal co-culture					
BM MSC	SCF, TPO, Flt-3L	No	7	15	(Andrade et al., 2010)
BM MSC	SCF, TPO, G-CSF	20% FBS	14	12	(Robinson et al., 2006)
BM MSC	SCF, Flt-3L, bFGF, LIF	No	26	35	(da Silva et al., 2005)
BM MSC	SCF, Flt-3L, bFGF, LIF	No	18	35	(da Silva et al., 2010)
BM MSC	SCF, IL-3, G-CSF	No	12	21.4	(Madkaikar et al., 2007)
BM MSC	SCF, Flt-3L, TPO, IL-3	No	10	50	(Zhang et al., 2006)
PMSC	SCF. TPO, Flt-3L, TPO	No	14	13	(Luan et al.,)
BM MSC	SCF, TPO, bFGF	No	7	35	(Walenda et al., 2011)

These discrepancies can be explained, as previously referred by the different isolation methods, but also by the medium formulations used and thus, the role of stroma in hematopoietic cell co-cultures remains a very controversial issue Hence, it is needed a systematic and comprehensive comparison between the different MSC-derived feeder

layer sources in order to comprehend the full potential in UCB-HSPC *ex vivo* expansion and maintenance, with concomitant engraftment ability.

Furthermore, AT-MSCs would benefit from the abundance of adipose tissue collection around the world (*e.g.* Liposuction surgeries), which in combination with the high yield of isolation protocols provides a fast, cost-effective source of MSCs. In this context, there are efforts towards the use of human origin, clinical-grade MSCs as feeder layers, prepared under GMP-compliant conditions, to overcome possible contamination risks from compounds of animal origin (*e.g.* Fetal bovine serum).

In addition, this system presents some difficulties regarding the clinical scenario by the risk of cross-contamination with feeder cells. However, the use of MSCs as feeder layer comes with further advantages in that regard, considering a co-transplantation scenario (i.e. as adjuvant) (Kim et al., 2013) and to reduce the incidence of GvHD (Lazarus et al., 2005).

I.5.4.2 Dynamic HSPC culture

Fully controlled bioreactor systems must be the solution towards the implementation of a robust manufacture production for human MSC in a cost-effective way. To achieve this goal, it is required to depict culture conditions that support the continued expansion/ maintenance and controlled differentiation of HSPCs, as choosing the design of scaled-up bioreactor systems that are able of maintaining these specific conditions.

Shear stress applied in the dynamic culture is a major concern for HSPC *ex vivo* expansion, as these cells have been shown to be sensitive to this factor *in vitro*. Interestingly, in a study recently published by Bixel and colleagues, it was possible to monitor HSC engraftment in vivo, as well as to measure the BM shear flow stress that cells are subjected to, which can be from 2.9 dyn/cm^2 in sinusoidal capillaries to 65.5 dyn/cm^2 in arterial vessels (Bixel et al., 2017). Although cells are subjected to high shear stress *in vivo*, *in vitro* behaviour has been shown to be different, possibly due to

molecular signalling and type of shear (flow dynamics) that is not reproducible in those systems.

Critical factors for bioreactor design strategy are partly defined by the cells' response to shear and mixing, the availability of compatible materials for bioreactor design, as well appropriate feeding schemes, featuring nutrients and exogenously added cytokines/growth factors (Csaszar et al., 2013). Bioreactor systems as perfusion, spinner flasks, fluidized bed, fixed bed, airlift, hollow fibers, rotating wall vessel (RWV) and roller bottles have been applied for HSPC expansion (reviewed in (Andrade et al., 2013b; Rodrigues et al., 2011))

I.6 FUTURE PERSPECTIVES AND CHALLENGES

Stem cell-based advanced therapy has become an emergent field to treat diseases that scarce of effective therapy. Efforts on developing innovative and effective expansion systems to meet clinical demand are being made. However, it is mandatory to fully understand the therapeutic properties of the cell-derived product for a specific clinical condition. Systematic comparative studies to address cell source, dosages and administration route needs to be performed to fully depict therapeutic potential. Furthermore, expansion systems have to rely on GMP standards for further translation to the clinic. It is vital to understand how the cells perform under these innovative culture conditions and there is an urge to bring these systems to pre-clinical trials to understand their efficacy. Furthermore, there are still many bioengineering challenges that need to be met, regarding not only cell culture (featuring optimization of the culture parameters affecting cell performance *in vitro* and in *vivo*) but also the bioprocessing (including separation) and scale-up.

I.7 REFERENCES

A. J. Friedenstein, I. I. Piatetzky-Shapiro, and Petrakova, K.V. (1966). Osteogenesis in transplants of bone marrow cells. J Embryol exp Morph *16*, 381-390.

Abaci, H.E., Devendra, R., Soman, R., Drazer, G., and Gerecht, S. (2012). Microbioreactors to manipulate oxygen tension and shear stress in the microenvironment of vascular stem and progenitor cells. Biotechnology and applied biochemistry *59*, 97-105.

Abdelrazik, H., Spaggiari, G.M., Chiossone, L., and Moretta, L. (2011). Mesenchymal stem cells expanded in human platelet lysate display a decreased inhibitory capacity on T- and NK-cell proliferation and function. European journal of immunology *41*, 3281-3290.

Aicher, A., Rentsch, M., Sasaki, K., Ellwart, J.W., Fandrich, F., Siebert, R., Cooke, J.P., Dimmeler, S., and Heeschen, C. (2007). Nonbone marrow-derived circulating progenitor cells contribute to postnatal neovascularization following tissue ischemia. Circulation research *100*, 581-589.

Akita, T., Murohara, T., Ikeda, H., Sasaki, K.-i., Shimada, T., Egami, K., and Imaizumi, T. (2003). Hypoxic Preconditioning Augments Efficacy of Human Endothelial Progenitor Cells for Therapeutic Neovascularization. Laboratory Investigation *83*, 65-73.

Alaiti, M.A., Ishikawa, M., and Costa, M.A. (2010). Bone marrow and circulating stem/progenitor cells for regenerative cardiovascular therapy. Translational research : the journal of laboratory and clinical medicine *156*, 112-129.

Alaiti, M.A., Ishikawa, M., Masuda, H., Simon, D.I., Jain, M.K., Asahara, T., and Costa, M.A. (2012). Up-regulation of miR-210 by vascular endothelial growth factor in ex vivo expanded CD34+ cells enhances cell-mediated angiogenesis. Journal of cellular and molecular medicine *16*, 2413-2421.

Almici, C., Carlo-Stella, C., Wagner, J.E., Mangoni, L., Garau, D., and Rizzoli, V. (1997). Biologic and phenotypic analysis of early hematopoietic progenitor cells in umbilical cord blood. Leukemia *2143-2149*.

Ando, J., and Yamamoto, K. (2009). Vascular mechanobiology: endothelial cell responses. Circ J *73*, 1131-1135.

Andrade-Zaldivar, H., Santos, L., and De Leon Rodriguez, A. (2008). Expansion of human hematopoietic stem cells for transplantation: trends and perspectives. Cytotechnology *56*, 151-160.

Andrade, P.Z., da Silva, C.L., dos Santos, F., Almeida-Porada, G., and Cabral, J.M. (2011). Initial CD34+ cell-enrichment of cord blood determines hematopoietic stem/progenitor cell yield upon ex vivo expansion. Journal of cellular biochemistry *112*, 1822-1831.

Andrade, P.Z., de Soure, A.M., Dos Santos, F., Paiva, A., Cabral, J.M., and da Silva, C.L. (2013a). Ex vivo expansion of cord blood haematopoietic stem/progenitor cells under physiological oxygen tensions: clear-cut effects on cell proliferation, differentiation and metabolism. Journal of tissue engineering and regenerative medicine.

Andrade, P.Z., dos Santos, F., Almeida-Porada, G., da Silva, C.L., and JM, S.C. (2010). Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells. Molecular bioSystems *6*, 1207-1215.

Andrade, P.Z., dos Santos, F., Cabral, J.M., and da Silva, C.L. (2013b). Stem cell bioengineering strategies to widen the therapeutic applications of haematopoietic stem/progenitor cells from umbilical cord blood. Journal of tissue engineering and regenerative medicine *9*, 988-1003.

Andrade, P.Z., dos Santos, F., Cabral, J.M., and da Silva, C.L. (2015). Stem cell bioengineering strategies to widen the therapeutic applications of haematopoietic

stem/progenitor cells from umbilical cord blood. Journal of tissue engineering and regenerative medicine *9*, 988-1003.

Andreas Reinisch, N.A.H., Anna C. Obenauf, Karl Kashofer, Eva Rohde, Katharina Schallmoser, Karin Flicker, Gerhard Lanzer, Werner Linkesch, Michael R. Speicher and Dirk Strunk (2009). Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. Blood *113*, 6716-6725.

Antal, C., Teletin, M., Wendling, O., Dgheem, M., Auwerx, J., and Mark, M. (2007). Tissue collection for systematic phenotyping in the mouse. Current protocols in molecular biology *Chapter 29*, Unit 29A 24.

Arora, N., Wenzel, P.L., McKinney-Freeman, S.L., Ross, S.J., Kim, P.G., Chou, S.S., Yoshimoto, M., Yoder, M.C., and Daley, G.Q. (2014). Effect of developmental stage of HSC and recipient on transplant outcomes. Developmental cell *29*, 621-628.

Asahara, T., Kawamoto, A., and Masuda, H. (2011). Concise review: Circulating endothelial progenitor cells for vascular medicine. Stem cells *29*, 1650-1655.

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M., and Isner, J.M. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circulation research *85*, 221-228.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Assmus, B., Schächinger, V., Teupe, C., Britten, M., Lehmann, R., Döbert, N., Grünwald, F., Aicher, A., Urbich, C., Martin, H.*, et al.* (2002). Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation *106*, 3009-3017.

AurÈlie, C., Christophe, D., Emilie, R., Denis, C., Bernadette, G., Laetitia, B., Annelise, B.-G., Olivier, P.-L., Georges, U., Lucile, A.*, et al.* (2008). A Cross-Talk Between Stromal Cell-Derived Factor-1 and Transforming Growth Factor-beta Controls the Quiescence/Cycling Switch of CD34^{<FONT SIZE='-1'>+</FONT>} Progenitors Through FoxO3 and Mammalian Target of Rapamycin. Stem Cells *26*, 3150-3161.

B. S. Lam, G.B.A. (2010). Hematopoietic stem cell lodgment in the adult bone marrow stem cell niche. International Journal of Laboratory Hematology *32*, 551-558.

Bai, J., Hu, Y., Wang, Y.R., Liu, L.F., Chen, J., Su, S.P., and Wang, Y. (2012). Comparison of human amniotic fluid-derived and umbilical cord Wharton's Jelly-derived mesenchymal stromal cells: Characterization and myocardial differentiation capacity. Journal of geriatric cardiology : JGC *9*, 166-171.

Bakhshi, T., Zabriskie, R.C., Bodie, S., Kidd, S., Ramin, S., Paganessi, L.A., Gregory, S.A., Fung, H.C., and Christopherson, K.W., 2nd (2008). Mesenchymal stem cells from the Wharton's jelly of umbilical cord segments provide stromal support for the maintenance of cord blood hematopoietic stem cells during long-term ex vivo culture. Transfusion *48*, 2638-2644.

Baksh D, Song L, and RS, T. (2004). Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. Journal of cellular and molecular medicine *8*, 301-316.

Baksh, D., Yao, R., and Tuan, R.S. (2007). Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem cells *25*, 1384-1392.

Ballen, K.K., Gluckman, E., and Broxmeyer, H.E. (2013). Umbilical cord blood transplantation: the first 25 years and beyond. Blood *122*, 491-498.

Banu, N., Rosenzweig, M., Kim, H., Bagley, J., and Pykett, M. (2001). Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix. Cytokine *13*, 349-358.

Barker, J.N., Weisdorf, D.J., DeFor, T.E., Blazar, B.R., McGlave, P.B., Miller, J.S., Verfaillie, C.M., and Wagner, J.E. (2005). Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. Blood *105*, 1343-1347.

Bartolucci, J., Verdugo, F.J., Gonzalez, P.L., Larrea, R.E., Abarzua, E., Goset, C., Rojo, P., Palma, I., Lamich, R., Pedreros, P.A.*, et al.* (2017). Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial (RIMECARD Trial [Randomized Clinical Trial of Intravenous Infusion Umbilical Cord Mesenchymal Stem Cells on Cardiopathy]). Circulation research *121*, 1192-1204.

Beckmann, J., Scheitza, S., Wernet, P., Fischer, J.C., and Giebel, B. (2007). Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. Blood *109*, 5494-5501.

Behbahan, I.S., Keating, A., and Gale, R.P. (2015). Bone Marrow Therapies for Chronic Heart Disease. Stem cells *33*, 3212-3227.

Berglund, A.K., Fortier, L.A., Antczak, D.F., and Schnabel, L.V. (2017). Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells. Stem cell research & therapy *8*, 288.

Bhattacharya, R., Senbanerjee, S., Lin, Z., Mir, S., Hamik, A., Wang, P., Mukherjee, P., Mukhopadhyay, D., and Jain, M.K. (2005). Inhibition of vascular permeability factor/vascular endothelial growth factor-mediated angiogenesis by the Kruppel-like factor KLF2. The Journal of biological chemistry *280*, 28848-28851.

Bianco, P., Cao, X., Frenette, P.S., Mao, J.J., Robey, P.G., Simmons, P.J., and Wang, C.Y. (2013). The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. Nature medicine *19*, 35-42.

Bixel, M.G., Kusumbe, A.P., Ramasamy, S.K., Sivaraj, K.K., Butz, S., Vestweber, D., and Adams, R.H. (2017). Flow Dynamics and HSPC Homing in Bone Marrow Microvessels. Cell reports *18*, 1804-1816.

Bonab, M.M., Alimoghaddam, K., Talebian, F., Ghaffari, S.H., Ghavamzadeh, A., and Nikbin, B. (2006). Aging of mesenchymal stem cell in vitro. BMC cell biology *7*, 14.

Bongso, A., and Fong, C.Y. (2013). The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton's jelly of the human umbilical cord. Stem cell reviews *9*, 226-240.

Bouchentouf, M., Forner, K., Cuerquis, J., Boulassel, M.R., Routy, J.P., Waller, E.K., Quyyumi, A.A., Paradis, P., Schiffrin, E.L., and Galipeau, J. (2011). A novel and simplified method of culture of human blood-derived early endothelial progenitor cells for the treatment of ischemic vascular disease. Cell transplantation *20*, 1431-1443.

Bourin, P., Bunnell, B.A., Casteilla, L., Dominici, M., Katz, A.J., March, K.L., Redl, H., Rubin, J.P., Yoshimura, K., and Gimble, J.M. (2013). Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). Cytotherapy *15*, 641-648.

Boyle, A.J., Whitbourn, R., Schlicht, S., Krum, H., Kocher, A., Nandurkar, H., Bergmann, S., Daniell, M., O'Day, J., Skerrett, D.*, et al.* (2006). Intra-coronary high-dose CD34+ stem cells in patients with chronic ischemic heart disease: a 12-month follow-up. International journal of cardiology *109*, 21-27.

Bullock, T.E., Wen, B., Marley, S.B., and Gordon, M.Y. (2007). Potential of CD34 in the regulation of symmetrical and asymmetrical divisions by hematopoietic progenitor cells. Stem cells *25*, 844-851.

Burt, R.K., Testori, A., Oyama, Y., Rodriguez, H.E., Yaung, K., Villa, M., Bucha, J.M., Milanetti, F., Sheehan, J., Rajamannan, N.*, et al.* (2010). Autologous peripheral

blood CD133+ cell implantation for limb salvage in patients with critical limb ischemia. Bone marrow transplantation *45*, 111-116.
Cabrita, G.J., Ferreira, B.S., da Silva, C.L., Goncalves, R., Almeida-Porada, G., and Cabral, J.M. (2003). Hematopoietic stem cells: from the bone to the bioreactor. Trends Biotechnol *21*, 233-240.
Cai, M., Shen, R., Song, L., Lu, M., Wang, J., Zhao, S., Tang, Y., Meng, X., Li, Z., and He, Z.X. (2016). Bone Marrow Mesenchymal Stem Cells (BM-MSCs) Improve Heart Function in Swine Myocardial Infarction Model through Paracrine Effects. Scientific reports *6*, 28250.
Campagnoli, C., Roberts, I.A., Kumar, S., Bennett, P.R., Bellantuono, I., and Fisk, N.M. (2001). Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood *98*, 2396-2402.
Can, A., and Karahuseyinoglu, S. (2007). Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. Stem Cells *25*, 2886-2895.
Caplan, A.I. (1991). Mesenchymal stem cells. Journal of Orthopaedic Research *9*, 641-650.
Caplan, A.I. (2007). Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol *213*, 341-347.
Caplan, A.I. (2017). Mesenchymal Stem Cells: Time to Change the Name! Stem cells translational medicine.
Caplan, A.I., and Correa, D. (2011a). The MSC: an injury drugstore. Cell stem cell *9*, 11-15.
Caplan, A.I., and Correa, D. (2011b). The MSC: An Injury Drugstore. Cell stem cell *9*, 11-15.
Carmelo, J.G., Fernandes-Platzgummer, A.M., Diogo, M.M., Silva, C.L.d., and Cabral, J.M.S. (2015). A Xeno-free Microcarrier-based Stirred Culture System for the Scalable Expansion of Human Mesenchymal Stem/Stromal Cells isolated from Bone Marrow and Adipose Tissue. Biotechnology Journal *10*, 1235-1247.
Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nature medicine *10*, 858-864.
Chamberlain, G., Fox, J., Ashton, B., and Middleton, J. (2007). Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem cells *11*, 2739-2749.
Chase, L.G., Yang, S., Zachar, V., Yang, Z., Lakshmipathy, U., Bradford, J., Boucher, S.E., and Vemuri, M.C. (2012). Development and characterization of a clinically compliant xeno-free culture medium in good manufacturing practice for human multipotent mesenchymal stem cells. Stem cells translational medicine *1*, 750-758.
Chavakis, E., Koyanagi, M., and Dimmeler, S. (2010). Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back. Circulation *121*, 325-335.
Cherry, R.S., and Papoutsakis, E.T. (1988). Physical mechanisms of cell damage in microcarrier cell culture bioreactors. *32*, 1001-1014.
Chou, S., Chu, P., Hwang, W., and Lodish, H. (2010). Expansion of human cord blood hematopoietic stem cells for transplantation. Cell Stem Cell *7*, 427-428.
Chou, S., Flygare, J., and Lodish, H.F. (2013). Fetal hepatic progenitors support long-term expansion of hematopoietic stem cells. Experimental hematology *41*, 479-490 e474.
Christoffersson, G., Vågesjö, E., Vandooren, J., Lidén, M., Massena, S., Reinert, R.B., Brissova, M., Powers, A.C., Opdenakker, G., and Phillipson, M. (2012). VEGF-A recruits a proangiogenic MMP-9–delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. Blood *120*, 4653-4662.
Colombo, E., Calcaterra, F., Cappelletti, M., Mavilio, D., and Della Bella, S. (2013). Comparison of Fibronectin and Collagen in Supporting the Isolation and Expansion

of Endothelial Progenitor Cells from Human Adult Peripheral Blood. PloS one *8*, e66734.

Corre, J., Barreau, C., Cousin, B., Chavoin, J.P., Caton, D., Fournial, G., Penicaud, L., Casteilla, L., and Laharrague, P. (2006). Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors. Journal of cellular physiology *208*, 282-288.

Coudriet, G.M., He, J., Trucco, M., Mars, W.M., and Piganelli, J.D. (2010). Hepatocyte Growth Factor Modulates Interleukin-6 Production in Bone Marrow Derived Macrophages- Implications for Inflammatory Mediated Diseases. PloS one *5*, 1-7.

Croughan, M.S., Hamel, J.F., and Wang, D.I.C. (1987). Hydrodynamic effects on animal cells grown in microcarrier cultures. Biotechnology and bioengineering *29*, 130-141.

Csaszar, E., Cohen, S., and Zandstra, P.W. (2013). Blood stem cell products: toward sustainable benchmarks for clinical translation. BioEssays : news and reviews in molecular, cellular and developmental biology *35*, 201-210.

Csaszar, E., Kirouac, D.C., Yu, M., Wang, W., Qiao, W., Cooke, M.P., Boitano, A.E., Ito, C., and Zandstra, P.W. (2012). Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell stem cell *10*, 218-229.

Cunha, B., Aguiar, T., Silva, M.M., Silva, R.J., Sousa, M.F., Pineda, E., Peixoto, C., Carrondo, M.J., Serra, M., and Alves, P.M. (2015). Exploring continuous and integrated strategies for the up- and downstream processing of human mesenchymal stem cells. Journal of biotechnology *213*, 97-108.

da Silva, C.L., Gonçalves, R., Crapnell, K.B., Cabral, J.M., Zanjani, E.D., and Almeida-Porada, G. (2005a). A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Experimental hematology *33*, 828-835.

da Silva, C.L., Goncalves, R., Crapnell, K.B., Cabral, J.M.S., Zanjani, E.D., and Almeida-Porada, G. (2005b). A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Exp Hematol *33*, 828-835.

da Silva, C.L., Goncalves, R., dos Santos, F., Andrade, P.Z., Almeida-Porada, G., and Cabral, J.M. (2010a). Dynamic cell-cell interactions between cord blood haematopoietic progenitors and the cellular niche are essential for the expansion of CD34+, CD34+CD38- and early lymphoid CD7+ cells. Journal of tissue engineering and regenerative medicine *4*, 149-158.

da Silva, C.L., Goncalves, R., Dos Santos, F., Andrade, P.Z., Almeida-Porada, G., and Cabral, J.M.S. (2010b). Dynamic cell-cell interactions between cord blood haematopoietic progenitors and the cellular niche are essential for the expansion of CD34(+), CD34(+)CD38(-) and early lymphoid CD7(+) cells. Journal of tissue engineering and regenerative medicine *4*, 149-158.

da Silva, C.L., Goncalves, R., Porada, C.D., Ascensao, J.L., Zanjani, E.D., Cabral, J.M., and Almeida-Porada, G. (2009a). Differences amid bone marrow and cord blood hematopoietic stem/progenitor cell division kinetics. Journal of cellular physiology *220*, 102-111.

da Silva, C.L., Gonçalves, R., Porada, C.D., Ascensão, J.L., Zanjani, E.D., Cabral, J.M.S., and Almeida-Porada, G. (2009b). Differences amid bone marrow and cord blood hematopoietic stem/progenitor cell division kinetics. Journal of Cellular Physiology *220*, 102-111.

Dahlberg, A., Delaney, C., and Bernstein, I.D. (2011a). Ex vivo expansion of human hematopoietic stem and progenitor cells. Blood.

Dahlberg, A., Delaney, C., and Bernstein, I.D. (2011b). Ex vivo expansion of human hematopoietic stem and progenitor cells. Blood *117*, 6083-6090.

David A. Ingram, L.E.M., Daniel B. Moore, Wayne Woodard, Amy Fenoglio and Mervin C., and Yoder (2005). Vessel wall–derived endothelial cells rapidly proliferate

because they contain a complete hierarchy of endothelial progenitor cells. Blood *105*, 2783-2786.

De Bruyn, C., Najar, M., Raicevic, G., Meuleman, N., Pieters, K., Stamatopoulos, B., Delforge, A., Bron, D., and Lagneaux, L. (2011). A rapid, simple, and reproducible method for the isolation of mesenchymal stromal cells from Wharton's jelly without enzymatic treatment. Stem Cells Dev *20*, 547-557.

de Lima, M., McMannis, J., Gee, A., Komanduri, K., Couriel, D., Andersson, B.S., Hosing, C., Khouri, I., Jones, R., Champlin, R.*, et al.* (2008). Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. Bone marrow transplantation *41*, 771-778.

de Soure, A.M., Fernandes-Platzgummer, A., da Silva, C.L., and Cabral, J.M. (2016). Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells. Journal of biotechnology *236*, 88-109.

De Toni, F., Poglio, S., Youcef, A.B., Cousin, B., Pflumio, F., Bourin, P., Casteilla, L., and Laharrague, P. (2011). Human adipose-derived stromal cells efficiently support hematopoiesis in vitro and in vivo: a key step for therapeutic studies. Stem cells and development *20*, 2127-2138.

Deans, R.J., and Moseley, A.B. (2000). Mesenchymal stem cells: biology and potential clinical uses. Experimental hematology *28*, 875-884.

Deborah Schop, Frank W. Janssen, Linda D.S. van Rijn, Hugo Fernandes, Rolf M. Bloem, Joost D. de Bruijn, and Dijkhuizen-Radersma, R.v. (2009). Growth, Metabolism, and Growth Inhibitors of Mesenchymal Stem Cells. Tissue Eng Part A *15*, 1877-1886.

Delaney, C., Heimfeld, S., Brashem-Stein, C., Voorhies, H., Manger, R.L., and Bernstein, I.D. (2010). Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nature medicine *16*, 232-236.

Dennis, J.E., Carbillet, J.-P., Caplan, A., and Charbord, P. (2002). The STRO-1+ Marrow Cell Population Is Multipotential. Cell Tissues Organs *170*, 73-82.

Dexter, T.M., Allen, T.D., Lajtha, L.G., Schofield, R., and Lord, B.I. (1973). Stimulation of differentiation and proliferation of haemopoietic cells in vitro. J Cell Physiol *82*, 461-473.

Dexter, T.M., Spooncer, E., Simmons, P., and Allen, T.D. (1984). Long-term marrow culture: an overview of techniques and experience. Kroc Found Ser *18*, 57-96.

Dib, N., Khawaja, H., Varner, S., McCarthy, M., and Campbell, A. (2011). Cell therapy for cardiovascular disease: a comparison of methods of delivery. Journal of cardiovascular translational research *4*, 177-181.

Ding, L., and Morrison, S.J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature *495*, 231-235.

Dixon, J.A., Gorman, R.C., Stroud, R.E., Bouges, S., Hirotsugu, H., Gorman, J.H., Martens, T.P., Itescu, S., Schuster, M.D., Plappert, T.*, et al.* (2009). Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation Journal *120*, 220-229.

Dmitrieva, R.I., Minullina, I.R., Bilibina, A.A., Tarasova, O.V., Anisimov, S.V., and Zaritskey, A.Y. (2012). Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells: Differences and similarities. Cell Cycle *11*, 377-383.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315-317.

Dong, Z., Chen, B., Fu, W., Wang, Y., Guo, D., Wei, Z., Xu, X., and Mendelsohn, F.O. (2013). Transplantation of purified CD34+ cells in the treatment of critical limb ischemia. Journal of vascular surgery *58*, 404-411 e403.

Dorrell, C., Gan, O.I., Pereira, D.S., Hawley, R.G., and Dick, J.E. (2000). Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral

transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. Blood *95*, 102-110.

Dos Santos, F., Andrade, P.Z., Boura, J.S., Abecasis, M.M., da Silva, C.L., and Cabral, J.M. (2010). Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. Journal of cellular physiology *223*, 27-35.

dos Santos, F., Andrade, P.Z., Eibes, G., da Silva, C.L., and Cabral, J.M. (2011). Ex Vivo Expansion of Human Mesenchymal Stem Cells on Microcarriers. Methods Mol Biol *698*, 189-198.

Dos Santos, F., Campbell, A., Fernandes-Platzgummer, A., Andrade, P.Z., Gimble, J.M., Wen, Y., Boucher, S., Vemuri, M.C., da Silva, C.L., and Cabral, J.M. (2014). A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. Biotechnology and bioengineering *111*, 1116-1127.

Douay, L. (2001a). Experimental culture conditions are critical for ex vivo expansion of hematopoietic cells. J Hematother Stem Cell Res *10*, 341-346.

Douay, L. (2001b). Experimental culture conditions are critical for ex vivo expansion of hematopoietic cells. J Hematother Stem Cell Res *10*, 341-346.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell stem cell *10*, 120-136.

Dragneva, G., Korpisalo, P., and Yla-Herttuala, S. (2013). Promoting blood vessel growth in ischemic diseases: challenges in translating preclinical potential into clinical success. Disease models & mechanisms *6*, 312-322.

Duttenhoefer, F., Freitas, R.L.d., Meury, T., Loibl, M., Benneker, L.M., Richards, R.G., and M. AliniS. Verrier1 (2013). 3D scaffolds co-seeded with human endothelial progenitor and mesenchymal stem cells- Evidence of prevascularisation within 7 days. European Cells and Materials *26*, 49-65.

Eggenhofer, E., Benseler, V., Kroemer, A., Popp, F.C., Geissler, E.K., Schlitt, H.J., Baan, C.C., Dahlke, M.H., and Hoogduijn, M.J. (2012). Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. Frontiers in immunology *3*, 297.

Eliasson, P., and Jonsson, J.I. (2010). The hematopoietic stem cell niche: low in oxygen but a nice place to be. Journal of cellular physiology *222*, 17-22.

Fadini, G.P., Losordo, D., and Dimmeler, S. (2012). Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. Circulation research *110*, 624-637.

Feng-Juan Lv, Rocky S. Tuan, Kenneth M.C. Cheung, and Leung, V.Y.L. (2014). Concise review: the surface markers and identity of human mesenchymal stem cells. Stem cells *32*, 1408-1419.

Flores-Guzman, P., Fernandez-Sanchez, V., Valencia-Plata, I., Arriaga-Pizano, L., Alarcon-Santos, G., and Mayani, H. (2013). Comparative in vitro analysis of different hematopoietic cell populations from human cord blood: in search of the best option for clinically oriented ex vivo cell expansion. Transfusion *53*, 668-678.

Frenette, P.S., Pinho, S., Lucas, D., and Scheiermann, C. (2013). Mesenchymal stem cell: keystone of the hematopoietic stem cell niche and a stepping-stone for regenerative medicine. Annual review of immunology *31*, 285-316.

Freyman, T., Polin, G., Osman, H., Crary, J., Lu, M., Cheng, L., Palasis, M., and Wilensky, R.L. (2006). A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. European heart journal *27*, 1114-1122.

Friedman, R., Betancur, M., Boissel, L., Tuncer, H., Cetrulo, C., and Klingemann, H. (2007). Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *13*, 1477-1486.

Galipeau, J. (2013). Concerns arising from MSC retrieval from cryostorage and effect on immune suppressive function and pharmaceutical usage in clinical trials. ISBT Science Series *8*, 100-101.

Gao, F., Chiu, S.M., Motan, D.A., Zhang, Z., Chen, L., Ji, H.L., Tse, H.F., Fu, Q.L., and Lian, Q. (2016). Mesenchymal stem cells and immunomodulation: current status and future prospects. Cell death & disease *7*, e2062.

Gao, L.R., Chen, Y., Zhang, N.K., Yang, X.L., Liu, H.L., Wang, Z.G., Yan, X.Y., Wang, Y., Zhu, Z.M., Li, T.C*., et al.* (2015). Intracoronary infusion of Wharton's jelly-derived mesenchymal stem cells in acute myocardial infarction: double-blind, randomized controlled trial. BMC medicine *13*, 162.

Gimble, J.M., Katz, A.J., and Bunnell, B.A. (2007). Adipose-derived stem cells for regenerative medicine. Circulation research *100*, 1249-1260.

Gluckman, E. (2009). History of cord blood transplantation. Bone marrow transplantation *44*, 621-626.

Godara, P., McFarland, C.D., and Nordon, R.E. (2008). Design of bioreactors for mesenchymal stem cell tissue engineering. Journal of Chemical Technology & Biotechnology *83*, 408-420.

Goncalves, R., Lobato da Silva, C., Cabral, J.M., Zanjani, E.D., and Almeida-Porada, G. (2006). A Stro-1(+) human universal stromal feeder layer to expand/maintain human bone marrow hematopoietic stem/progenitor cells in a serum-free culture system. Experimental hematology *34*, 1353-1359.

Gothot A, Pyatt R, McMahel J, Rice S, and EF, S. (1998). Assessment of proliferative and colony-forming capacity after successive in vitro divisions of single human CD34+ cells initially isolated in G0. Experimental hematology *26*, 562-570.

Grochot-Przeczek, A., Dulak, J., and Jozkowicz, A. (2013). Therapeutic angiogenesis for revascularization in peripheral artery disease. Gene *525*, 220-228.

Gronthos, S., and Simmons, P.J. (1995). The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood *85*, 929-940.

Gronthos, S., Zannettino, A.C., Hay, S.J., Shi, S., Graves, S.E., Kortesidis, A., and Simmons, P.J. (2003). Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci *116*, 1827-1835.

Grove, J.E., Bruscia, E., and Krause, D.S. (2004). Plasticity of Bone Marrow-Derived Stem Cells. Stem Cells *22*, 487-500.

Gulati, R., Jevremovic, D., Peterson, T.E., Chatterjee, S., Shah, V., Vile, R.G., and Simari, R.D. (2003). Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circulation research *93*, 1023-1025.

Hans-Georg Kopp, S.T.A., Andrea T. Hooper, and Shahin Rafii (2005). The Bone Marrow Vascular Niche: Home of HSC Differentiation and Mobilization. Physiology *20*, 349-356.

Hare, J.M., Traverse, J.H., Henry, T.D., Dib, N., Strumpf, R.K., Schulman, S.P., Gerstenblith, G., DeMaria, A.N., Denktas, A.E., Gammon, R.S*., et al.* (2009). A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. Journal of the American College of Cardiology *54*, 2277-2286.

Hashemi, S.M., Ghods, S., Kolodgie, F.D., Parcham-Azad, K., Keane, M., Hamamdzic, D., Young, R., Rippy, M.K., Virmani, R., Litt, H*., et al.* (2008). A placebo controlled, dose-ranging, safety study of allogeneic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. European heart journal *29*, 251-259.

Hass, R., Kasper, C., Bohm, S., and Jacobs, R. (2011a). Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. Cell communication and signaling : CCS *9*, 12.

Hass, R., Kasper, C., Böhm, S., and Jacobs, R. (2011b). Different populations and sources of human mesenchymal stem cells (MSC)- A comparison of adult and neonatal tissue-derived MSC. Cell Communication and Signaling *9*.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Barbara Ferris, Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.S.*, et al.* (2002). Recruitment of Stem and Progenitor Cells from the Bone Marrow Niche Requires MMP-9 Mediated Release of Kit-Ligand. Cell *109*, 625-637.

Hemeda, H., Giebel, B., and Wagner, W. (2014). Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells. Cytotherapy *16*, 170-180.

Hewitt, C.J., Lee, K., Nienow, A.W., Thomas, R.J., Smith, M., and Thomas, C.R. (2011). Expansion of human mesenchymal stem cells on microcarriers. Biotechnology letters *33*, 2325-2335.

Hill, J.M., Zalos, G., Halcox, J.P.J., Schenke, W.H., Waclawiw, M.A., Quyyumi, A.A., and Finkel, T. (2003). Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med *348*, 593-600.

Hoch, A.I., and Leach, J.K. (2014). Concise review: optimizing expansion of bone marrow mesenchymal stem/stromal cells for clinical applications. Stem cells translational medicine *3*, 643-652.

Hofbauer, P., Riedl, S., Witzeneder, K., Hildner, F., Wolbank, S., Groeger, M., Gabriel, C., Redl, H., and Holnthoner, W. (2014). Human platelet lysate is a feasible candidate to replace fetal calf serum as medium supplement for blood vascular and lymphatic endothelial cells. Cytotherapy *16*, 1238-1244.

Hofmeister, C.C., Zhang, J., Knight, K.L., Le, P., and Stiff, P.J. (2007). Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone marrow transplantation *39*, 11-23.

Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Deans, R.J., Krause, D.S., Keating, A., and International Society for Cellular, T. (2005). Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy *7*, 393-395.

Houtgraaf, J.H., de Jong, R., Kazemi, K., de Groot, D., van der Spoel, T.I., Arslan, F., Hoefer, I., Pasterkamp, G., Itescu, S., Zijlstra, F.*, et al.* (2013). Intracoronary infusion of allogeneic mesenchymal precursor cells directly after experimental acute myocardial infarction reduces infarct size, abrogates adverse remodeling, and improves cardiac function. Circulation research *113*, 153-166.

Hristov, M., Erl, W., and Weber, P.C. (2003). Endothelial progenitor cells: mobilization, differentiation, and homing. Arteriosclerosis, thrombosis, and vascular biology *23*, 1185-1189.

Hristov, M., Schmitz, S., Nauwelaers, F., and Weber, C. (2012). A flow cytometric protocol for enumeration of endothelial progenitor cells and monocyte subsets in human blood. Journal of immunological methods *381*, 9-13.

Hung, S.C., Pochampally, R.R., Chen, S.C., Hsu, S.C., and Prockop, D.J. (2007). Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. Stem cells *25*, 2363-2370.

Hur, J., Yang, H.M., Yoon, C.H., Lee, C.S., Park, K.W., Kim, J.H., Kim, T.Y., Kim, J.Y., Kang, H.J., Chae, I.H.*, et al.* (2007). Identification of a novel role of T cells in postnatal vasculogenesis: characterization of endothelial progenitor cell colonies. Circulation *116*, 1671-1682.

Hur, J., Yoon, C.H., Kim, H.S., Choi, J.H., Kang, H.J., Hwang, K.K., Oh, B.H., Lee, M.M., and Park, Y.B. (2004). Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arteriosclerosis, thrombosis, and vascular biology *24*, 288-293.

III, L.M., Eid, S., Lingas, K., Reese, J., Meyerson, H., Tong, A., de Lima, M., and Huang, A.Y. (2016). Intra-osseous Co-transplantation of CD34-selected Umbilical Cord Blood and Mesenchymal Stromal Cells. Hematol Med Oncol, 25-29.

Ingram, D.A., Mead, L.E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M.J., Gilley, D., and Yoder, M.C. (2004). Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood *104*, 2752-2760.

Jaalouk, D.E., and Lammerding, J. (2009). Mechanotransduction gone awry. Nature reviews Molecular cell biology *10*, 63-73.

Janic, B., Guo, A.M., Iskander, A.S., Varma, N.R., Scicli, A.G., and Arbab, A.S. (2010). Human cord blood-derived AC133+ progenitor cells preserve endothelial progenitor characteristics after long term in vitro expansion. PloS one *5*, e9173.

Jauniaux, E., Watson, A.L., Hempstock, J., Bao, Y.-P., Skepper, J.N., and Burton, G.J. (2000). Onset of Maternal Arterial Blood Flow and Placental Oxidative Stress. The American journal of pathology *157*, 2111-2122.

Jazwa, A., Tomczyk, M., Taha, H.M., Hytonen, E., Stoszko, M., Zentilin, L., Giacca, M., Yla-Herttuala, S., Emanueli, C., Jozkowicz, A.*, et al.* (2013). Arteriogenic therapy based on simultaneous delivery of VEGF-A and FGF4 genes improves the recovery from acute limb ischemia. Vascular Cell *5*.

Jing, D., Fonseca, A.-V., Alakel, N., Fierro, F.A., Muller, K., Bornhauser, M., Ehninger, G., Corbeil, D., and Ordemann, R. (2010a). Hematopoietic stem cells in co-culture with mesenchymal stromal cells - modeling the niche compartments in vitro. Haematologica *95*, 542-550.

Jing, D., Fonseca, A.V., Alakel, N., Fierro, F.A., Muller, K., Bornhauser, M., Ehninger, G., Corbeil, D., and Ordemann, R. (2010b). Hematopoietic stem cells in co-culture with mesenchymal stromal cells--modeling the niche compartments in vitro. Haematologica *95*, 542-550.

Joswig, A.J., Mitchell, A., Cummings, K.J., Levine, G.J., Gregory, C.A., Smith, R., 3rd, and Watts, A.E. (2017). Repeated intra-articular injection of allogeneic mesenchymal stem cells causes an adverse response compared to autologous cells in the equine model. Stem cell research & therapy *8*, 42.

Juhl, M., Tratwal, J., Follin, B., Sondergaard, R.H., Kirchhoff, M., Ekblond, A., Kastrup, J., and Haack-Sorensen, M. (2016). Comparison of clinical grade human platelet lysates for cultivation of mesenchymal stromal cells from bone marrow and adipose tissue. Scandinavian journal of clinical and laboratory investigation *76*, 93-104.

Jung, S., Sen, A., Rosenberg, L., and Behie, L.A. (2012). Human mesenchymal stem cell culture: rapid and efficient isolation and expansion in a defined serum-free medium. Journal of tissue engineering and regenerative medicine *6*, 391-403.

Kalka, C., and Baumgartner, I. (2008). Gene and stem cell therapy in peripheral arterial occlusive disease. Vascular medicine *13*, 157-172.

Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W.M., Silver, M., Kearney, M., Li, T., Isner, J.M., and Asahara, T. (2000). Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proceedings of the National Academy of Sciences of the United States of America *97*, 3422-3427.

Kanelidis, A.J., Premer, C., Lopez, J., Balkan, W., and Hare, J.M. (2017). Route of Delivery Modulates the Efficacy of Mesenchymal Stem Cell Therapy for Myocardial Infarction: A Meta-Analysis of Preclinical Studies and Clinical Trials. Circulation research *120*, 1139-1150.

Keating, A. (2012). Mesenchymal stromal cells: new directions. Cell stem cell *10*, 709-716.

Kern, S., Eichler, H., Stoeve, J., Kluter, H., and Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem cells *24*, 1294-1301.

Kiel, M.J., and Morrison, S.J. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol *8*, 290-301.

Kim, H.-J., Jang, S.Y., Park, J.-I., Byun, J., Kim, D.-I., Do, Y.-S., Kim, J.-M., Kim, S., Kim, B.-M., Kim, W.o.-B.*, et al.* (2004). Vascular endothelial growth factor-induced angiogenic gene therapy in patients with peripheral artery disease. Experimental and Molecular Medicine *36*, 336-344.

Kim, J.E., Yoo, C., Kim, S., Lee, D.H., Kim, S.W., Lee, J.S., and Suh, C. (2011). Optimal timing of G-CSF administration for effective autologous stem cell collection. Bone marrow transplantation *46*, 806-812.

Kinoshita, M., Fujita, Y., Katayama, M., Baba, R., Shibakawa, M., Yoshikawa, K., Katakami, N., Furukawa, Y., Tsukie, T., Nagano, T.*, et al.* (2012). Long-term clinical outcome after intramuscular transplantation of granulocyte colony stimulating factor-mobilized CD34 positive cells in patients with critical limb ischemia. Atherosclerosis *224*, 440-445.

Kirouac, D.C., and Zandstra, P.W. (2008). The systematic production of cells for cell therapies. Cell Stem Cell *3*, 369-381.

Klein, D., Hohn, H.-P., Kleff, V., Tilki, D., and Ergün1, S. (2010). Vascular wall-resident stem cells. Histol Histopathol *25*, 681-689.

Klingemann, H., Matzilevich, D., and Marchand, J. (2008). Mesenchymal Stem Cells - Sources and Clinical Applications. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie *35*, 272-277.

Kolf, C.M., Cho, E., and Tuan, R.S. (2007). Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis research & therapy *9*, 204.

Kumar, S., and Geiger, H. (2017). HSC Niche Biology and HSC Expansion Ex Vivo. Trends in molecular medicine *23*, 799-819.

Kwon, S.M., Lee, J.H., Lee, S.H., Jung, S.Y., Kim, D.Y., Kang, S.H., Yoo, S.Y., Hong, J.K., Park, J.H., Kim, J.H.*, et al.* (2014). Cross talk with hematopoietic cells regulates the endothelial progenitor cell differentiation of CD34 positive cells. PloS one *9*, e106310.

Lackman, F., Capewell, V., Gagnon, R., and Richardson, B. (2001). Fetal umbilical cord oxygen values and birth to placental weight ratio in relation to size at birth. American journal of obstetrics and gynecology *185*, 674-682.

Lakshmipathy, U., and Verfaillie, C. (2005). Stem cell plasticity. Blood reviews *19*, 29-38.

Lalu, M.M., McIntyre, L., Pugliese, C., Fergusson, D., Winston, B.W., Marshall, J.C., Granton, J., Stewart, D.J., and Group, C.C.C.T. (2012). Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PloS one *7*.

Lawall, H., Bramlage, P., and Amann, B. (2011). Treatment of peripheral arterial disease using stem and progenitor cell therapy. Journal of vascular surgery *53*, 445-453.

Lazarus, H.M., Koc, O.N., Devine, S.M., Curtin, P., Maziarz, R.T., Holland, H.K., Shpall, E.J., McCarthy, P., Atkinson, K., Cooper, B.W.*, et al.* (2005). Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *11*, 389-398.

Le Blanc, K., Tammik, C., Rosendahl, K., Zetterberg, E., and Ringdén, O. (2003). HLA expression and immunologic propertiesof differentiated and undifferentiated mesenchymal stem cells. Experimental hematology *31*, 890-896.

Lee, J.H., Lee, S.H., Yoo, S.Y., Asahara, T., and Kwon, S.M. (2013a). CD34 Hybrid Cells Promote Endothelial Colony-Forming Cell Bioactivity and Therapeutic Potential for Ischemic Diseases. Arteriosclerosis, thrombosis, and vascular biology *33*, 1622-1634.

Lee, J.W., Lee, S.H., Youn, Y.J., Ahn, M.S., Kim, J.Y., Yoo, B.S., Yoon, J., Kwon, W., Hong, I.S., Lee, K.*, et al.* (2014). A randomized, open-label, multicenter trial for the

safety and efficacy of adult mesenchymal stem cells after acute myocardial infarction. Journal of Korean medical science *29*, 23-31.

Lee, R.H., Pulin, A.A., Seo, M.J., Kota, D.J., Ylostalo, J., Larson, B.L., Semprun-Prieto, L., Delafontaine, P., and Prockop, D.J. (2009). Intravenous hMSCs Improve Myocardial Infarction in Mice because Cells Embolized in Lung Are Activated to Secrete the Anti-inflammatory Protein TSG-6. Cell stem cell *5*, 54-63.

Lee, S.H., Lee, J.H., Yoo, S.Y., Hur, J., Kim, H.-S., and Kwon, S.M. (2013b). Hypoxia inhibits cellular senescence to restore the therapeutic potential of old human endothelial progenitor cells via the hypoxia-inducible factor-1α-TWIST-p21 axis. Arteriosclerosis, thrombosis, and vascular biology *33*.

Leemhuis, T., Wells, S., Scheffold, C., Edinger, M., and Negrin, R.S. (2005). A phase I trial of autologous cytokine-induced killer cells for the treatment of relapsed Hodgkin disease and non-Hodgkin lymphoma. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *11*, 181-187.

Leri, A., and Anversa, P. (2013). Stem cells: Bone-marrow-derived cells and heart failure—the debate goes on. Nat Rev Cardiol *10*, 372-373.

Li, K., Lee, S.M., Su, R.J., Zhang, X.B., Yuen, P.M., Li, C.K., Yang, M., Tsang, K.S., James, A.E., Tse, Y.H.*, et al.* (2005). Multipotent neural precursors express neural and hematopoietic factors, and enhance ex vivo expansion of cord blood CD34+ cells, colony forming units and NOD/SCID-repopulating cells in contact and noncontact cultures. Leukemia *19*, 91-97.

Lin, G., Liu, G., Banie, L., Wang, G., Ning, H., Lue, T.F., and Lin, C.S. (2011). Tissue distribution of mesenchymal stem cell marker Stro-1. Stem cells and development *20*, 1747-1752.

Lippross, S., Loibl, M., Hoppe, S., Meury, T., Benneker, L., Alini, M., and Verrier, S. (2011). Platelet released growth factors boost expansion of bone marrow derived CD34(+) and CD133(+) endothelial progenitor cells for autologous grafting. Platelets *22*, 422-432.

Losordo, D.W., Kibbe, M.R., Mendelsohn, F., Marston, W., Driver, V.R., Sharafuddin, M., Teodorescu, V., Wiechmann, B.N., Thompson, C., Kraiss, L.*, et al.* (2012). A randomized, controlled pilot study of autologous CD34+ cell therapy for critical limb ischemia. Circulation Cardiovascular interventions *5*, 821-830.

Magin, A.S., Korfer, N.R., Partenheimer, H., Lange, C., Zander, A., and Noll, T. (2009). Primary cells as feeder cells for coculture expansion of human hematopoietic stem cells from umbilical cord blood--a comparative study. Stem cells and development *18*, 173-186.

Majka, M., Sulkowski, M., Badyra, B., and Musialek, P. (2017). Concise Review: Mesenchymal Stem Cells in Cardiovascular Regeneration: Emerging Research Directions and Clinical Applications. Stem cells translational medicine *6*, 1859-1867.

Makkar, R.R., Price, M.J., Lill, M., Frantzen, M., Takizawa, K., Kleisli, T., Zheng, J., Kar, S., McClelan, R., Miyamota, T.*, et al.* (2005). Intramyocardial injection of allogeneic bone marrow-derived mesenchymal stem cells without immunosuppression preserves cardiac function in a porcine model of myocardial infarction. Journal of cardiovascular pharmacology and therapeutics *10*, 225-233.

Masuda, H., Alev, C., Akimaru, H., Ito, R., Shizuno, T., Kobori, M., Horii, M., Ishihara, T., Isobe, K., Isozaki, M.*, et al.* (2011). Methodological development of a clonogenic assay to determine endothelial progenitor cell potential. Circulation research *109*, 20-37.

Masuda, H., Iwasaki, H., Kawamoto, A., Akimaru, H., Ishikawa, M., Ii, M., Shizuno, T., Sato, A., Ito, R., Horii, M.*, et al.* (2012). Development of serum-free quality and quantity control culture of colony-forming endothelial progenitor cell for vasculogenesis. Stem cells translational medicine *1*, 160-171.

Masuda, H., Tanaka, R., Fujimura, S., Ishikawa, M., Akimaru, H., Shizuno, T., Sato, A., Okada, Y., Iida, Y., Itoh, J.*, et al.* (2014). Vasculogenic conditioning of peripheral blood mononuclear cells promotes endothelial progenitor cell expansion and phenotype transition of anti-inflammatory macrophage and T lymphocyte to cells with regenerative potential. Journal of the American Heart Association *3*, e000743.

Mata, M.F., Lopes, J.P., Ishikawa, M., Alaiti, M.A., Cabral, J.M., da Silva, C.L., and Costa, M.A. (2015). Scaling up the ex vivo expansion of human circulating CD34(+)progenitor cells with upregulation of angiogenic and anti-inflammatory potential. Cytotherapy *17*, 1777-1784.

Mattiucci, D., Maurizi, G., Izzi, V., Cenci, L., Ciarlantini, M., Mancini, S., Mensa, E., Pascarella, R., Vivarelli, M., Olivieri, A.*, et al.* (2018). Bone marrow adipocytes support hematopoietic stem cell survival. Journal of cellular physiology *233*, 1500-1511.

McAdams, T.A., Winter, J.N., Miller, W.M., and Papoutsakis, E.T. (1996). Hematopoietic cell culture therapies (Part II): Clinical aspects and applications. Trends in biotechnology *14*, 388-396.

Medina, R.J., Barber, C.L., Sabatier, F., Dignat-George, F., Melero-Martin, J.M., Khosrotehrani, K., Ohneda, O., Randi, A.M., Chan, J.K., Yamaguchi, T.*, et al.* (2017). Endothelial Progenitors: A Consensus Statement on Nomenclature. Stem cells translational medicine.

Medina, R.J., O'Neill, C.L., O'Doherty, T.M., Knott, H., Guduric-Fuchs, J., Gardiner, T.A., and Stitt, A.W. (2011). Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8. Molecular medicine *17*, 1045-1055.

Meirelles , L.S., Fontes, A.M., Covas, D.T., and Caplan, A.I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine & growth factor reviews *20*, 419-427.

Mendelson, A., and Frenette, P.S. (2014). Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nature medicine *20*, 833-846.

Mikkola, H.K., and Orkin, S.H. (2006). The journey of developing hematopoietic stem cells. Development *133*, 3733-3744.

Mimeault, M., Hauke, R., and Batra, S.K. (2007). Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clinical pharmacology and therapeutics *82*, 252-264.

Mohyeldin, A., Garzon-Muvdi, T., and Quinones-Hinojosa, A. (2010). Oxygen in stem cell biology: a critical component of the stem cell niche. Cell stem cell *7*, 150-161.

Moon, S.H., Kim, S.M., Park, S.J., Kim, H., Bae, D., Choi, Y.S., and Chung, H.M. (2013). Development of a xeno-free autologous culture system for endothelial progenitor cells derived from human umbilical cord blood. PloS one *8*, e75224.

Morrison, S.J., and Scadden, D.T. (2014). The bone marrow niche for haematopoietic stem cells. Nature *505*, 327-334.

Motaln, H., Schichor, C., and Lah, T.T. (2010). Human mesenchymal stem cells and their use in cell-based therapies. Cancer *116*, 2519-2530.

Mund, J.A., Estes, M.L., Yoder, M.C., Ingram, D.A., Jr., and Case, J. (2012). Flow cytometric identification and functional characterization of immature and mature circulating endothelial cells. Arteriosclerosis, thrombosis, and vascular biology *32*, 1045-1053.

Musialek, P., Mazurek, A., Jarocha, D., Tekieli, L., Szot, W., Kostkiewicz, M., Banys, R.P., Urbanczyk, M., Kadzielski, A., Trystula, M.*, et al.* (2015). Myocardial regeneration strategy using Wharton's jelly mesenchymal stem cells as an off-the-shelf 'unlimited' therapeutic agent: results from the Acute Myocardial Infarction First-in-Man Study. Postepy w kardiologii interwencyjnej = Advances in interventional cardiology *11*, 100-107.

Nagahama, R., Matoba, T., Nakano, K., Kim-Mitsuyama, S., Sunagawa, K., and Egashira, K. (2012). Nanoparticle-mediated delivery of pioglitazone enhances therapeutic neovascularization in a murine model of hindlimb ischemia. Arteriosclerosis, thrombosis, and vascular biology *32*, 2427-2434.

Nagata, S. (1975). Mixing: principles and applications. Kodansha,Tokyo.

Nakao, N., Nakayama, T., Yahata, T., Muguruma, Y., Saito, S., Miyata, Y., Yamamoto, K., and Naoe, T. (2010). Adipose tissue-derived mesenchymal stem cells facilitate hematopoiesis in vitro and in vivo: advantages over bone marrow-derived mesenchymal stem cells. The American journal of pathology *177*, 547-554.

Newsholme;, E.A., Crabtree;, B., Salleh;, M., and Ardawi, M. (1985). Glutamine metabolism in lymphocytes its biochemical, physiological and clinical importance. Quarterly Journal of Experimental Physiology *70*, 473-489.

Ng, C.P., Sharif, A.R., Heath, D.E., Chow, J.W., Zhang, C.B., Chan-Park, M.B., Hammond, P.T., Chan, J.K., and Griffith, L.G. (2014). Enhanced ex vivo expansion of adult mesenchymal stem cells by fetal mesenchymal stem cell ECM. Biomaterials *35*, 4046-4057.

Nicklin, P., Bergman, P., Zhang, B., Triantafellow, E., Wang, H., Nyfeler, B., Yang, H., Hild, M., Kung, C., Wilson, C.*, et al.* (2009). Bidirectional transport of amino acids regulates mTOR and autophagy. Cell *136*, 521-534.

Nienow, A.W. (2006). Reactor engineering in large scale animal cell culture. Cytotechnology *50*, 9-33.

Nienow, A.W., Rafiq, Q.A., Coopman, K., and Hewitt, C.J. (2014). A potentially scalable method for the harvesting of hMSCs from microcarriers. Biochemical Engineering Journal *85*, 79-88.

Niiyama, H., Huang, N.F., Rollins, M.D., and Cooke, J.P. (2009). Murine model of hindlimb ischemia. Journal of visualized experiments : JoVE.

Nishiwaki, S., Nakayama, T., Saito, S., Mizuno, H., Ozaki, T., Takahashi, Y., Maruyama, S., Nishida, T., Murata, M., Kojima, S.*, et al.* (2012). Efficacy and safety of human adipose tissue-derived mesenchymal stem cells for supporting hematopoiesis. International journal of hematology *96*, 295-300.

Noort, W.A., Kruisselbrink, A.B., in't Anker, P.S., Kruger, M., van Bezooijen, R.L., de Paus, R.A., Heemskerk, M.H., Lowik, C.W., Falkenburg, J.H., Willemze, R.*, et al.* (2002). Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol *30*, 870-878.

Norgren, L., Hiatt, W.R., Dormandy, J.A., Nehler, M.R., Harris, K.A., Fowkes, F.G., and Group, T.I.W. (2007). Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Journal of vascular surgery *45 Suppl S*, S5-67.

Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick, J.E. (2011). Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science *333*, 218-221.

Numaguchi, Y., Sone, T., Okumura, K., Ishii, M., Morita, Y., Kubota, R., Yokouchi, K., Imai, H., Harada, M., Osanai, H.*, et al.* (2006). The impact of the capability of circulating progenitor cell to differentiate on myocardial salvage in patients with primary acute myocardial infarction. Circulation *114*, I114-119.

O, E., Lee, B.H., Ahn, H.Y., Shin, J.C., Kim, H.K., Kim, M., Park, I.Y., Park, Y.G., and Joe, Y.A. (2011). Efficient nonadhesive ex vivo expansion of early endothelial progenitor cells derived from CD34+ human cord blood fraction for effective therapeutic vascularization. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *25*, 159-169.

Obi, S., Masuda, H., Shizuno, T., Sato, A., Yamamoto, K., Ando, J., Abe, Y., and Asahara, T. (2012). Fluid shear stress induces differentiation of circulating phenotype endothelial progenitor cells. J Appl Physiol *303*, 595-606.

Obi, S., Yamamoto, K., Shimizu, N., Kumagaya, S., Masumura, T., Sokabe, T., Asahara, T., and Ando, J. (2009). Fluid shear stress induces arterial differentiation of endothelial progenitor cells. J Appl Physiol *106*, 203-211.

Oburoglu, L., Tardito, S., Fritz, V., de Barros, S.C., Merida, P., Craveiro, M., Mamede, J., Cretenet, G., Mongellaz, C., An, X.*, et al.* (2014). Glucose and glutamine metabolism regulate human hematopoietic stem cell lineage specification. Cell stem cell *15*, 169-184.

Oh, I.H., and Kwon, K.R. (2010). Concise review: multiple niches for hematopoietic stem cell regulations. Stem Cells *28*, 1243-1249.

Oikonomopoulos, A., van Deen, W.K., Manansala, A.R., Lacey, P.N., Tomakili, T.A., Ziman, A., and Hommes, D.W. (2015). Optimization of human mesenchymal stem cell manufacturing: the effects of animal/xeno-free media. Scientific reports *5*, 16570.

Orkin, S.H., and Zon, L.I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. Cell *132*, 631-644.

Ott, I., Keller, U., Knoedler, M., Götze, K.S., Doss, K., Fischer, P., Urlbauer, K., Debus, G., Bubnoff, N.v., Rudelius, M.*, et al.* (2005). Endothelial-like cells expanded from CD34+ blood cells improve left ventricular function after experimental myocardial infarction. FASEB journal : official publication of the Federation of American Societies for Experimental Biology.

Ouma, G.O., Zafrir, B., Mohler, E.R., 3rd, and Flugelman, M.Y. (2013). Therapeutic angiogenesis in critical limb ischemia. Angiology *64*, 466-480.

Pai, M.H., Shih, Y.M., Shih, J.M., and Yeh, C.L. (2016). Glutamine Administration Modulates Endothelial Progenitor Cell and Lung Injury in Septic Mice. Shock *46*, 587-592.

Papaioannou, T.G., and Stefanadis, C. (2005). Vascular Wall Shear Stress: Basic Principles and Methods. Hellenic Journal of Cardiology *46*, 9-15.

Parsa, H., Ronaldson, K., and Vunjak-Novakovic, G. (2015). Bioengineering methods for myocardial regeneration. Advanced drug delivery reviews.

Passier, R. (2003). Origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovascular research *58*, 324-335.

Passweg, J.R., Baldomero, H., Peters, C., Gaspar, H.B., Cesaro, S., Dreger, P., Duarte, R.F., Falkenburg, J.H.F., Farge-Bancel, D., Gennery, A.*, et al.* (2014). Hematopoietic SCT in Europe: data and trends in 2012 with special consideration of pediatric transplantation. Bone marrow transplantation *49*, 744-750.

Patel, S.D., Papoutsakis, E.T., Winter, J.N., and Miller, W.M. (2000). The lactate issue revisited: novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures. Biotechnol Prog *16*, 885-892.

Perin, E.C., Sanz-Ruiz, R., Sanchez, P.L., Lasso, J., Perez-Cano, R., Alonso-Farto, J.C., Perez-David, E., Fernandez-Santos, M.E., Serruys, P.W., Duckers, H.J.*, et al.* (2014). Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial. American heart journal *168*, 88-95 e82.

Perin, E.C., and Silva, G.V. (2011). Cell-based therapy for chronic ischemic heart disease--a clinical perspective. Cardiovascular therapeutics *29*, 211-217.

Persichini, T., Funari, A., Colasanti, M., and Sacchetti, B. (2017). Clonogenic, myogenic progenitors expressing MCAM/CD146 are incorporated as adventitial reticular cells in the microvascular compartment of human post-natal skeletal muscle. PloS one *12*, e0188844.

Piatkowski, A., Grieb, G., Simons, D., Bernhagen, J., and van der Hulst, R.R. (2013). Endothelial progenitor cells--potential new avenues to improve neoangiogenesis and reendothelialization. International review of cell and molecular biology *306*, 43-81.

Pietras, E.M., Warr, M.R., and Passegue, E. (2011). Cell cycle regulation in hematopoietic stem cells. The Journal of cell biology *195*, 709-720.

Pill, K., Hofmann, S., Redl, H., and Holnthoner, W. (2015). Vascularization mediated by mesenchymal stem cells from bone marrow and adipose tissue: a comparison. Cell regeneration *4*, 8.

Pilz, G.A., Braun, J., Ulrich, C., Felka, T., Warstat, K., Ruh, M., Schewe, B., Abele, H., Larbi, A., and Aicher, W.K. (2011). Human mesenchymal stromal cells express CD14 cross-reactive epitopes. Cytometry Part A : the journal of the International Society for Analytical Cytology *79*, 635-645.

Pineault, N., and Abu-Khader, A. (2015). Advances in umbilical cord blood stem cell expansion and clinical translation. Exp Hematol *43*, 498-513.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Pittenger, M.F., and Martin, B.J. (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. Circulation research *95*, 9-20.

Ploemacher, R.E., van der Sluijs, J.P., Voerman, J.S., and Brons, N.H. (1989). An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood *74*, 2755-2763.

Poltavtseva, R.A., Nikonova, Y.A., Selezneva, II, Yaroslavtseva, A.K., Stepanenko, V.N., Esipov, R.S., Pavlovich, S.V., Klimantsev, I.V., Tyutyunnik, N.V., Grebennik, T.K.*, et al.* (2014). Mesenchymal stem cells from human dental pulp: isolation, characteristics, and potencies of targeted differentiation. Bulletin of experimental biology and medicine *158*, 164-169.

Ponte, A.L., Marais, E., Gallay, N., Langonne, A., Delorme, B., Herault, O., Charbord, P., and Domenech, J. (2007). The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem cells *25*, 1737-1745.

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. Cell *146*, 873-887.

Prockop, D.J., and Oh, J.Y. (2012). Medical therapies with adult stem/progenitor cells (MSCs): A backward journey from dramatic results in vivo to the cellular and molecular explanations. Journal of cellular biochemistry *113*, 1460-1469.

Psaltis, P.J., Paton, S., See, F., Arthur, A., Martin, S., Itescu, S., Worthley, S.G., Gronthos, S., and Zannettino, A.C. (2010). Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. Journal of cellular physiology *223*, 530-540.

Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P.*, et al.* (2005). Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. British Journal of Haematology *129*, 118-129.

Rafiq, Q.A., Coopman, K., Nienow, A.W., and Hewitt, C.J. (2016). Systematic microcarrier screening and agitated culture conditions improves human mesenchymal stem cell yield in bioreactors. Biotechnol J *11*, 473-486.

Rani, S., Ryan, A.E., Griffin, M.D., and Ritter, T. (2015). Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications. Molecular therapy : the journal of the American Society of Gene Therapy *23*, 812-823.

Reddy, K., Khaliq, A., and Henning, R.J. (2015). Recent advances in the diagnosis and treatment of acute myocardial infarction. World journal of cardiology *7*, 243-276.

Rehman, J. (2003). Peripheral Blood "Endothelial Progenitor Cells" Are Derived From Monocyte/Macrophages and Secrete Angiogenic Growth Factors. Circulation *107*, 1164-1169.

Riether, C., Schurch, C.M., and Ochsenbein, A.F. (2015). Regulation of hematopoietic and leukemic stem cells by the immune system. Cell Death Differ *22*, 187-198.

Rodig, N., Ryan, T., Allen, J.A., Pang, H., Grabie, N., Chernova, T., Greenfield, E.A., Liang, S.C., Sharpe, A.H., Lichtman, A.H.*, et al.* (2003). Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. European journal of immunology *33*, 3117-3126.

Rodrigo, S.F., van Ramshorst, J., Hoogslag, G.E., Boden, H., Velders, M.A., Cannegieter, S.C., Roelofs, H., Al Younis, I., Dibbets-Schneider, P., Fibbe, W.E.,

et al. (2013). Intramyocardial injection of autologous bone marrow-derived ex vivo expanded mesenchymal stem cells in acute myocardial infarction patients is feasible and safe up to 5 years of follow-up. Journal of cardiovascular translational research *6*, 816-825.

Rodrigues, C.A., Fernandes, T.G., Diogo, M.M., da Silva, C.L., and Cabral, J.M. (2011). Stem cell cultivation in bioreactors. Biotechnology advances *29*, 815-829.

Rohde, E., Bartmann, C., Schallmoser, K., Reinisch, A., Lanzer, G., Linkesch, W., Guelly, C., and Strunk, D. (2007). Immune cells mimic the morphology of endothelial progenitor colonies in vitro. Stem cells *25*, 1746-1752.

Rojewski, M.T., Fekete, N., Baila, S., Nguyen, K., Furst, D., Antwiler, D., Dausend, J., Kreja, L., Ignatius, A., Sensebe, L.*, et al.* (2013). GMP-compliant isolation and expansion of bone marrow-derived MSCs in the closed, automated device quantum cell expansion system. Cell transplantation *22*, 1981-2000.

Roy, S., Tripathy, M., Mathur, N., Jain, A., and Mukhopadhyay, A. (2012). Hypoxia improves expansion potential of human cord blood-derived hematopoietic stem cells and marrow repopulation efficiency. European journal of haematology *88*, 396-405.

Ruster, B., Gottig, S., Ludwig, R.J., Bistrian, R., Muller, S., Seifried, E., Gille, J., and Henschler, R. (2006). Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood *108*, 3938-3944.

Sadeghi, A., Pauler, L., Anneren, C., Friberg, A., Brandhorst, D., Korsgren, O., and Totterman, T.H. (2011). Large-scale bioreactor expansion of tumor-infiltrating lymphocytes. Journal of immunological methods *364*, 94-100.

Samsonraj, R.M., Raghunath, M., Nurcombe, V., Hui, J.H., van Wijnen, A.J., and Cool, S.M. (2017). Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. Stem cells translational medicine *6*, 2173-2185.

Samsonraj, R.M., Rai, B., Sathiyanathan, P., Puan, K.J., Rotzschke, O., Hui, J.H., Raghunath, M., Stanton, L.W., Nurcombe, V., and Cool, S.M. (2015). Establishing criteria for human mesenchymal stem cell potency. Stem cells *33*, 1878-1891.

Sanchez-Dominguez, R., Pereira-Mendez, S., Gomez, A., Torrabadella, M., Azqueta, C., Querol, S., Barquinero, J., and Gimeno, R. (2012). Notch signals contribute to preserve the multipotentiality of human CD34(+)CD38(-)CD45RA(-)CD90(+) hematopoietic progenitors by maintaining T cell lineage differentiation potential. Experimental hematology *40*, 983-993 e984.

Sanganalmath, S.K., and Bolli, R. (2013). Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions. Circulation research *113*, 810-834.

Santos, F., Andrade, P.Z., Abecasis, M.M., Gimble, J.M., Chase, L.G., Campbell, A.M., Boucher, S., Vemuri, M.C., Silva, C.L., and Cabral, J.M. (2011). Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions. Tissue engineering Part C, Methods *17*, 1201-1210.

Scheubel, R.J., Zorn, H., Silber, R.-E., Kuss, O., Morawietz, H., Holtz, J., and Simm, A. (2003). Age-dependent depression in circulating endothelial progenitor cells inpatients undergoing coronary artery bypass grafting. Journal of the American College of Cardiology *42*, 2073–2080.

Schlechta, B., Wiedemann, D., Kittinger, C., Jandrositz, A., Bonaros, N.E., Huber, J.C., Preisegger, K.-H., and Kocher, A.A. (2010). Ex-Vivo Expanded Umbilical Cord Blood Stem Cells Retain Capacity for Myocardial Regeneration. Circulation Journal *74*, 188-194.

Schmidt-Lucke, C., Fichtlscherer, S., Aicher, A., Tschope, C., Schultheiss, H.P., Zeiher, A.M., and Dimmeler, S. (2010). Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. PloS one *5*, e13790.

Schmidt-Mende, J., Hellström-Lindberg, E., Joseph, B., and Zhivotovsky, B. (2000). Freezing induces artificial cleavage of apoptosis-related proteins in human bone marrow cells. Journal of immunological methods *245*, 91-94.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

Schop, D., Janssen, F.W., van Rijn, L.D.S., Fernandes, H., Bloem, R.M., de Bruijn, J.D., and van Dijkhuizen-Radersma, R. (2009). Growth, Metabolism, and Growth Inhibitors of Mesenchymal Stem Cells. Tissue Eng Part A *15*, 1877-1886.

Segers, V.F., and Lee, R.T. (2008). Stem-cell therapy for cardiac disease. Nature *451*, 937-942.

Segers, V.F., Van Riet, I., Andries, L.J., Lemmens, K., Demolder, M.J., De Becker, A.J., Kockx, M.M., and De Keulenaer, G.W. (2006). Mesenchymal stem cell adhesion to cardiac microvascular endothelium:

activators and mechanisms. Am J Physiol Heart Circ Physiol *290*, 1370-1377.

Seita, J., and Weissman, I.L. (2010). Hematopoietic stem cell: self-renewal versus differentiation. Wiley interdisciplinary reviews Systems biology and medicine *2*, 640-653.

Senegaglia, A.C., Barboza, L.A., Dallagiovanna, B., Aita, C.A., Hansen, P., Rebelatto, C.L., Aguiar, A.M., Miyague, N.I., Shigunov, P., Barchiki, F.*, et al.* (2010). Are purified or expanded cord blood-derived CD133+ cells better at improving cardiac function? Experimental biology and medicine *235*, 119-129.

Shake, J.G., Gruber, P.J., Baumgartner, W.A., Senechal, G., Meyers, J., Redmond, J.M., Pittenger, M.F., and Martin, B.J. (2002). Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg *73*, 1919-1925.

Shi, Q., Rafii, S., Wu, M.H.-D., Wijelath, E.S., Yu, C., Ishida, A., Fujita, Y., Kothari, S., Mohle, R., Sauvage, L.R.*, et al.* (1998). Evidence for circulating bone marrow-derived endothelial cells. Blood *92*, 362-367.

Shoji, M., Sata, M., Fukuda, D., Tanaka, K., Sato, T., Iso, Y., Shibata, M., Suzuki, H., Koba, S., Geshi, E.*, et al.* (2004). Temporal and spatial characterization of cellular constituents during neointimal hyperplasia after vascular injury: Potential contribution of bone-marrow-derived progenitors to arterial remodeling. Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology *13*, 306-312.

Shpall, E.J., Quinones, R., Giller, R., Zeng, C., Baron, A.E., Jones, R.B., Bearman, S.I., Nieto, Y., Freed, B., Madinger, N.*, et al.* (2001). Transplantation of Ex Vivo Expanded Cord Blood. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *8*, 368-376.

Sibov, T.T., Severino, P., Marti, L.C., Pavon, L.F., Oliveira, D.M., Tobo, P.R., Campos, A.H., Paes, A.T., Amaro, E., Jr., L, F.G.*, et al.* (2012). Mesenchymal stem cells from umbilical cord blood: parameters for isolation, characterization and adipogenic differentiation. Cytotechnology *64*, 511-521.

Sidney, L.E., Branch, M.J., Dunphy, S.E., Dua, H.S., and Hopkinson, A. (2014). Concise Review: Evidence for CD34 as a Common Marker for Diverse Progenitors. Stem cells *32*, 1380-1389.

Simões, I.N., Boura, J.S., dos Santos, F., Andrade, P.Z., Cardoso, C.M., Gimble, J.M., da Silva, C.L., and Cabral, J.M. (2013). Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. Biotechnol J *8*, 448-458.

Singh, K.P., and Sharma, A.M. (2014). Critical limb ischemia: current approach and future directions. Journal of cardiovascular translational research *7*, 437-445.

Somerville, R.P., Devillier, L., Parkhurst, M.R., Rosenberg, S.A., and Dudley, M.E. (2012). Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE(R) bioreactor. Journal of translational medicine *10*, 69.

Sorrell, J.M., and Caplan, A.I. (2010). Topical delivery of mesenchymal stem cells and their function in wounds. Stem cell research & therapy *1*, 30.

Spence SE, Keller JR, Ruscetti FW, McCauslin CS, Gooya JM, Funakoshi S, Longo DL, and WJ., M. (1998). Engraftment of ex vivo expanded and cycling human cord blood hematopoietic progenitor cells in SCID mice. Experimental hematology *26*, 507-514.

Squillaro, T., Peluso, G., and Galderisi, U. (2016). Clinical Trials With Mesenchymal Stem Cells: An Update. Cell transplantation *25*, 829-848.

Stolberg, S., and McCloskey, E.K. (2009). Can shear stress direct stem cell fate? Biotechnol Prog *25*, 10-19.

Su, S.T., Yeh, C.L., Hou, Y.C., Pai, M.H., and Yeh, S.L. (2017). Dietary glutamine supplementation enhances endothelial progenitor cell mobilization in streptozotocin-induced diabetic mice subjected to limb ischemia. The Journal of nutritional biochemistry *40*, 86-94.

Sugita, N., Moriguchi, Y., Sakaue, M., Hart, D.A., Yasui, Y., Koizumi, K., Chijimatsu, R., Shimomura, S., Ikeda, Y., Yoshikawa, H.*, et al.* (2016). Optimization of human mesenchymal stem cell isolation from synovial membrane: Implications for subsequent tissue engineering effectiveness. Regenerative Therapy *5*, 79-85.

Sundrud, M.S., Koralov, S.B., Feuerer, M., Calado, D.P., Kozhaya, A.E., Rhule-Smith, A., Lefebvre, R.E., Unutmaz, D., Mazitschek, R., Waldner, H.*, et al.* (2009). Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. Science *324*, 1334-1338.

Suzuki, J.I., Shimamura, M., Suda, H., Wakayama, K., Kumagai, H., Ikeda, Y., Akazawa, H., Isobe, M., Komuro, I., and Morishita, R. (2015). Current therapies and investigational drugs for peripheral arterial disease. Hypertension research : official journal of the Japanese Society of Hypertension.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Takahashi, M., Matsuoka, Y., Sumide, K., Nakatsuka, R., Fujioka, T., Kohno, H., Sasaki, Y., Matsui, K., Asano, H., Kaneko, K.*, et al.* (2014). CD133 is a positive marker for a distinct class of primitive human cord blood-derived CD34-negative hematopoietic stem cells. Leukemia *28*, 1308-1315.

Taljaard, M., Ward, M.R., Kutryk, M.J., Courtman, D.W., Camack, N.J., Goodman, S.G., Parker, T.G., Dick, A.J., Galipeau, J., and Stewart, D.J. (2010). Rationale and design of Enhanced Angiogenic Cell Therapy in Acute Myocardial Infarction (ENACT-AMI): the first randomized placebo-controlled trial of enhanced progenitor cell therapy for acute myocardial infarction. American heart journal *159*, 354-360.

Tanaka, R., Vaynrub, M., Masuda, H., Ito, R., Kobori, M., Miyasaka, M., Mizuno, H., Warren, S.M., and Asahara, T. (2013). Quality-control culture system restores diabetic endothelial progenitor cell vasculogenesis and accelerates wound closure. Diabetes *62*, 3207-3217.

Tao, H., Han, Z., Han, Z.C., and Li, Z. (2016). Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. Stem cells international *2016*, 1314709.

Teng, X., Chen, L., Chen, W., Yang, J., Yang, Z., and Shen, Z. (2015). Mesenchymal Stem Cell-Derived Exosomes Improve the Microenvironment of Infarcted Myocardium Contributing to Angiogenesis and Anti-Inflammation. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology *37*, 2415-2424.

Tepper, O.M., Carr, J., Allen, R.J., Jr., Chang, C.C., Lin, C.D., Tanaka, R., Gupta, S.M., Levine, J.P., Saadeh, P.B., and Warren, S.M. (2010). Decreased circulating progenitor cell number and failed mechanisms of stromal cell-derived factor-1alpha mediated bone marrow mobilization impair diabetic tissue repair. Diabetes *59*, 1974-1983.

Thomas;, E.D., Lochte;, H.L., Lu;, W.C., and Ferrebee, J.H. (1957). Intravenous Infusion of Bone Marrow in Patients Receiving Radiation and Chemotherapy. N Engl J Med *257*, 491-496.

Timmins, N.E., Palfreyman, E., Marturana, F., Dietmair, S., Luikenga, S., Lopez, G., Fung, Y.L., Minchinton, R., and Nielsen, L.K. (2009). Clinical scale ex vivo manufacture of neutrophils from hematopoietic progenitor cells. Biotechnology and bioengineering *104*, 832-840.

Tipnis, S., and Viswanathan, C. (2010). Umbilical cord matrix derived mesenchymal stem cells can change the cord blood transplant scenario. Int J Stem Cells *3*, 103-118.

Tiwari, A., Wong, C.S., Nekkanti, L.P., Deane, J.A., McDonald, C., Jenkin, G., and Kirkland, M.A. (2016). Impact of Oxygen Levels on Human Hematopoietic Stem and Progenitor Cell Expansion. Stem cells and development.

Tran, C., and Damaser, M.S. (2015). Stem cells as drug delivery methods: application of stem cell secretome for regeneration. Advanced drug delivery reviews *82-83*, 1-11.

Troyer, D.L., and Weiss, M.L. (2008). Concise Review: Wharton's Jelly-Derived Cells Are a Primitive Stromal Cell Population. STEM CELLS *26*, 591-599.

van der Bogt, K.E., Schrepfer, S., Yu, J., Sheikh, A.Y., Hoyt, G., Govaert, J.A., Velotta, J.B., Contag, C.H., Robbins, R.C., and Wu, J.C. (2009). Comparison of transplantation of adipose tissue- and bone marrow-derived mesenchymal stem cells in the infarcted heart. Transplantation *87*, 642-652.

Vellasamy, S., Sandrasaigaran, P., Vidyadaran, S., George, E., and Ramasamy, R. (2012). Isolation and characterisation of mesenchymal stem cells derived from human placenta tissue. World journal of stem cells *4*, 53-61.

Venkat, R.V., Stock, L.R., and Chalmers, J.J. (1996). Study of hydrodynamics in microcarrier culture spinner vessels: A particle tracking velocimetry approach. Biotechnology and bioengineering *49*, 456-466.

Wagers, A.J., and Weissman, I.L. (2004). Plasticity of Adult Stem Cells. Cell *116*, 639-648.

Wagner, W., Saffrich, R., and Ho, A.D. (2008). The Stromal Activity of Mesenchymal Stromal Cells. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie *35*, 185-193.

Wakabayashi, T., Naito, H., Suehiro, J.I., Lin, Y., Kawaji, H., Iba, T., Kouno, T., Ishikawa-Kato, S., Furuno, M., Takara, K.*, et al.* (2018). CD157 Marks Tissue-Resident Endothelial Stem Cells with Homeostatic and Regenerative Properties. Cell stem cell.

Wang, D., Jin, Y., Ding, C., Zhang, F., Chen, M., Yang, B., Shan, Q., Zou, J., and Cao, K. (2011). Intracoronary delivery of mesenchymal stem cells reduces proarrhythmogenic risks in swine with myocardial infarction. Irish journal of medical science *180*, 379-385.

Wang, X.X., Zhang, F.R., Shang, Y.P., Zhu, J.H., Xie, X.D., Tao, Q.M., Zhu, J.H., and Chen, J.Z. (2007). Transplantation of autologous endothelial progenitor cells may be beneficial in patients with idiopathic pulmonary arterial hypertension: a pilot randomized controlled trial. Journal of the American College of Cardiology *49*, 1566-1571.

Watt, S.M., Athanassopoulos, A., Harris, A.L., and Tsaknakis, G. (2010). Human endothelial stem/progenitor cells, angiogenic factors and vascular repair. Journal of the Royal Society, Interface / the Royal Society *7 Suppl 6*, S731-751.

Watt, S.M., Gullo, F., van der Garde, M., Markeson, D., Camicia, R., Khoo, C.P., and Zwaginga, J.J. (2013). The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. British medical bulletin *108*, 25-53.

Wei, X., Yang, X., Han, Z.P., Qu, F.F., Shao, L., and Shi, Y.F. (2013). Mesenchymal stem cells: a new trend for cell therapy. Acta pharmacologica Sinica *34*, 747-754.

Whiteley, J., Bielecki, R., Li, M., Chua, S., Ward, M.R., Yamanaka, N., Stewart, D.J., Casper, R.F., and Rogers, I.M. (2014). An Expanded Population of CD34+ Cells from Frozen Banked Umbilical Cord Blood Demonstrate Tissue Repair Mechanisms of Mesenchymal Stromal Cells and Circulating Angiogenic Cells in an Ischemic Hind Limb Model. Stem cell reviews *10*, 338-350.

Wilcox, R.A., Feldman, A.L., Wada, D.A., Yang, Z.Z., Comfere, N.I., Dong, H., Kwon, E.D., Novak, A.J., Markovic, S.N., Pittelkow, M.R.*, et al.* (2009). B7-H1 (PD-L1, CD274) suppresses host immunity in T-cell lymphoproliferative disorders. Blood *114*, 2149-2158.

Wise, D.R., and Thompson, C.B. (2010). Glutamine addiction: a new therapeutic target in cancer. Trends in biochemical sciences *35*, 427-433.

Wu, R., Hu, X., and Wang, J. (2018). Concise Review: Optimized Strategies for Stem Cell-Based Therapy in Myocardial Repair: Clinical Translatability and Potential Limitation. Stem cells.

Wu, Y., Ip, J.E., Huang, J., Zhang, L., Matsushita, K., Liew, C.C., Pratt, R.E., and Dzau, V.J. (2006). Essential role of ICAM-1/CD18 in mediating EPC recruitment, angiogenesis, and repair to the infarcted myocardium. Circulation research *99*, 315-322.

Yamamoto, K., Takahashi, T., Asahara, T., Ohura, N., Sokabe, T., Kamiya, A., and Ando, J. (2003). Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. J Appl Physiol *95*, 2081-2088.

Yamanaka, S. (2010). Patient-Specific Pluripotent Stem Cells Become Even More Accessible. Cell stem cell *7*, 1-2.

Yang, Z., von Ballmoos, M.W., Diehm, N., Baumgartner, I., Kalka, C., and Di Santo, S. (2009). Call for a reference model of chronic hind limb ischemia to investigate therapeutic angiogenesis. Vascular pharmacology *51*, 268-274.

Yao, Y., Huang, J., Geng, Y., Qian, H., Wang, F., Liu, X., Shang, M., Nie, S., Liu, N., Du, X.*, et al.* (2015). Paracrine action of mesenchymal stem cells revealed by single cell gene profiling in infarcted murine hearts. PloS one *10*, e0129164.

Yi, W., Sun, Y., Wei, X., Gu, C., Dong, X., Kang, X., Guo, S., and Dou, K. (2010). Proteomic profiling of human bone marrow mesenchymal stem cells under shear stress. Molecular and cellular biochemistry *341*, 9-16.

Yoder, M.C., Mead, L.E., Prater, D., Krier, T.R., Mroueh, K.N., Li, F., Krasich, R., Temm, C.J., Prchal, J.T., and Ingram, D.A. (2007). Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem-progenitor cell principals. Blood *109*, 1801-1809.

Yong, K., Keng, C.T., Tan, S., Loh, E., Chang, K., Tan, T., Hong, W., and Chen, Q. (2016). Human CD34loCD133lo fetal liver cells support the expansion of human CD34hiCD133hi hematopoietic stem cells. Cellular & molecular immunology *13*, 605-614.

Yoo, S.Y., and Kwon, S.M. (2013). Angiogenesis and its therapeutic opportunities. Mediators of inflammation *2013*, 127170.

Yu, B., Zhang, X., and Li, X. (2014). Exosomes derived from mesenchymal stem cells. International journal of molecular sciences *15*, 4142-4157.

Zhang, C.C., and Sadek, H.A. (2014). Hypoxia and metabolic properties of hematopoietic stem cells. Antioxidants & redox signaling *20*, 1891-1901.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W., Ross, J., Haug, J., Johnson, T., Feng, J.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhang, L., and Xu, Q. (2014). Stem/Progenitor cells in vascular regeneration. Arteriosclerosis, thrombosis, and vascular biology *34*, 1114-1119.

Zhang, Y., and Gao, Y. (2016). Novel chemical attempts at ex vivo hematopoietic stem cell expansion. International journal of hematology *103*, 519-529.

CHAPTER II - MATERIALS AND METHODS

II.1 ISOLATION OF MONONUCLEAR (MNC) CELLS FROM THE UMBILICAL CORD BLOOD (UCB)

Umbilical cord blood (UCB) samples were kindly provided by Centro Hospitalar de Lisboa Norte, Hospital Santa Maria, Serviço de Ginecologia e Obstetrícia, and collected after maternal consent. Low density UCB MNC were separated by a Ficoll-Hypaque (GE Healthcare) density gradient (1.077 g/ml), and were then washed twice in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®-Life Technologies) containing 1% of Antibiotic-Antimycotic (AA, contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®; Gibco®-Life Technologies). UCB MNC were then resuspended in freezing medium (Gibco®-Life Technologies) in a concentration of $50x10^6$ cells/mL in 1mL CryoTube™ vials (Nunc), and cryopreserved in liquid/vapour phase nitrogen tanks until further use.

II.2 ISOLATION OF MESENCHYMAL STEM/STROMAL CELLS (MSC) FROM BONE MARROW (BM)

Bone marrow (BM) aspirates were collected from healthy donors after informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal. BM mesenchymal stem/stromal cells (MSCs) were isolated based on adherence to plastic. Briefly, low density BM mononuclear cells (MNCs) were obtained by a Ficoll-Hypaque density gradient, and were then washed twice in DMEM (supplemented with 10% Fetal Bovine Serum (FBS, Gibco®-Life Technologies) and AA solution (Gibco®-Life Technologies). Then, BM MNC were plated at a density of $2x10^5$ cells/cm^2 in cell treated culture flasks (BD Falcon) in low glucose DMEM with 10% FBS-MSC (FBS, MSC qualified, Gibco®-Life Technologies) and AA, at 37ºC and 5% CO_2 in a humidified incubator. The non-adherent cell fraction was removed and fresh medium was added 72h after plating. Medium replacement was done every 3-4 days until 70-80% confluence

was reached. Exhausted medium was then removed from the flasks, cells were washed with phosphate buffered saline (PBS, Gibco®-Life Technologies), and detached from the flask by incubation with the dissociation reagent Accutase® (Sigma) for 7 min at 37ºC. Cell detachment was verified using an inverted microscope, and enzymatic activity was stopped by adding DMEM with 10% FBS and AA. The resultant cell suspension was then transferred to conical tubes (BD Falcon), centrifuged for 7 min at 1250 rpm and resuspended in culture media. Cell count was performed using Trypan Blue (Gibco®-Life Technologies) exclusion method. Cells were then plated into new culture treated flasks in DMEM containing 10% FBS-MSC and AA, at a density range of $1x10^5$ – $3x10^5$ cells/cm^2 and their immunophenotypic characterization confirmed by flow cytometry. Fetal Liver (FL) Stro-1 derived MSCs and BM-Stro-1-MSCs were kindly provided by Professor Graça Almeida-Porada, University of Nevda Reno, USA and kept cryopreserved until further use.

II.3 ISOLATION OF UMBILICAL CORD-DERIVED STROMAL/STEM CELLS (UCM MSC)

Human UCM MSC were obtained from umbilical cord (UC) units kindly provided by Centro Hospitalar de Lisboa Norte, Hospital Santa Maria, Serviço de Ginecologia e Obstetrícia, upon informed maternal consent. Briefly, after washing UC with PBS and eliminating the blood clots, the UC was cut into smaller pieces and arteries and veins were removed. The remaining tissue was then minced, digested with 0.1 % Collagenase type II (Sigma) for 4h at 37ºC, and filtered with a 100 µm nylon mesh Steriflip® (Millipore). After washing the cell suspension with DMEM supplemented with 1% AA, the cell number and viability was determined using the Trypan Blue exclusion method, and cells were plated at an initial density of $10x10^3$ cells/cm^2. Cells were cultured in cell culture flasks with low glucose DMEM supplemented with 10%FBS-MSC and 1% AA, and kept at 37ºC and 5% CO_2 in a humidified atmosphere. After 48 h of culture, the non-adherent cells were removed and cells were maintained by replacing the medium every 3-4 days.

At 70-80% confluence, MSC were detached from the flasks by adding Accutase® solution for 7 min at 37ºC. Cell number and viability were determined using the Trypan Blue exclusion method, and cells were replated at an initial density of $3x10^3$ cells/cm^2. The immunophenotypic analysis of the isolated UCM MSC was done by flow cytometry.

II.4 CD34$^+$ ENRICHMENT OF HUMAN UCB CELLS

To obtain a suspension of purified Hematopoietic Stem and Progenitor Cells (HSPCs) and circulating progenitor cells (CAPC), UCB MNCs were picked from the cryopreserved samples and thawed in DMEM with 20%FBS supplemented with DNase I (10µg/mL, Roche™) to avoid clump formation. A maximum of 250 x 10^6 cells (5 vials) were thawed per 50 mL of thawing medium. UCB MNCs were then enriched for CD34$^+$ cells trough magnetic activated cell sorting (MACS®, Miltenyi Biotech), following manufacturer's instructions.

II.5 CELL LINES

Murine MS-5 stromal cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ ACC 441), and propagated in low glucose DMEM containing 10% FBS and1% AA.

Human Umbilical Vein Endothelial Cells (HUVEC-2) were obtained from BD and maintained in EGM-2 Bullet Kit CC-3162 (complete medium contains hEGF, Hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, Heparin and 2% FBS, Lonza).

All cell lines were cultured at 37ºC under 5% CO_2 in a humidified incubator. Medium was changed every 3-4 days and passage was done as described previously in chapter II.2.

II.6 CELL CRYOPRESERVATION AND THAWING

Upon expansion, cells were cryopreserved at an average cell density of 0.5-1x10^6 cells/mL (MSCs) or at 50x10^6 cells/mL (MNCs) in 1 ml CryoTube™ vials. Cells were centrifuged at 1250 rpm for 7 min and resuspended in Recovery™ Cell Culture Freezing Medium (Gibco®-Life Technologies) in 1 mL cryovials, which were kept for 24 h in a -80ºC freezer, in a Mr. Frosty/CoolCell® container, before being stored in a liquid/vapor phase nitrogen container.

For MSCs, cells were thawed by quickly warming the 1 mL cryovial in a water bath at 37ºC and diluting the cell suspension using 5 mL of DMEM supplemented with 20% FBS or MACs buffer, for MSCs/Cell lines and MNCs respectively. Cells were then centrifuged at 1250 rpm for 7 min and resuspended in the appropriate medium.

Cell number and viability was determined using the Trypan Blue exclusion method.

II.7 EX-VIVO EXPANSION OF CD34$^+$ STEM/PROGENITOR CELLAS

CD34$^+$ Circulating angiogenic progenitor cells (CAPCs) cells isolated from mobilized peripheral blood of healthy donors (n=5) were obtained from AllCells™ (CA, USA) and were cultured for 7 days in 30 mL Cell Culture Bags (VueLife™ FEP Bag, American Fluoroseal Corporation, MD, USA) at a density of 20x10^3 cells/mL in QQ medium composed by CellGro® SCGM (CellGenix, NH, USA) Serum-Free Media supplemented with SCF (100ng/mL), Flt-3 ligand (100ng/mL), TPO (20ng/mL), IL-6 (20ng/mL) and VEGF (50ng/mL) (Peprotech Inc., NJ, USA) supplemented with 1% of penicillin/streptomycin. The cells were incubated at 37ºC in a fully humidified incubator with atmospheric air supplemented with 5% CO_2.

CD34$^+$ cells isolated from frozen mononuclear cells (MNCs) from UCB, by magnetic activated cell sorting (MACS, Miltenyi), were cultured in both static (12 well plate) and

dynamic (50mL Spinner flask, StemSpan™) systems at a density of $20x10^3$ cells/mL in QQ medium composed by either SCGM (CellGenix) (only for static) or StemSpan™ SFEM II medium (StemCell Technologies) with SCF (100 ng/mL), Flt-3 ligand (100 ng/mL), TPO (20 ng/mL), IL-6 (20 ng/mL) and VEGF (50 ng/mL) for 10 and 12 days, respectively. In the dynamic system, cells were cultured in StemSpan™ spinner flasks with 30 mL working volume and 30 rpm agitation. The cells were incubated at 37ºC and 5% CO_2 (Normoxia) or at 5% O_2 (Hypoxia).

Cell number and viability were determined by Trypan Blue exclusion method.

II.8 IMMUNOPHENOTYPIC ANALYSIS

Characterization of pre- and post-expanded $CD34^+$ cells (CAPCs) was performed by flow cytometry using BD™ LSR Cell Analyser (BD Biosciences, CA, USA) and Cell Quest™ software (BD Biosciences) after staining with mouse anti-human monoclonal antibodies against specific surface markers: CD3-AF700, CD11b-PB, CD14-PerCP (R&D), CD16-AF700, CD18-FITC, CD19-APC, CD31-PB, CD34-PerCP, CD45-FITC, CD105-FITC, CD133-APC (Miltenyi Biotec, CA, USA). Antibodies were purchased from Biolegend (CA, USA), if not stated otherwise. Cells were stained with monoclonal antibodies for 20 min at 4°C following FcR blocking, washed twice using buffer, and analyzed. Isotype controls were also prepared for every experiment.

For HSC expansion experiments, both fresh $CD34^+$-enriched cells and expanded $CD34^+$-enriched cells were analysed by flow cytometry (FACScalibur, Becton Dickinson), using a panel of monoclonal antibodies (FITC-, or PE-conjugated) against: CD90 for HSCs; CD34 and CD133 for stem/progenitor cells; CD41 for megakaryocyte lineage, and CD14 for monocytic lineage; CD15 and CD33 for myeloid lineage; CD7 for early lymphoid cells. A minimum of 3×10^4 cells/tube were incubated with these monoclonal antibodies for 15 min in the dark at room temperature. Cells were washed afterwards with PBS and fixed with 1% paraformaldehyde (Sigma). Appropriate isotype controls were also prepared for

every experiment to exclude the possibility of non-specific binding of antibodies to Fc receptors.

MSC were characterized by flow cytometry using specific monoclonal antibodies according to criteria defined in the literature (Dominici et al., 2006). Over 95% of the population should express CD73, CD90 and CD105 and not express (less than 2%) CD14, CD19, CD31, CD34, CD45, CD80 and HLA-DR.

A minimum of 10 000 events was collected for each sample. Analysis was performed using FlowJo software.

II.9 QUANTITATIVE PCR

To assess the gene expression of angiogenic, pro-inflammatory and anti-inflammatory markers of the expanded cells (*vs.* non-expanded cells), quantitative PCR (qPCR) was performed. A quantity of 10 ng of total RNA (RNAqueous®-Micro Kit, Life Technologies) was used for reverse transcription (RT) reactions from each sample following manufacturer's protocol (iScript™, Bio-Rad, CA, USA). Reaction mixtures (15 µl) were incubated in a thermal cycler (Veriti® 96-Well Thermal Cycler; Applied Biosystems, CA, USA) for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C and then maintained at 4°C. For miRNA-210 measurement, TaqMan Small RNA kit was used (Applied Biosystems). Quantitative PCR assays were performed using a TaqMan assay kit (Applied Biosystems). qPCR was performed with a StepOnePlus™ Real-Time PCR System (Applied Biosystems). All reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min; all were performed in triplicate. The GAPDH and RNU48 (for miRNA-210) were used as internal controls to normalize differences in total RNA levels in each sample. A threshold cycle (Ct) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the ΔΔCt values and were expressed as $2^{\Delta\Delta Ct}$.

The value of each control sample was set at 1 and was used to calculate the fold of difference in the target gene.

II.10 *IN VITRO* TUBE FORMATION ASSAY

To evaluate the angiogenic potential of the cells, pre- and post- expanded cells were tested in the tube formation assay by co-culturing these with human umbilical vein endothelial cells (HUVECs) on either ECMatrix™ (Millipore, MA, USA) or Matrigel™ (BD). $1x10^3$ cells from each group (pre-expanded and post-expanded) were co-cultured with $1.5x10^4$ HUVECs in 50 µl of EBM-2 (Lonza, NJ, USA) medium with 2% FBS (Gibco®). A quantity of 50 µl of the cell suspension incubated at 37°C for 5 min. was applied onto ECMatrix™ or Matrigel™ (50 µl/well) in 96-well plate (3-10 wells/group). As a control, HUVEC were cultured alone. After incubation for 18 hours, a photomicrograph per well was taken under light microscopy (Leica DM IL LED with EC3 camera system, IL, USA), then the number of branch points was counted using the ImageJ (NIH) software.

II.11 HUMAN MSC CULTURE USING SERUM-SUPPLEMENTED MEDIUM UNDER STATIC CONDITIONS

Human MSC were plated at a cell density of $3\text{-}6x10^3$ cells/cm^2 in the appropriate treated plastic ware (either T-flasks or well-plates, BD Falcon), using low glucose DMEM supplemented with 10% FBS MSC. Complete culture medium changes were performed every 3-4 days and cells were passaged when they reached 70-80% cell confluence. Culture medium was removed and cells were washed once with PBS and then incubated with Accutase® for 7 min at 37ºC. Cell detachment was verified using a microscope, and enzymatic activity was stopped with DMEM supplemented with 10% FBS. The resultant

cell suspension was transferred to conical tubes and centrifuged for 7 min at 1250 rpm. Cell number and viability was then determined using the Trypan Blue exclusion method and cells were replated using the method described previously

II.12 HUMAN MSC CULTURE USING SERUM/XENOGENIC-FREE MEDIUM UNDER STATIC CONDITIONS

Human MSC were plated at a cell density of 3-6x10^3 cells/cm^2 on CELLstart™ pre-coated culture flasks using StemPro® MSC SFM or StemPro® MSC SFM XenoFree (Gibco®-Life Technologies). At 70-80% confluence, MSC were detached from the flasks by adding TryPLE (Gibco®-Life Technologies) solution for 7 min at 37ºC. After cell detachment had been verified using a microscope, enzymatic agent was diluted with DMEM containing 10% FBS, and the resultant cell suspension was transferred to conical tubes and centrifuged for 7 min at 1250 rpm. Cell number and viability were assessed using the Trypan Blue exclusion method, and cells were replated using the previously described method

II.13 HUMAN MSC EXPANSION IN SPINNER FLASKS USING XENO-FREE (XF) MEDIUM

BM MSC spinner flask expansion was performed under XF conditions based on our established protocol which was further optimized (Carmelo et al., 2015). Briefly, cells were expanded on microcarriers under dynamic conditions in spinner flask cultures, using Bellco® spinner flasks (Bellco Glass, Inc.) with a working volume of 100 mL or 250 mL, equipped with 90º paddles (normal paddles) and a magnetic stir bar. The flasks were previously coated with Sigmacote (Sigma) to minimize microcarrier and cell attachment to their inner walls. Prior to their use, the flasks were sterilized by autoclaving and

washed with culture medium StemPro® MSC SFM XenoFree. The initial cell density was $1x10^5$ cell/mL. Nonporous plastic microcarriers (SoloHill Engineering, Inc.) were prepared according to manufacturer's instructions. Then, plastic microcarriers were coated with a CELLstart™ CTS™ solution (diluted 1:100 in PBS with Ca^{2+} and Mg^{2+}) for 2 h at 37º C, with an intermittent agitation (2 min at 750 rpm, 10 min nonagitated) using a Thermomixer® confort (Eppendorf AG), and afterward equilibrated in prewarmed StemPro MSC SFM XenoFree medium. After laying the microcarriers inside the spinner flask, MSC previously expanded in a XF monolayer culture for at least one passage on CELLstart™ pre-coated T-175 flasks, were inoculated and culture medium was added to make up half of the working volume. The spinner flasks were placed inside the humidified incubator at 37º C and 5% CO_2. The standard agitation for the adhesion step started with 24 h of continuous low agitation (30 rpm). After that 24 h step, continuous agitation was set at 40 rpm. At day 3, culture medium was added up to the final working volume and from then on 25% of the volume was renewed every day for a total of 7-8 days of expansion. At the end of the cell expansion, cells were dissociated from the microcarriers by adding 40 or 80 mL of TryPLE (1x) (Gibco) into the spinner flaks (100 mL and 250 mL, respectively) and incubated at 37º C for 7 min at 50 rpm. To assure that there were no microcarriers present in the infusion solution, the cell *plus* microcarrier suspension was firstly filtered through a Steriflip® 100 µm filtration unit (Millipore) and secondly through a 30 µm separation filter (Miltenyi Biotec).

II.14 ESTABLISHMENT OF MSC-DERIVED FEEDER LAYERS FOR HEMATOPOIETIC SUPPORTIVE STUDIES

To establish a MSC-derived stromal feeder layer capable of supporting HSPC expansion, confluent cultures of BM/AT/UCM/Stro-1/FL MSC, were growth arrested by treatment with DMEM-10%FBS supplemented with mitomycin C (0.5 µg/mL), for 2.5 hours, at 37ºC, 5% CO_2.

II.15 EXPANSION OF CD34$^+$-ENRICHED CELLS (HSPC) UNDER STATIC CONDITIONS.

Suspensions of CD34$^+$-enriched cells were cultured (30 x 10^3 cells/mL) on top of a previously growth arrested human MSC-derived feeder layer, for 7 days without medium change, at 37ºC, 5%CO_2. CD34$^+$-enriched cells were also cultured in the absence of a stromal feeder layer (noStr) as a control condition. Quality Biological Serum-Free medium (QBSF-60; Quality Biological, Inc.) was used, supplemented with a specific cytokine cocktail, optimized in our lab (Andrade et al., 2010), referred as Z9: SCF (60 ng/ml), Flt-3L (50 ng/ml), TPO (55ng/ml) and bFGF (5 ng/ml).

II.16 EXPANSION OF CD34$^+$-ENRICHED CELLS (HSPC) UNDER DYNAMIC CONDITIONS

CD34$^+$-enriched cells were cultured in spinner flasks, in prototype SP medium enriched with cytokines from Company X, with or without stromal cells (MSC), in a 15 mL working volume spinner flask at an initial seeding density of 50 x 10^3 cells/mL. Agitation regimen was set to 40 rpm, intermittent for the first 12 h (5 min agitating and 4h stopped), and set to continuous agitation thereafter. At day 3, 50% of medium was changed with cell retention. HSC-MSC co-culture was performed with previously expanded microcarrier-adherent BM-MSC (see **II.13**) (100 x 10^3 cells/mL). MSC were previously treated with mitomycin inside the spinner flask, replacing the culture medium by 80 mL (minimal volume to cover paddles) of DMEM-10%FBS, supplemented with mitomycin C (0.5 µg/mL), for 2.5 hours and 30-40 rpm at 37ºC, 5% CO_2. After treatment, cells were washed by letting microcarriers settle, 3x with 20 mL of DMEM in a flacon tube. Cell count was performed at that time for microcarrier adhered MSC inoculation. Dynamic cultures were kept for 7 days at 37ºC, 5%CO_2,

II.17 CLONOGENIC ASSAYS

Colony forming unit in culture (CFU-C) assays are short-term, semi-solid colony assays. Three different scores were attributed to the colonies according to their composition, size, and color: Colony-forming unit–granulocyte macrophage (CFU–GM), colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte (CFU-Mix) and burst forming unit–erythroid (BFU–E).

Both fresh (day-0) and expanded (day-7) UCB CD34+-enriched cells are analysed to evaluate their clonogenic potential. Fold increase in clonogenic potential from day-0 to day-7 was measured for each condition. The clonogenic assays were performed by plating 1 x 10^3 fresh cells at day-0, and 5 x 103 expanded cells at day-7, in MethoCult GF H4434 (Stem Cell Technologies). The assay was done on 4-well plates with $2cm^2$ each. Three of the wells were loaded with the cell sample, the fourth was loaded with purified water to provide a steady source of humidity throughout the assay. The clonogenic cultures were maintained at 37ºC and 5% CO^2. After 14 days, the colonies were counted and categorized according to established criteria.

II.18 COBBLESTONE AREA-FORMING CELL (CAFC) ASSAY

Cobblestone-Area Forming Cells (CAFC) assay requires a stromal layer. Both fresh $CD34^+$ enriched cells and expanded cells (1 x 10^3 cells) were cultured on top of a confluent, mitomycin-treated (50 µg/ml in DMEM 10% FBS; for 2.5 hours at 37ºC, 5%CO_2) monolayer of cells in the MS-5 murine fibroblast cell line in long-term culture medium (Myelocult™, StemCell Technologies), supplemented with 10^{-6}M hydrocortisone (Sigma) in 24-well plates. Cobblestone areas of more than five tightly packed cells underneath the stromal layer were microscopically screened and scored 14 days after seeding (Denning-Kendall et al., 2003).

II.19 METABOLITE ANALYSIS IN DYNAMIC CULTURE

Metabolites from dynamic spinner flask cultures (MSC and CAPCs) were monitored daily. Supernatant samples from spinner flasks were retrieved and analyzed in an automatic analyzer YSI7100MBS (Yellow Springs Instrument®) to determine the concentration of glucose and glutamine (nutrients) and lactate (metabolite) throughout culture. Specific consumption/production rates (q_{MET}, pmol/day/cell) and the apparent yield of lactate from glucose ($Y'_{lact/gluc}$) were calculated according to equations II.1 and II.2, respectively.

$$q_{MET} = \frac{\Delta MET}{\Delta t \times \Delta X_v} \quad \textbf{(II.1)}$$

$$Y'_{lactate/glucose} = \frac{q_{lactate}}{q_{glucose}} \quad \textbf{(II.2)}$$

II.20 SHEAR STRESS ESTIMATION

Shear stress estimation for the dynamic cultures in spinner flasks can be calculated through Nagata's model (Croughan et al., 1987; Nagata, 1975).The average shear stress, τ (dyn/cm^2) is estimated by equation II.3, where ρ is the culture medium density (Kg.m^{-3}), ε is the energy dissipation per mass of fluid (W.Kg^{-1}) and υ is the kinematic viscosity of the fluid (m^2.s^{-1})

$$\tau = 5.33\rho(\varepsilon\upsilon)^{1/2} \quad \textbf{(II.3)}$$

The maximum dissipation energy, ε, occurs in the impeller zone and it can be approximated to the mean specific energy dissipation in the vessel, given by equation II.4, in which N_p is the dimensionless power number, D_i the agitator diameter (m), N the

agitation rate (revolutions per second, rps) and V_L the volume of medium in the vessel (m^3).

$$\varepsilon = \frac{N_p D_i N^2}{V_L} \quad \textbf{(II.4)}$$

If the flow is not fully turbulent, the power number N_p depends on agitation rate, and impeller shape. For spinner flask impellers N_p can be estimated by Nagata correlations, which were developed for unbaffled tanks with two-blade paddle impellers, given by equation II.5.

$$N_p = \frac{K_1}{Re} + K_2 \left[\frac{10^3 + 1.2Re^{0.66}}{10^3 + 3.2Re^{0.66}}\right]^{K_4} \quad \textbf{(II.5)}$$

And where K_1 to K_4 is given by equations II.6a - II.6d, where W_i is the impeller width (m) and D_t the spinner flask diameter (m).

$$K_1 = 14 + \left(\frac{W_i}{D_t}\right)\left[670\left(\frac{D_i}{D_t} - 0.6\right)^2 + 185\right] \quad \textbf{(II.6a)}$$

$$K_2 = 10^{K_3} \quad \textbf{(II.6b)}$$

$$K_3 = 1.3 - 4\left(\frac{W_i}{D_t} - 0.5\right)^2 - 1.14\left(\frac{D_i}{D_t}\right) \quad \textbf{(II.6c)}$$

$$K_4 = 1.1 + 4\left(\frac{W_i}{D_t}\right) - 2.5\left(\frac{D_i}{D_t} - 0.5\right)^2 - 7\left(\frac{W_i}{D_t}\right)^4 \quad \textbf{(II.6d)}$$

Finally, the Reynolds number is given by equation II.7.

$$Re = \frac{N D_i^2}{v} \quad \textbf{(II.7)}$$

On microcarrier cell culture, the impact of shear stress generated by agitation was determined based on Kolmogorov's theory of isotropic turbulence, assuming that for complete off-bottom suspension of microcarriers, virtually all stirred vessels must be agitated under turbulent regime. Per this theory, fluid perturbations at the impeller region create a cascade of eddies with microscale of turbulence, λ_K (m) that can be c **(II.8)** l through equation II.8.

$$\lambda_K = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4}$$

It is then commonly postulated that if the size of the biological entity is smaller than λ_K, damage will not occur. The smallest eddies in the vessel, known as Kolmogorov-scale eddies, given by the minimum value of λ_{Kmin}, are obtained for the maximum ε_{max}, which occurs in the impeller zone, and can be calculated by substituting vessel volume (V_L) by swept volume V_s in equation II.4. In the same manner, maximum shear stress τ_{max} is given by inputting at ε_{max} in equation II.3.

Swept volume can be calculated through equation II.9.

$$V_s = \pi \times \left(\frac{D_i}{2}\right)^2 \times W_i \qquad \textbf{(II.9)}$$

II.21 *IN VIVO* STUDY TO ATTEST THE SAFETY AND STABILITY OF EXPANDED CD34+ CELLS

II.21.1 EX-VIVO EXPANSION OF CIRCULATING ANGIOGENIC PROGENITOR CELLS (CD34+) FROM MPB

CD34+ cells isolated from mobilized peripheral blood of healthy donors (n=3) were obtained from Allcells™ and were cultured for 7 days in 6 well plates (Primaria, BD) at a density of $20x10^3$ cells/mL in QQc media as described in II.7.. The cells were incubated

at 37ºC, 21% O_2 and 5% CO_2. Cell number and viability were determined by Trypan Blue (Gibco) exclusion method.

II.21.2 ANIMALS

Male BALB/c nude mice with 6 to 8 weeks old and weighing 30-40 g were used in this study. A total of 48 mice were used (6 replicates, 2 conditions, 3 donors and 1 control). All animal studies were conformed to national and institutional guidelines. The protocols were approved by the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Case Western Reserve University (CWRU), School of Medicine, based on Guide for the Care and Use of Laboratory Animals.

II.21.3 SURGERY

Unilateral hind limb ischemia was surgically induced. Firstly, the mice were weighted and anesthetized by intraperitoneal injection of xylazine (40 mg/kg) and ketamine (100 mg/kg). The anesthetized mouse was placed under the stereotactic microscope and hands and feet were fixed with adhesive tape. Surgical field on the left leg (**Figure II-1**A) of the mouse was prepared by gently scrubbing with povidone iodine swabsticks (PDI Dwo-Swab Povidione Iodine Swabstick®). Then, a longitudinal 5 mm incision was done, beginning at the inguinal crease along the femoral vessels visible through the skin using ThoughCut® Scissors and Dumont Forceps (**Figure II-1** B and C). The connective tissue was griped to the right of the femoral bundle of artery, vein and nerve distal to the origin of the deep femoral branch with Dumont Tip Forceps and gently tugged caudally to slightly stretch and separate the femoral branch. Then, the femoral artery was separated from the nerve and vein by dissecting bluntly through the connective tissue, creating an opening of 1-2 mm (**Figure II-1** D and E). The artery ligation was done by penetrating the opening between the femoral vein and artery with the thread and pulling the tip from the opening between the artery and the vein (**Figure II-1** F). The femoral artery was then occluded by using double surgical knots (**Figure II-1** G). Mice underwent left femoral

artery ligation and transection at two points (**Figure II-1** H): proximally at inguinal ligament level and distally before bifurcation of popliteal and saphenous arteries and the epigastric artery was cauterized. Finally, the incision was closed with Autoclips® and the mouse was transferred to a heating pad until it recovered from the anesthesia.

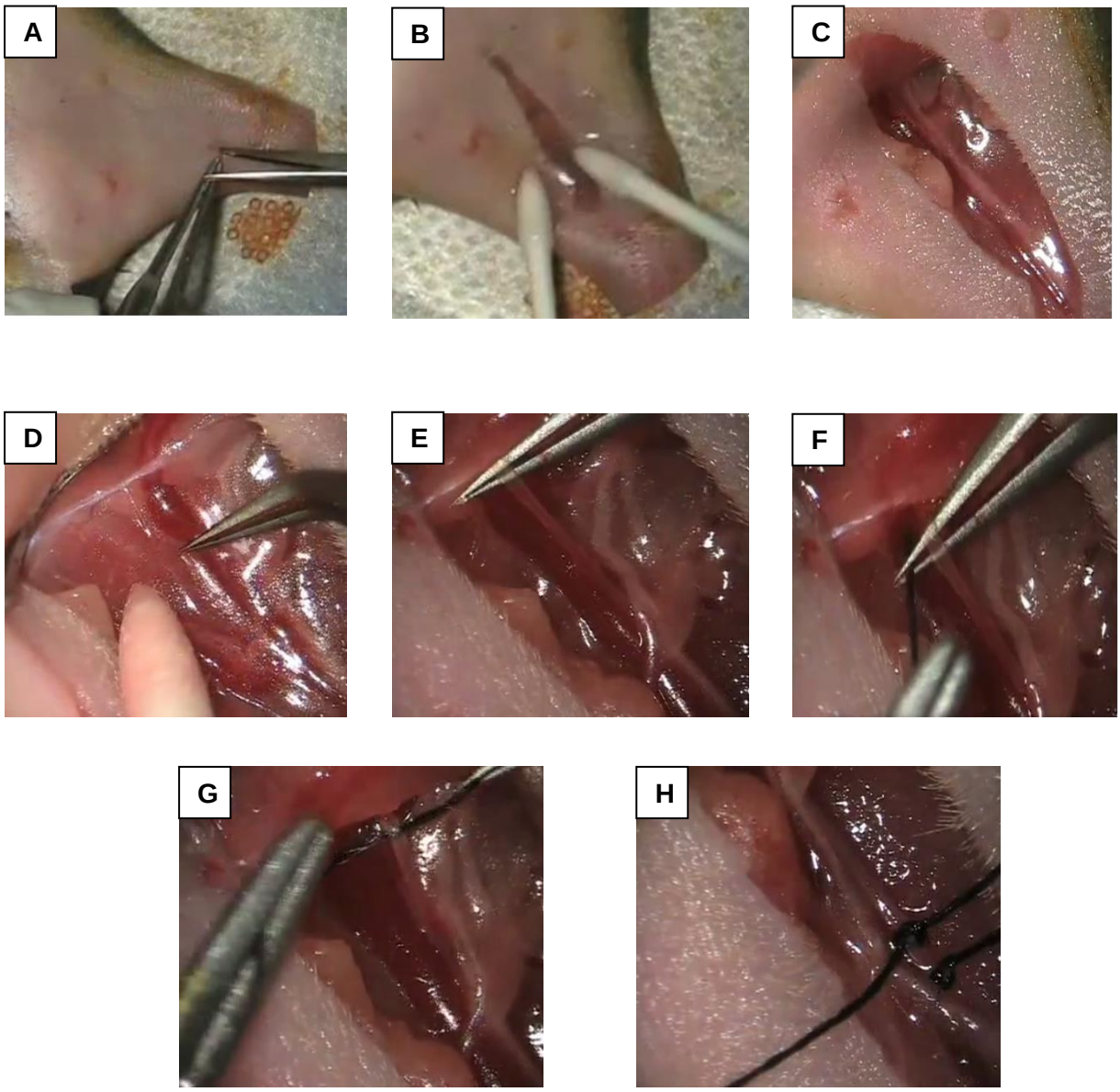


Figure II-1 *- Ventral aspect of mouse left upper hind limb and prepare for incision (A). Surgical skin incision (B) and femoral bundle exposure (C). Blunt dissection of the connective tissue (D) and separation of femoral artery and vein (E). Insertion of the surgical threads underneath the femoral artery (F) and occlude artery with surgical knot (G). Two point ligation of the artery (H), where the artery will be cut. Adapted from (Niiyama et al., 2009)*

II.21.4 CELL INFUSION

One day after surgery, mice were weighted and $2.5x10^4$ $CD34^+$ cells (pre and post expanded) suspended in 30 µL of PBS, or PBS only (control group) was injected into the left adductor muscles.

II.21.5 CLINICAL OBSERVATION

Mice were clinically observed for a total period of 12 weeks. Once a week, a clinical observation was done, registering several aspects: body weight, response to external stimuli, cage movement, shortness of breath, skin appearance, ear color and fecal observation.

II.21.6 ORGAN COLLECTION FOR HISTOLOGY

At the end of the 12 week period, mice were sacrificed in a CO_2 chamber and the following organs collected: brain, left and right adductor muscles, left and right gastrocnemicus muscles, heart, lungs, liver, spleen and kidneys as previously described (Antal et al., 2007). Briefly, the organs were cut and then placed with the proximal part facing the bottom of the cassette, which had been covered with Tissue Freezing Media (TFM®, Triangle Biomedical Sciences, Inc., Durham, NC, USA). Some organs (lungs, liver and spleen) do not have a direction, so a section was cut and placed against the cassette. Immediately after, cassettes were filled with TFM® and snapped frozen in liquid nitrogen. Samples were stored at -80ºC for future histological analysis.

II.22 DATA ANALYSIS

All data is presented as mean ± standard error of the mean (SEM) of the values obtained for different cell donors, and was analyzed with SPSS Statistics software (IBM, U.S.A).

The results were statistically analyzed by performing a paired t-test when the data was normally distributed (upon Shapiro-Wilk testing) for comparison of data between pre- and post-expanded cells. When this requirement was not satisfied, the non-parametric Wilcoxon test was used. The comparisons among three groups were made using one-way analysis of variance (ANOVA). Post hoc analysis was performed by Tukey's test. $P< 0.05$ was considered to be statistically significant.

II.23 REFERENCES

Andrade, P.Z., dos Santos, F., Almeida-Porada, G., da Silva, C.L., and JM, S.C. (2010). Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells. Molecular bioSystems *6*, 1207-1215.

Antal, C., Teletin, M., Wendling, O., Dgheem, M., Auwerx, J., and Mark, M. (2007). Tissue collection for systematic phenotyping in the mouse. Current protocols in molecular biology *Chapter 29*, Unit 29A 24.

Carmelo, J.G., Fernandes-Platzgummer, A.M., Diogo, M.M., Silva, C.L.d., and Cabral, J.M.S. (2015). A Xeno-free Microcarrier-based Stirred Culture System for the Scalable Expansion of Human Mesenchymal Stem/Stromal Cells isolated from Bone Marrow and Adipose Tissue. Biotechnology Journal *10*, 1235-1247.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315-317.

Niiyama, H., Huang, N.F., Rollins, M.D., and Cooke, J.P. (2009). Murine model of hindlimb ischemia. Journal of visualized experiments : JoVE.

CHAPTER III - SCALING-UP THE *EX VIVO* EXPANSION OF HUMAN CIRCULATING CD34^{+}PROGENITOR CELLS WITH UP-REGULATION OF ANGIOGENIC AND ANTI-INFLAMMATORY POTENTIAL.

This chapter is based on the published article: Mata, MF, Lopes, JP, Ishikawa, M, Alaiti, MA, Cabral, JM, da Silva, CL, Costa MA. Scaling-up the *ex vivo* expansion of human circulating CD34^{+}progenitor cells with up-regulation of angiogenic and anti-inflammatory potential. Cytotherapy. 2015 Dec;17(12):1777-84

III.1 SUMMARY

The therapeutic application of $CD34^+$ Circulating Progenitor Cells (CAPCs) (which includes endothelial progenitor cells (EPCs)) has been hampered by the quantity and quality of isolated circulating $CD34^+$ cells from patient's peripheral blood. Our group had previously established a suspension culture system for human $CD34^+$ cells, with increased quantity and quality (QQ) of the angiogenic cell product. Here, we firstly tested the safety and stability of $CD34^+$ derived from mobilized peripheral blood (mPB) in an *in vivo* model of limb ischemia and, secondly, successfully scaled-up the expansion process by using culture bags since there is the need to move towards a dynamic and fully controlled bioreactor system in order to meet GMP standards and attain clinically meaningful cell doses in a time- and cost-effective way. It was shown that $CD34^+$ cells isolated from mPB of healthy donors expanded *ex vivo* for 7 days in QQ medium (serum-free) in cell culture plates were safe and stable regarding clinical observation of the injected animals during the 3-month period monitoring as no adverse effects were found.

Additionally, these cells were expanded *ex vivo* in cell culture bags (30 mL) and pre and post expansion cells were characterized by flow cytometry, qPCR and their angiogenic potential assessed by *in vitro* tube formation assay. Our data shows effective expansion of the cultured population (7-fold), while maintaining the stem/progenitor content and increasing the endothelial population. Moreover, post-expanded cells showed higher tube formation capacity compared to pre-expanded cells. In addition, an up-regulation of the anti-inflammatory gene expression and a down-regulation of pro-inflammatory genes were observed, which suggests that the increase in angiogenic potential is not paired with an increase in the inflammatory profile. Hence, the QQ expansion method was successfully scaled-up to cell culture bags, able to meet GMP standards, and expanded cells presented a higher *in vitro* angiogenic profile.

III.2 BACKGROUND

Peripheral arterial disease (PAD) is a common syndrome that affects approximately 15-20% of the world population older than 70 years old, characterized by pain, chronic skin ulceration, gangrene, amputation, infection, and ultimately death (Norgren et al., 2007). Revascularization either by endovascular means or by open surgery, combined with the best possible risk factor modification has not yet been able to allow limb salvage and relief of ischemic rest pain for a large majority of patients (Kalka and Baumgartner, 2008). In this context, stem/progenitor cell-based therapies have emerged, given their potential to proliferate, differentiate, and form new blood vessels or to support other cell types involved in the neovascularization process (Alaiti et al., 2010; Perin and Silva, 2011). Initial preclinical studies evaluated pluripotent embryonic stem cells (ESCs) and lineage-committed progenitor cells as $CD34^+$ progenitor cells, that were previously defined as hematopoietic stem/progenitor cells, but also serve as an enriched source for endothelial progenitor cells (EPCs) found within the bone marrow (BM), mobilized peripheral blood (mPB) and umbilical cord blood (UCB) (Asahara et al., 1997; Ingram et al., 2004; Shi et al., 1998). Early clinical trials have suggested some potential benefits of $CD34^+$-based therapy in patients with ischemic arterial peripheral or coronary disease (Assmus et al., 2002; Dong et al., 2013; Kinoshita et al., 2012; Perin and Silva, 2011). However, some controversial issues have emerged when analyzing the clinical benefits of $CD34^+$-therapy, which might be due to the variety of protocols used for cell isolation and the type of cells administered to patients (*i.e.* either enriched progenitor fractions or whole mononuclear cell (MNC) fraction).

Although CD34 enrichment using MACS technology represents a feasible strategy to isolate $CD34^+$ cells as EPC containing cell fraction (Schmidt-Lucke et al., 2010), its frequency in peripheral blood is very low (less than 0.1%), limiting the cell numbers that can be obtained (Scheubel et al., 2003). In addition, in the setting of autologous therapy,

one must take into account that the quality of the cells (*i.e.* their angiogenic potential) is affected by increasing age and disease (Numaguchi et al., 2006).

In this context, an effective therapeutic product based on $CD34^+$ progenitor cells mandates their *ex-vivo* expansion, not only to achieve clinically meaningful cell numbers, but mostly due to the superior quality of cultured cells when compared to freshly isolated $CD34^+$ cells, which has been reported to result in an higher extent of neovascularization after transplantation (Asahara et al., 2011; Kalka et al., 2000). To this end, different EPC-enriched culture methods have been developed, including adherent culture on either collagen or fibronectin coated plates (Colombo et al., 2013), which despite the high performance for vasculogenesis attained in animal models, is time consuming, as it takes several weeks to isolate the cells (Hur et al., 2004) and enhances cellular senescence. In addition, to harvest cells as the final product, cells will be incubated with trypsin/EDTA and scraped when cultured as adherent cells. From the practical standpoint, this kind of manual procedure is time consuming. Considering a translational scenario to the clinical bedside, one should take into consideration that the amount of labor as well as cost and time to achieve meaningful cell numbers remains challenging when using adherent cultures. Thus, alternative suspension culture methods have been developed, where a higher quantity and quality of the final cell product is claimed with efficient neovascularization in ischemic animal models (Bouchentouf et al., 2011; Masuda et al., 2012; Masuda et al., 2014; O et al., 2011). Nevertheless, despite the proven efficacy and quality of the cells, it is mandatory to access the stability and safety of the post-expanded cells before moving towards a clinical therapy, and in that line *in vitro* and *in vivo* studies must be performed. Hence, it is crucial to test the safety and stability of these cells *in vivo* with an appropriate animal model before proceeding to clinical trials.

Here, we investigated the safety and stability of post expanded $CD34^+$ cells in a mouse model of hind limb ischemia where, after surgery, cells (pre and post expanded) were injected in the adductor muscle and the animals were clinically observed for 3 months.

After this period, the animals were euthanized and the organs collected for histologic studies.Moreover, there is the need to comply with GMP standards when scaling-up the suspension $CD34^+$ Circulating Angiogenic Progenitor Cells (CAPCs) culture. Cell culture bags, widely used in clinical trials for T cell expansion, as well as other immune cell types (Leemhuis et al., 2005; Sadeghi et al., 2011; Somerville et al., 2012; Timmins et al., 2009), combined with defined culture reagents (ex. serum-free medium) represent a suitable option for the scale-up of $CD34^+$ CAPC culture in a clinical setting.

III.3 RESULTS

III.3.1.1 Safety and stability of the expanded cells

The safety and stability of post-expanded peripheral blood mobilized $CD34^+$ cells from three healthy donors were evaluated in a mouse model of unilateral hind limb ischemia induced surgically. Pre and post expanded cells (2.5×10^4) as well as PBS only (control group) were injected one day after surgery into the adductor muscle of the left leg (where the surgery was performed) and clinical observation of several aspects was registered for a period of 12 weeks. The graphs for the weight measurements of the animals for this period of time are represented in **Figure III-1**.

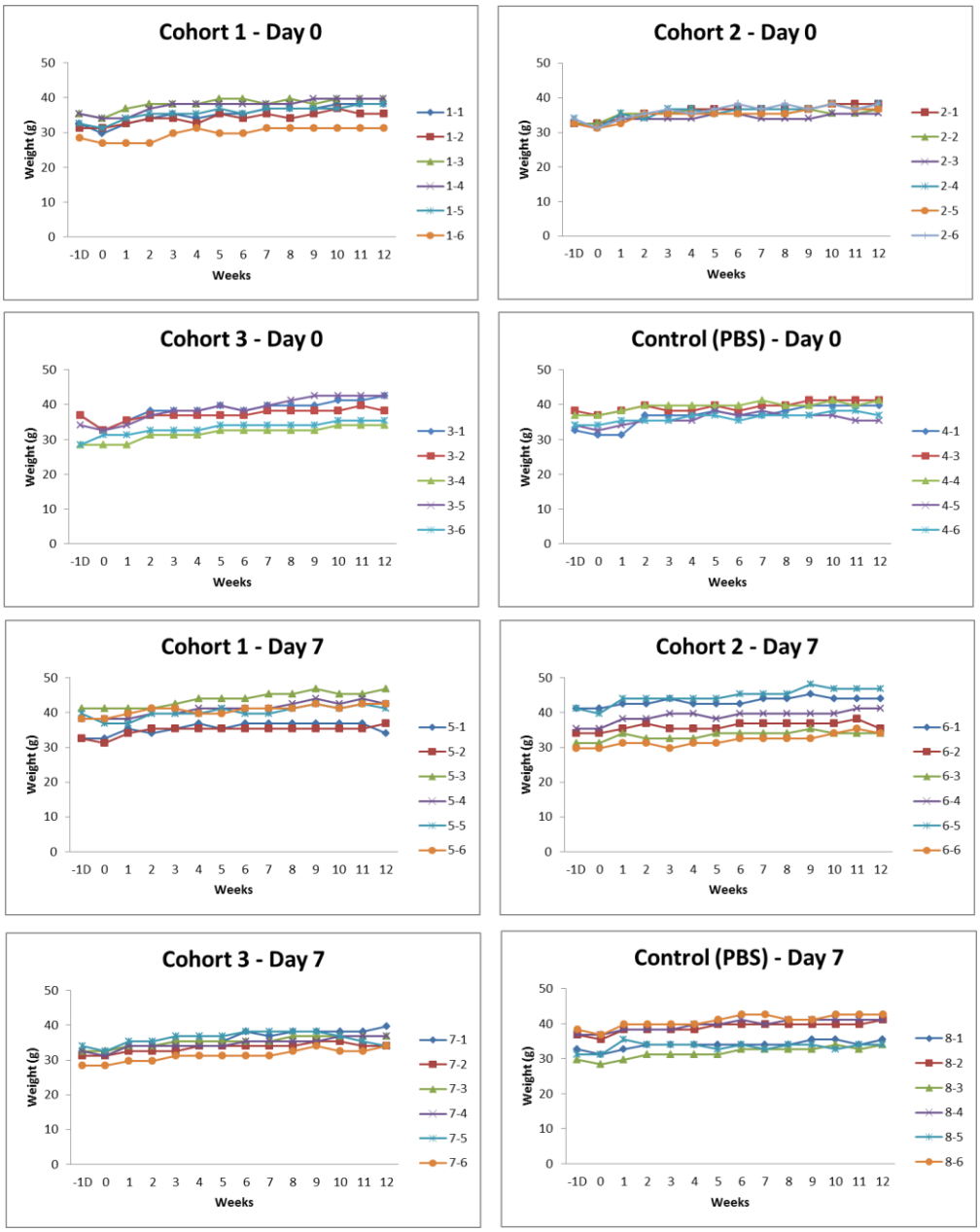


Figure III-1 *- Graphic representation of the weight measurement of the animals throughout the 12 weeks period. Day 0 represent the pre expanded cells and day 7 the post expanded cells. Three different donors (Cohorts 1, 2 and 3) and control group (PBS only). Day -1D*

There were no major changes in the mouse weight, as well as the other physical aspects noted (response to external stimuli, cage movement, shortness of breath, skin appearance, ear color and fecal observation). The only aspect that must be pointed here is that in both groups of day 0 Cohort 3 and Control (PBS), there was an animal that did not survive but it was due to surgical complications where the vein was accidentally pinched, creating a severe hemorrhage.

III.3.1.2 Scale-up of cell expansion into cell culture bags

The expansion of $CD34^+$ cells from mPB of healthy donors was successfully achieved in cell culture bags, with an average increase of Total Nucleated Cells (TNC) of 7.5-fold ($p<0.05$) (**Figure III-2**A) leading to an average total cell number of $4x10^6$. In addition, an increase in the total number of CD34+ cells of 5.3-fold ($p<0.05$) and 8.5-fold ($p<0.05$) for EPCs, defined as CD34+KDR+CD45dim (11), was attained (**Figure III-2**B).

Moreover, pre- and post-expanded cells were characterized by flow cytometric analysis of stem/progenitor, endothelial and mono/lymphoid markers (**Figure III-2**C, D and E). Regarding the stem/progenitor content of the expanded population (**Figure III-2**C), the percentage of $CD34^+$ cells decreased from 97% to 68% ($p<0.05$) and the expression of CD133 decreased from 86% to 62% ; for the $CD34^+/CD133^+$ cells, it was observed a decrease from 88% to 47% . The levels of expression of the lineage marker $CD45^+$ cells did not significantly change (~98%) with the cell culture. On the other hand, the percentage of progenitor/endothelial markers (**Figure III-2**D) increased significantly with culture, with an increase in $CD105^+$ content from 0.70% in uncultured cells to 12% for the expanded cells ($p<0.05$). Concerning the expression of CD31, there was an increase from 0.68% to 51% ($p<0.05$). Concomitantly, the expression of other hematopoietic markers (**Figure III-2**E), namely from lymphoid lineage, was maintained at low levels and did not change significantly or even tended to decrease ($CD19^+$ was 1.8% for the pre-expanded cells and 0.25% for the post-expanded cells). On the other hand, considering a monocytic phenotype, $CD14^+$ expression was higher in post-expanded than in pre-expanded cells (0.22% to 1.3%, $p<0.05$), while CD18+ content rose from 7.7% to 66% ($p<0.05$), which was concurrent with the increase in the expression of endothelial specific markers (Gulati et al., 2003; Wu et al., 2006).

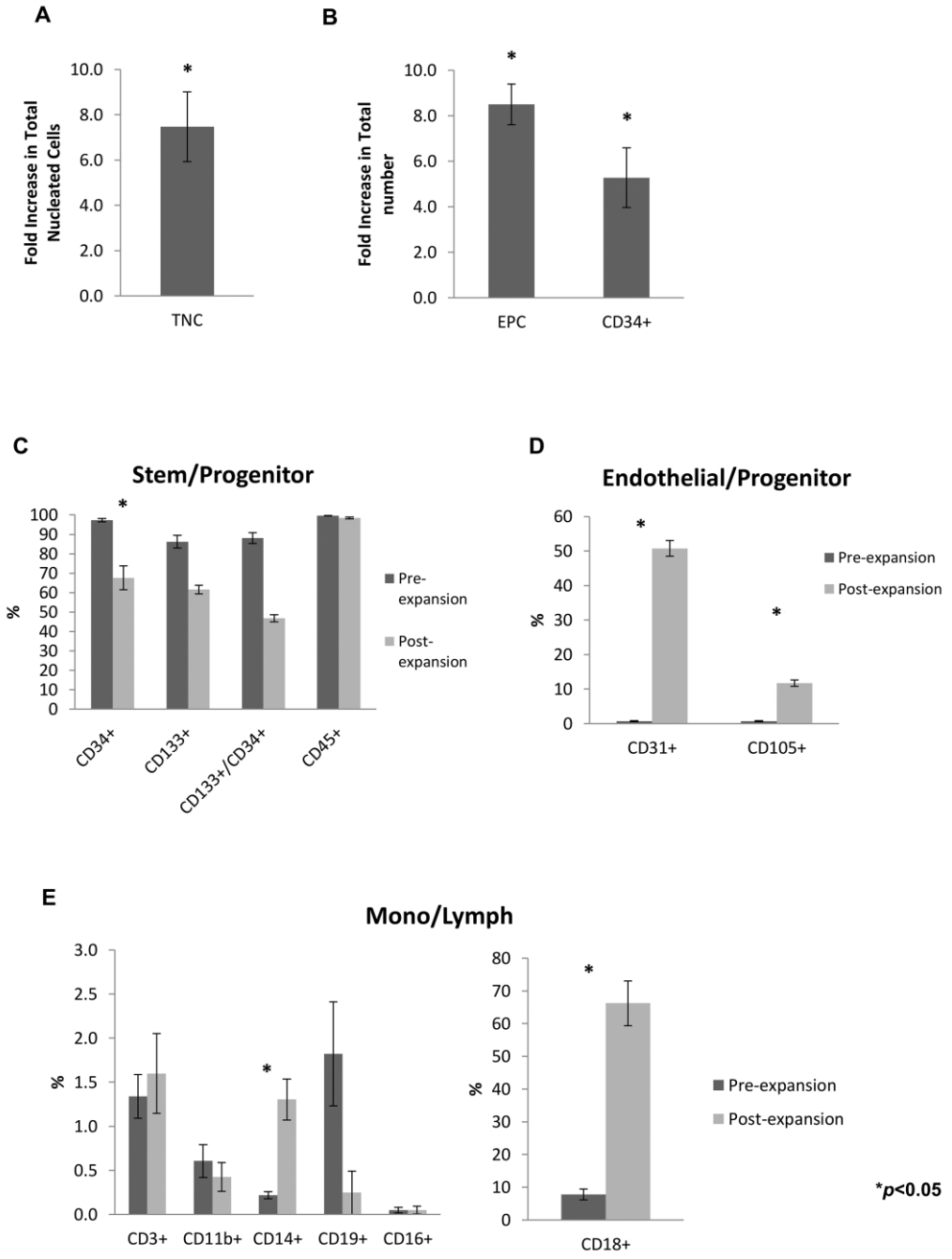


Figure III-2 *- Fold Change in total cells and characterization of pre- and post-expanded cells analyzed through Flow Cytometry (n=5). (A) Fold Increase in total nucleated cells. (B) Fold increase in total EPCs and CD34+ cells. (C) Percentage of expression of stem/progenitor markers. (D) Percentage of expression of endothelial/progenitor markers. (E) Percentage of expression of monocytic/lymphoid markers. Results are given as mean ± SEM (*p<0.05).*

Pre- and post-expanded cells were also characterized for the expression of angiogenic, anti- and pro-inflammatory and senescence/apoptotic markers through qPCR (**Figure**

III-3). The results show that there was an upregulation of pro-angiogenic gene expression with the culture (**Figure III-3**A). It was observed a 9.5-fold increase for mir-210 ($p<0.05$) and an increase of 2.5-fold for VEGFA ($p<0.05$). Regarding the pro-angiogenic CXCL12, MMP9 and PDGFB transcription, it was also observed an increase of 3.7-fold, 4.0-fold and 14-fold, respectively, although with no statistical significance. There was a decrease in the expression of KLF2 (by 0.48-fold, $p<0.05$). An upregulation of the anti-inflammatory gene expression (**Figure III-3**B), with a 3.9-fold increase for IL-10 ($p<0.05$) and a 4.4-fold increase for IL1RN ($p<0.05$) was also observed. Although without statistical significance, HGF transcript level displayed an increasing trend, while the expression of pro-inflammatory factors did not change significantly (**Figure III-3**C) as it is the case of ANG1, ANG2 and M-CSF, or even decreased substantially as seen for TNFα (0.30-fold, $p<0.05$), IL-1B (0.75-fold, $p<0.01$), IL6 (0.16-fold, $p<0.01$) and G-CSF (0.42-fold, $p<0.05$).

Finally, regarding the markers for senescence (P16) and apoptosis (Annexin V), there was no significant change in expression between the cultured and uncultured cells (**Figure III-3**D).

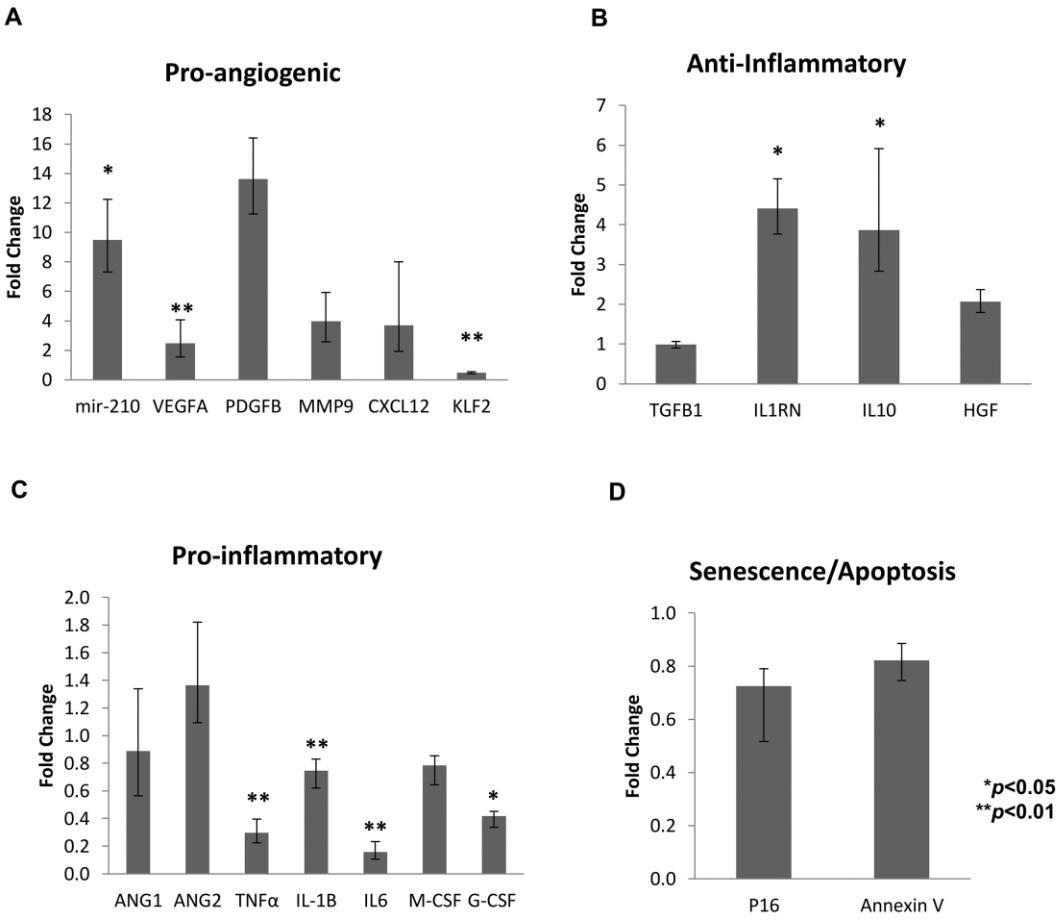


Figure III-3- *Q-PCR characterization of pre- and post-expanded cells given as fold change of the post-expanded/pre-expanded ratio (n=4 – 5). (A) Fold change in pro-angiogenic gene expression. (B) Fold change in anti-inflammatory gene expression. (C) Fold change in anti-inflammatory gene expression. (D) Fold change in senescence/apoptosis gene expression. Results are given as mean ± SEM (*p<0.05; **p<0.01).*

To access the angiogenic potential of pre- and post-expanded cells, an in vitro co-culture tube formation assay was pursued, where HUVECs were used as control (**Figure III-4**). Expanded CD34+ cells were not only able to form tubes in vitro (**Figure III-4**A) but also showed a higher angiogenic capacity (~1.20-fold, p<0.01) compared to the pre-expanded cells (**Figure III-4**B).

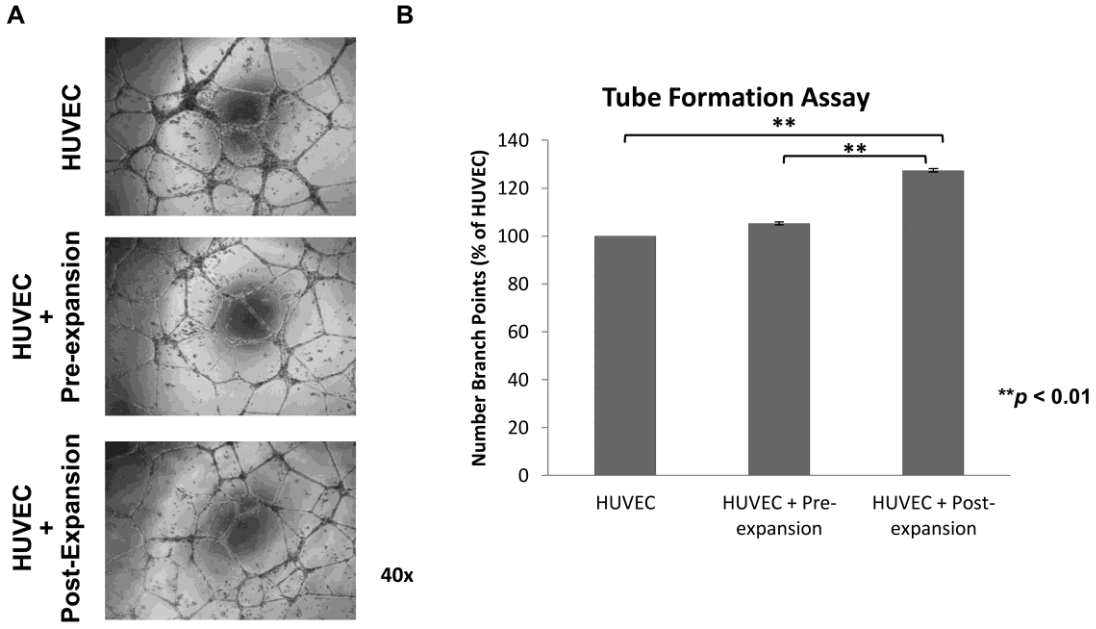


Figure III-4*- Assessment of the angiogenic potential in vitro through tube formation assay by the co-culture of pre- and post-expanded cells (1x10^3) with HUVEC (1.5x10^4) (n=5). (A) Representative photomicrograph obtained in the assay for the control (HUVEC only) and co-culture of HUVEC + pre-expanded cells and HUVEC + post-expanded cells after 18 hours of incubation. (B) Percentage of the number of branch points relative to HUVEC (100%). Results are given as mean ± SEM (**p<0.01).*

III.4 DISCUSSION AND CONCLUSIONS

III.4.1.1 Safety and stability of the expanded cells

Despite that in terms of the physical outcomes there were no adverse situations, it is important to access further the effect of the cell treatment in the tissues, i. e. if the injected cells produced any complications that are not visible at naked eye through clinical observation, such as tissue necrosis or tumor growth. It is also important to evaluate, for the preclinical safety, where these cells are located after transplantation and in that way several organs were collected for histological analysis by a professional and accredited laboratory (Tissue Resources Core Facility of the Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio), since this study was part of a proposition for a ‘’first in human’’ clinical trial.

Taken together, these results, along with the *in vitro* analysis (FACS, qPCR (angiogenic, pro-inflammatory and anti-inflammatory profile), telomerase activity, tube formation assay and karyotyping) shown previously by our lab, primarily indicate that the previously optimized expanded $CD34^+$ cells treatment into a mouse model of hind limb ischemia is safe, stable and has higher therapeutic potentiality. However, as mentioned before, in order to fully attest the safety of these cells to move towards a clinical trial, the toxicological analysis must be performed through the histologic study of the several organs.

III.4.1.2 Scale-up of cell expansion into cell culture bags

In this study, we successfully scaled-up a serum-free mPB-derived $CD34^+$ cell culture with the previously established and optimized QQ medium formulation (Masuda et al., 2012) using cell culture bags (30 mL volume) resulting in a 7.5 fold increase in total cells. Moreover, this method introduced high levels of stem/progenitor cells and increased the endothelial committed fraction, resulting in an effective expansion and fortification of the final cell product for angiogenic cell therapy. To our knowledge, this is the first report that confirms the feasibility of mPB-$CD34^+$ cell expansion and priming in a GMP-compatible culture system, featuring serum-free medium culture conditions and a disposable bag, resulting in a cell product with increased angiogenic and anti-inflammatory properties, that can further be translated to the clinical setting.

Therapeutic neovascularization has the potential to add to the treatment of ischemic diseases, either through administration of soluble factors, such as cytokines, or through cell-based therapies. $CD34^+$ cells (which includes EPCs) have been suggested to play an important role in vasculogenesis and arteriogenesis (Alaiti et al., 2010). Thus, their transplantation opened unprecedented opportunities for the treatment of such diseases. The mechanism of action of these cells can rely on paracrine signaling, where they

participate in activation of the endogenous wound repair machine, or can contribute directly to tissue regeneration by engrafting and differentiating into new blood vessels.

A consensus definition of these cells is not yet established in the field. Very briefly, EPCs can be characterized into two subsets: hematopoietic (which includes early EPC (eEPC)) and non-hematopoietic (which includes late outgrowth EPC (oEPC)) (Asahara et al., 2011). The eEPCs are angiogenic in that they can extend vasculature from pre-existing blood vessels, acting through paracrine signaling. These cells arise from hematopoietic cells (monocytes, myeloid progenitor cells, and T lymphocytes) and are further characterized by limited *ex vivo* proliferative capacity. Regarding human oEPCs, they are truly endothelial lineage committed cells, with studies demonstrating the ability of these cells to form *de novo* blood vessels (Yoder et al., 2007). Nevertheless, it is believed that both hematopoietic and endothelial lineages come from a common embryological ancestor, the hemangioblast; and a potential common precursor in adulthood may persist, (Ingram et al., 2004). Hence, the results obtained herein suggest that the expanded cell product has a hematopoietic origin, due to the expression of the lineage marker CD45 that may suggest the mechanism of action of these cells to be through paracrine signaling, corroborated by the gene expression analysis.

The differences throughout the literature regarding EPC characterization may be due to the variety of cell isolation processes performed and even the culture methodologies used. Indeed, the lack of standardization is one of the major problems that must be surpassed to achieve an optimized and GMP-compliant protocol for cell product manufacturing. It is also important to address the cost-effectiveness of bringing such therapy from bench to bedside, namely in terms of cell processing cost. Therefore, optimization of processing steps (cell isolation and cultivation), time and cost is mandatory. The suspension culture method represents a better manufacturing choice, compared to adherent culture, since it significantly reduces time, labor and costs of processing and could be easily scaled-up to a GMP culture system. In addition, culturing

cells in suspension utilizing a closed culture bag system reduces the product handling (i.e. no enzymatic steps are needed for harvesting and consecutive passaging), which will diminish the contamination risk, as well as cell stress that can promote apoptosis and senescence.

Importantly, the final cell product showed higher expression of pro-angiogenic and anti-inflammatory genes, with a decrease in expression of pro-inflammatory genes. Thus, we can conclude the increase in angiogenic potential of the cultured cells is not paired with an increase in their inflammatory profile. The up-regulation of both mir-210 and VEGFA was expected since it was shown that VEGF treatment enhanced the production of certain microRNAs, more specifically of mir-210 in UCB derived CD34+ cells (Alaiti et al., 2012). Concomitantly with the increase in VEGFA, MMP9 demonstrated a tendency for a higher transcript level after culture (although without statistical significance), as it has been suggested that the angiogenic initiation may be through MMP9-mediated activation of VEGF (Christoffersson et al., 2012). Also, MMP9 was shown to participate in the mobilization of progenitors from BM by shedding soluble forms of membrane-bound cytokines (Heissig et al., 2002). On the other hand, the decrease on the expression of KLF2 of the cultured cells is in agreement with the angiogenic profile of the final cell product, more specifically with the increase of VEGFA, since it has been reported that KLF2 has powerful anti-angiogenic effects, as its overexpression inhibited VEGF-mediated angiogenesis and ability of endothelial cell activation (Bhattacharya et al., 2005). Although without statistical significance, the anti-inflammatory marker HGF tended to increase its expression after culture. Actually, the increase in HGF is in agreement with the expression decrease of the pro-inflammatory cytokine IL-6, along with an increase in the anti-inflammatory cytokine IL-10, as previously described (Coudriet et al., 2010).

From the flow cytometric analysis, our results show that there might be a shift of the cultured cells towards endothelial lineage, promoted by QQ-culture conditions. The

higher expression of monocyte markers of the expanded cells is concurrent with the endothelial specific markers. Actually, it has been shown that blocking CD18, using a neutralizing antibody in EPCs, significantly reduced EPC recruitment to the ischemic myocardium, attenuated neovascularization, and worsened pathological remodeling (Wu et al., 2006). Moreover, it has been suggested that the majority of early EPCs arise from a $CD14^+$ subpopulation of PBMCs (Gulati et al., 2003). We also showed that *ex vivo* expanded cells displayed a higher *in vitro* angiogenic potential compared to uncultured cells demonstrated by an increased tube formation capacity. This result emphasizes the suggested angiogenic fortification that the final product acquires through the *ex-vivo* expansion process. In the setting of a cell-based angiogenic therapy for the treatment of ischemic diseases, such as critical limb ischemia, doses between $1x10^5$ to $1x10^6$ cells/kg have been studied (Kinoshita et al., 2012; Losordo et al., 2012; Masuda et al., 2014). Considering an average adult of 80 kg, and the fold increase in total nucleated cells attained in this work, a system with a 50-500mL capacity would be needed in order to achieve clinically meaningful cell numbers. Nevertheless, further pre-clinical studies on testing different cell doses vs. therapeutic effect are needed to allow translation into the clinical setting. Indeed, considering the translation of the cell product developed herein, there is the need to fully characterize it in terms of safety and stability by using *in vivo* models, pathological analysis and karyotyping. Overall, we anticipate that this system can be easily adapted to further pursue to a fully dynamic and controlled manufacturing system (bioreactor) in order to meet clinically relevant cell needs in a more cost-effective and robust way. Some preliminary data on the expansion of these cells in spinner flasks can be found on Chapter VI.

III.5 REFERENCES

Alaiti, M.A., Ishikawa, M., and Costa, M.A. (2010). Bone marrow and circulating stem/progenitor cells for regenerative cardiovascular therapy. Translational research : the journal of laboratory and clinical medicine *156*, 112-129.

Alaiti, M.A., Ishikawa, M., Masuda, H., Simon, D.I., Jain, M.K., Asahara, T., and Costa, M.A. (2012). Up-regulation of miR-210 by vascular endothelial growth factor in ex vivo expanded CD34+ cells enhances cell-mediated angiogenesis. Journal of cellular and molecular medicine *16*, 2413-2421.

Asahara, T., Kawamoto, A., and Masuda, H. (2011). Concise review: Circulating endothelial progenitor cells for vascular medicine. Stem cells *29*, 1650-1655.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Assmus, B., Schächinger, V., Teupe, C., Britten, M., Lehmann, R., Döbert, N., Grünwald, F., Aicher, A., Urbich, C., Martin, H.*, et al.* (2002). Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation *106*, 3009-3017.

Bhattacharya, R., Senbanerjee, S., Lin, Z., Mir, S., Hamik, A., Wang, P., Mukherjee, P., Mukhopadhyay, D., and Jain, M.K. (2005). Inhibition of vascular permeability factor/vascular endothelial growth factor-mediated angiogenesis by the Kruppel-like factor KLF2. The Journal of biological chemistry *280*, 28848-28851.

Bouchentouf, M., Forner, K., Cuerquis, J., Boulassel, M.R., Routy, J.P., Waller, E.K., Quyyumi, A.A., Paradis, P., Schiffrin, E.L., and Galipeau, J. (2011). A novel and simplified method of culture of human blood-derived early endothelial progenitor cells for the treatment of ischemic vascular disease. Cell transplantation *20*, 1431-1443.

Christoffersson, G., Vågesjö, E., Vandooren, J., Lidén, M., Massena, S., Reinert, R.B., Brissova, M., Powers, A.C., Opdenakker, G., and Phillipson, M. (2012). VEGF-A recruits a proangiogenic MMP-9–delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. Blood *120*, 4653-4662.

Colombo, E., Calcaterra, F., Cappelletti, M., Mavilio, D., and Della Bella, S. (2013). Comparison of Fibronectin and Collagen in Supporting the Isolation and Expansion of Endothelial Progenitor Cells from Human Adult Peripheral Blood. PloS one *8*, e66734.

Coudriet, G.M., He, J., Trucco, M., Mars, W.M., and Piganelli, J.D. (2010). Hepatocyte Growth Factor Modulates Interleukin-6 Production in Bone Marrow Derived Macrophages- Implications for Inflammatory Mediated Diseases. PloS one *5*, 1-7.

Dong, Z., Chen, B., Fu, W., Wang, Y., Guo, D., Wei, Z., Xu, X., and Mendelsohn, F.O. (2013). Transplantation of purified CD34+ cells in the treatment of critical limb ischemia. Journal of vascular surgery *58*, 404-411 e403.

Gulati, R., Jevremovic, D., Peterson, T.E., Chatterjee, S., Shah, V., Vile, R.G., and Simari, R.D. (2003). Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circulation research *93*, 1023-1025.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Barbara Ferris, Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.S.*, et al.* (2002). Recruitment of Stem and Progenitor Cells from the Bone Marrow Niche Requires MMP-9 Mediated Release of Kit-Ligand. Cell *109*, 625-637.

Hur, J., Yoon, C.H., Kim, H.S., Choi, J.H., Kang, H.J., Hwang, K.K., Oh, B.H., Lee, M.M., and Park, Y.B. (2004). Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arteriosclerosis, thrombosis, and vascular biology *24*, 288-293.

Ingram, D.A., Mead, L.E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M.J., Gilley, D., and Yoder, M.C. (2004). Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood *104*, 2752-2760.

Kalka, C., and Baumgartner, I. (2008). Gene and stem cell therapy in peripheral arterial occlusive disease. Vascular medicine *13*, 157-172.

Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W.M., Silver, M., Kearney, M., Li, T., Isner, J.M., and Asahara, T. (2000). Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proceedings of the National Academy of Sciences of the United States of America *97*, 3422-3427.

Kinoshita, M., Fujita, Y., Katayama, M., Baba, R., Shibakawa, M., Yoshikawa, K., Katakami, N., Furukawa, Y., Tsukie, T., Nagano, T.*, et al.* (2012). Long-term clinical outcome after intramuscular transplantation of granulocyte colony stimulating factor-mobilized CD34 positive cells in patients with critical limb ischemia. Atherosclerosis *224*, 440-445.

Leemhuis, T., Wells, S., Scheffold, C., Edinger, M., and Negrin, R.S. (2005). A phase I trial of autologous cytokine-induced killer cells for the treatment of relapsed Hodgkin disease and non-Hodgkin lymphoma. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *11*, 181-187.

Losordo, D.W., Kibbe, M.R., Mendelsohn, F., Marston, W., Driver, V.R., Sharafuddin, M., Teodorescu, V., Wiechmann, B.N., Thompson, C., Kraiss, L.*, et al.* (2012). A randomized, controlled pilot study of autologous CD34+ cell therapy for critical limb ischemia. Circulation Cardiovascular interventions *5*, 821-830.

Masuda, H., Iwasaki, H., Kawamoto, A., Akimaru, H., Ishikawa, M., Ii, M., Shizuno, T., Sato, A., Ito, R., Horii, M.*, et al.* (2012). Development of serum-free quality and quantity control culture of colony-forming endothelial progenitor cell for vasculogenesis. Stem cells translational medicine *1*, 160-171.

Masuda, H., Tanaka, R., Fujimura, S., Ishikawa, M., Akimaru, H., Shizuno, T., Sato, A., Okada, Y., Iida, Y., Itoh, J.*, et al.* (2014). Vasculogenic conditioning of peripheral blood mononuclear cells promotes endothelial progenitor cell expansion and phenotype transition of anti-inflammatory macrophage and T lymphocyte to cells with regenerative potential. Journal of the American Heart Association *3*, e000743.

Norgren, L., Hiatt, W.R., Dormandy, J.A., Nehler, M.R., Harris, K.A., Fowkes, F.G., and Group, T.I.W. (2007). Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Journal of vascular surgery *45 Suppl S*, S5-67.

Numaguchi, Y., Sone, T., Okumura, K., Ishii, M., Morita, Y., Kubota, R., Yokouchi, K., Imai, H., Harada, M., Osanai, H.*, et al.* (2006). The impact of the capability of circulating progenitor cell to differentiate on myocardial salvage in patients with primary acute myocardial infarction. Circulation *114*, I114-119.

O, E., Lee, B.H., Ahn, H.Y., Shin, J.C., Kim, H.K., Kim, M., Park, I.Y., Park, Y.G., and Joe, Y.A. (2011). Efficient nonadhesive ex vivo expansion of early endothelial progenitor cells derived from CD34+ human cord blood fraction for effective therapeutic vascularization. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *25*, 159-169.

Perin, E.C., and Silva, G.V. (2011). Cell-based therapy for chronic ischemic heart disease--a clinical perspective. Cardiovascular therapeutics *29*, 211-217.

Sadeghi, A., Pauler, L., Anneren, C., Friberg, A., Brandhorst, D., Korsgren, O., and Totterman, T.H. (2011). Large-scale bioreactor expansion of tumor-infiltrating lymphocytes. Journal of immunological methods *364*, 94-100.

Scheubel, R.J., Zorn, H., Silber, R.-E., Kuss, O., Morawietz, H., Holtz, J., and Simm, A. (2003). Age-dependent depression in circulating endothelial progenitor cells inpatients undergoing coronary artery bypass grafting. Journal of the American College of Cardiology *42*, 2073–2080.

Schmidt-Lucke, C., Fichtlscherer, S., Aicher, A., Tschope, C., Schultheiss, H.P., Zeiher, A.M., and Dimmeler, S. (2010). Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. PloS one *5*, e13790.

Shi, Q., Rafii, S., Wu, M.H.-D., Wijelath, E.S., Yu, C., Ishida, A., Fujita, Y., Kothari, S., Mohle, R., Sauvage, L.R.*, et al.* (1998). Evidence for circulating bone marrow-derived endothelial cells. Blood *92*, 362-367.

Somerville, R.P., Devillier, L., Parkhurst, M.R., Rosenberg, S.A., and Dudley, M.E. (2012). Clinical scale rapid expansion of lymphocytes for adoptive cell transfer therapy in the WAVE(R) bioreactor. Journal of translational medicine *10*, 69.

Timmins, N.E., Palfreyman, E., Marturana, F., Dietmair, S., Luikenga, S., Lopez, G., Fung, Y.L., Minchinton, R., and Nielsen, L.K. (2009). Clinical scale ex vivo manufacture of neutrophils from hematopoietic progenitor cells. Biotechnology and bioengineering *104*, 832-840.

Wu, Y., Ip, J.E., Huang, J., Zhang, L., Matsushita, K., Liew, C.C., Pratt, R.E., and Dzau, V.J. (2006). Essential role of ICAM-1/CD18 in mediating EPC recruitment, angiogenesis, and repair to the infarcted myocardium. Circulation research *99*, 315-322.

Yoder, M.C., Mead, L.E., Prater, D., Krier, T.R., Mroueh, K.N., Li, F., Krasich, R., Temm, C.J., Prchal, J.T., and Ingram, D.A. (2007). Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem-progenitor cell principals. Blood *109*, 1801-1809.

CHAPTER IV - EFFECT OF EXPANDED MESENCHYMAL STEM/STROMAL CELLS ON THE INTRACORONARY MICROCIRCULATION OF A PIG MODEL.

This chapter is based on the articles:

António Fiarresga, Márcia F. Mata, Sandra Cavaco-Gonçalves, Mafalda Selas, Irina N. Simões, Eunice Oliveira, Belmira Carrapiço, Nuno Cardim, Joaquim M.S. Cabral, Rui Cruz Ferreira, Cláudia Lobato da Silva. Intracoronary delivery of human mesenchymal/stromal stem cells: insights from coronary microcirculation invasive assessment in a swine model. PLoS ONE 10(10): e0139870

António Fiarresga, Márcia F. Mata, Raquel M. S. Cunha, Diogo S. Pinto, Sandra Cavaco-Gonçalves, Mafalda Selas, Eunice Oliveira, Hugo Pissarra, Belmira Carrapiço, Maria C. Peleteiro, Nuno Cardim, Joaquim M.S. Cabral, Rui C. Ferreira, Cláudia L. da Silva. The index of microcirculatory resistance for optimizing the use of intracoronary delivery of mesenchymal stem/stromal cells in an acute myocardial infarction model (manuscript under preparation)

IV.1 SUMMARY

Herein, the work was developed in a collaboration with António Fiarresga, MD, inserted in his PhD work (António José Fiarresga PhD Thesis, "*Terapia Celular Com Células Mesenquimais Por Via Intracoronária: Contributos Do Estudo Invasivo Da Microcirculação Coronária*", Tese de Doutoramento em Medicina na Especialidade de Investigação Clínica, Faculdade de Ciências Médicas, Universidade Nova de Lisboa. (April 2016)). The focus was on the expansion of Bone Marrow mesenchymal stem/stromal cells (BM-MSC) and their immunophenotypic and morphological characterization.

The work was divided into 2 phases, being the first one the study of intracoronary (IC) BM-MSC expanded under static conditions in low-glucose DMEM containing 10% fetal bovine serum (FBS) on the microcirculation of a healthy pig model and the second the comparison of the infusion of cells expanded under different systems (static; FBS – 'conventional'; dynamic; xeno-free (XF) – 'innovative') on the microcirculation of an induced acute myocardial (AMI) pig model.

In the first phase of the studies, the number of cells needed to infuse the pigs (n=9) were successfully obtained under expansion on plastic flasks. Their identity was confirmed by immunophenotyping and differentiation ability . The aim was to assess if the index of microcirculatory resistance (IMR) was able to detect changes on microcirculation upon MSC IC administration in a healthy pig model. It was observed an increase on IMR after cell administration in comparison with the control group, hence providing evidence of microcirculatory disruption upon IC MSC administration in a large animal model, which closely resembles human anatomy, physiology and function.

For the second phase, the number of cells needed for transplantation (n=6) were successfully achieved, for both 'conventional' cells using plastic flasks and 'innovative'' cells using spinner flasks and their identity was confirmed by immunophenotyping. It was

seen a difference in size between the cells expanded under the two methods (12±0.3 and 16±0.5, for ‘’innovative’’ and ‘’conventional’’ respectively, $p<0.05$). Upon IC administration, it was seen a microcirculatory disruption measured by IMR of the groups receiving cells *versus* the control group, but no difference was observed between the two groups that received cells expanded under the different systems. At the end point, four weeks after, IMR decreased to values similar to the baseline and histology results showed no difference on the ischemic heart tissue and number of capillaries, and hence no regenerative function was seen after cell administration.

IV.2 BACKGROUND

Acute myocardial infarction (AMI) is a common cardiovascular event and is one of the major death causes in both developed and developing countries with concomitant major causes of disability, loss of productivity and increased health costs worldwide (Reddy et al., 2015). After an AMI, it is known to occur left ventricular remodeling, which can lead to congestive heart failure (HF), and terminal heart disease. Despite advances in the treatment and prevention of AMI, the number of patients with HF continues to grow and remains associated with an increased death risk (Alaiti et al., 2010). Current therapeutic strategies rely mainly on the recovery of the ischemic myocardium by restoration of blood flow or reperfusion. However, the only standard therapy for HF that addresses the fundamental problem of cardiomyocyte loss is cardiac transplantation, which is limited by the inadequate supply of donor hearts and the need for subsequent immunosuppression (Parsa et al., 2015). Consequently, there is a need for the development of therapies for the myocardial regeneration and prevention of its degeneration.

In this context, a variety of therapies have been explored using combinations of cells, biomaterials, cocktails of factors, and bioengineered cardiac tissues for the repair of the damaged myocardium, either by *de novo* cardiomyogenesis or by promotion of neovasculogenesis (Chavakis et al., 2010; Perin and Silva, 2011; Yoo and Kwon, 2013). Stem cell-based therapies hold a huge promise to regenerate tissues/organs based either on cell engraftment into the target tissue and/or cell's trophic activity (*i.e.* delivery of soluble factors) (Samsonraj et al., 2017). MSC, in particular, have been extensively investigated in cardiac regenerative therapy trials during the past decade due to their angiogenic properties (Kanelidis et al., 2017; Squillaro et al., 2016; Watt et al., 2013). Although results of early-phase trials are encouraging, the efficacy demonstrated has not been uniform and further research on the regenerative properties of MSC is required for a safer and more effective MSC-based therapy for AMI.

Importantly, most of the clinical trials in heart regenerative medicine used the intracoronary (IC) route for cell delivery, as they allow for the delivery of high number of cells into the myocardial region (Dib et al., 2011). Nonetheless, IC delivery of cultured MSC is still a matter for debate, as these cells are larger (10-20 µm) than the heart capillaries (5-10 µm), hence increasing the risk of vessel obstruction (Sanganalmath and Bolli, 2013).

The main focus of this work was to evaluate the safety of IC delivery of BM-MSC by measurement of the microvascular obstruction upon administration of different cell dosages and their regenerative potential in a swine AMI model. Two different MSC populations were studied: cells expanded *ex-vivo* using an innovative protocol established in our lab (Carmelo et al., 2015) involving cultivation under stirred conditions with xenogeneic-free (XF) culture medium, which are expected to have an increased pro-angiogenic potential and a smaller size compared to cells cultivated using the traditional expansion protocol with FBS-supplemented medium.

IV.3 RESULTS

IV.3.1 PHASE 1

For the purpose of this study, BM-MSC administered into the animals were previously expanded onto tissue culture flasks through consecutive passaging and maintained their characteristic spindle shape morphology (**Figure IV-1 A**), immunophenotype (**Figure IV-1 D**), as well as their multilineage differentiation ability (**Figure IV-1 B and C**).

The number of cells needed for the infusion in the animal model was successfully achieved (Dose of 1 million MSCs/Kg, equivalent to an average total of $30x10^6$ cells/animal).

No difference was observed on the measured hemodynamic parameters. However, a significant difference on the IMR was observed after 5 and 30 min post cell infusion in

comparison with the baseline and between groups at 30 min post cell infusion (Annex ***Figure IV-7***).

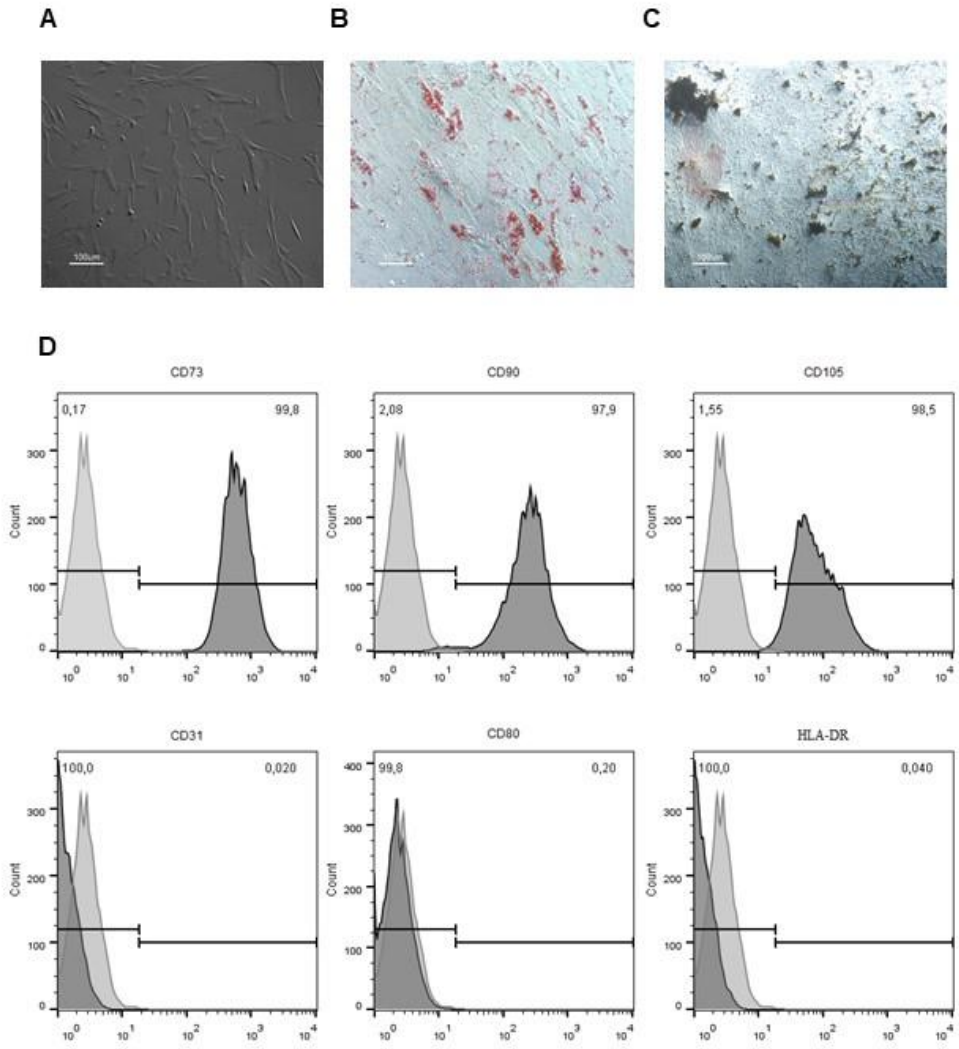


Figure IV-1 - *Characterization of bone marrow (BM)-derived mesenchymal stem/stromal cells (MSC) expanded on tissue culture flasks using DMEM with 10% FBS. (A) Morphology of cultured cells and adipogenic (B) and osteogenic (C) differentiation ability assessed by optical microscopy (D) Immunophenotype of cultured MSC assessed by flow cytometry. Percentage (%) displayed in the upper right corner of each histogram indicates the expression of each antigen*

IV.3.2 PHASE 2

The aim of this phase was to assess the acute and long-term effects on microcirculation of intracoronary delivery of BM-MSC in the acute myocardial infarction (MI) setting, using IMR and comparing two types of different sized cells expanded under different strategies.

Expansion data for cells cultured with FBS- containing medium is represented on **Table IV-1**. In the majority of cases, we were able to successfully expand cells from both donors in order to achieve the cell number needed for infusion. However, due to some restrictions based with unpredictable aspects when working with animals (*i.e.* such as mortality upon induced infarct) and time restrictions, there were exceptions when (i) the cells were cultured only for a short period of time upon thawing or (ii) when the target total number of cells was not achieved by culture, cells at the same passage available in the cell bank were thawed and mixed with the expanded cells before administration.

***Table IV-1**- Expansion data for "Conventional" BM-MSC (donor, passage, days in culture, initial and final cell number, fold increase (FI) in total cell number and population doublings (PDs)) for each administration for cells from both donors (M72A07 and M48A08). PD=log_{10}(Fold Increase)/log_{10}(2).*

Date of delivery	Donor	Passage	Days in culture	Initial cell number	Final cell number	Fold increase	PDs
10.2.15	M72A07	7	4	3.66E+07	4.43E+07	1.2	0.28
17.3.15	M72A07	6	7	2.33E+07	5.31E+07	2.3	1.19
24.3.15	M72A07	6	4	4.29E+07	5.20E+07	1.2	0.28
29.4.15	M48A08	6	2	6.76E+07	4.82E+07	-	-
13.5.15	M48A08	3	12	6.93E+06	7.57E+07	6.8	3.45
			8	4.23E+06			4.16
9.6.15	M48A08	5	7	1.53E+07	3.12E+07	2.0	1.03

An average fold increase of 1.6±0.4 and 2.9±2.0 for M72A07 and M48A08, respectively, was attained. Regarding population doublings, the average values obtained were 0.6±0.4 for M72A07 and 2.2±1.0 for M48A08. Overall, the ‘’conventional’’ method resulted in a fold increase of 2.3±1.0 and a population doubling of 1.5±0.6.

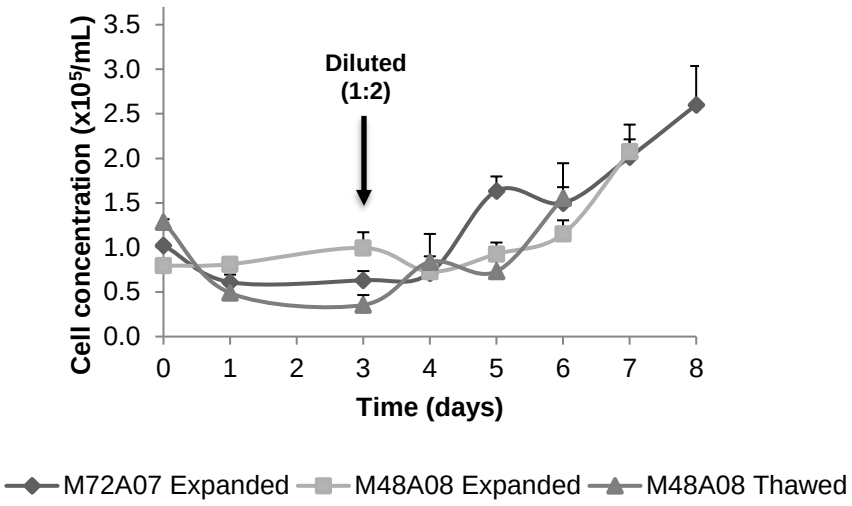


Figure IV-2 *- Ex-vivo expansion of BM-MSC from the two donors in a XF microcarrier-based culture system in spinner flasks (agitation of 40 rpm). Cells were seeded at a cell density of 1×10^5 cell/mL with 20 g/L of SoloHill plastic microcarriers pre-coated with CELLstart in StemPro MSC SFM XenoFree medium. Cells that were directly inoculated into the spinner after thawing are also represented (M48A08 Thawed). Values are represented as mean±SEM (standard error of the mean) (n=3-8).*

The cell concentration throughout expansion time and seeding efficiency are represented in ***Figure IV-2*** and ***Figure IV-3***, respectively, when using the microcarrier-based expansion method ("innovative")**.** After 24 hours, 61±8% (M72A07 donor cells), 93±5% (M48A08 donor cells) and 38±7% (M48A08 *thawed*) of the initial cells adhered to the microcarriers. The exponential growth of BM-MSC yielded, at day 7, an average concentration of 2.0x10^5 cell/mL (±0.2 for M72A07 and ±0.3 for M48A08), in a 80 and 100 ml working volume. Again, due to time restrictions and unpredictable factors, there were situations where cells were directly thawed and inoculated into the spinner (referred in the figure as "*Thawed*"). In these cases, 38±7% of the cells adhered to the microcarriers and it was possible to achieve a concentration of 1.6±0.4 x10^5 cell/mL at day 6. Fold increase values (in total cell number) of 7.1±0.3 (M72A07 *Expanded*), 4.4±0.4 (M48A08 *Expanded*) and 3.2±0.3 (M48A08 *Thawed*) were attained.

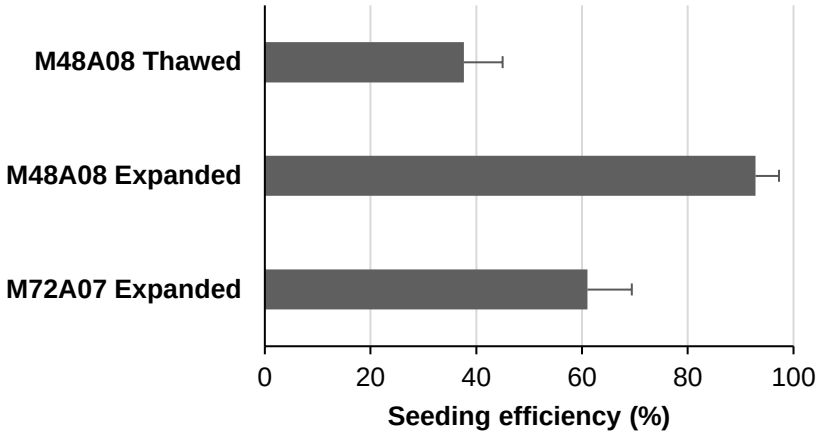


Figure IV-3 *- Seeding efficiency (%) for cells from the different donors expanded under spinner flasks ("innovative"), given by (number of cells at day 1)/(number of cells seeded) x 100. Results presented as mean±SEM (n=3-8).*

The effect of mechanical forces in the spinner flask cultures, which can influence MSC behavior and function, was evaluated by calculating the size of potentially damaging eddies and shear stress in the cultures, according to Kolmogorov's theory of isotropic turbulence (Santos et al., 2011) and are represented on **Table IV-2,** which contains the values for each culture condition. Maximum shear stress and minimum microscale eddies are known to occur in the impeller zone. These were calculated using the impeller swept volume to calculate the maximum energy dissipation that occurs in these systems. To note that in this case, for the final volume of 80 mL and 100 mL are the same as the same spinner flask was used on both situations.

Table IV-2 *- Values of average and maximum shear stress, τ (dyn/cm^2) and average and minimum size of Kolmogorov-scale turbulent eddies, λK (μm) for each condition in dynamic culture.*

		250 mL	100 mL	80 mL
τ (dyn/cm^2)	**1/2 volume, 30 rpm**	1.44	1.50	1.68
	1/2 volume, 40 rpm	2.14	2.23	2.49
	Total volume, 40 rpm	1.51	1.58	1.76
λK (μm)	**1/2 volume, 30 rpm**	178	174	165
	1/2 volume, 40 rpm	146	143	135
	Total volume, 40 rpm	173	170	161
τ_{max} (dyn/cm^2)	**30 rpm**	2.10	1.69	
	40 rpm	3.11	2.50	
λK_{min} (μm)	**30 rpm**	147	164	
	40 rpm	121	135	

It was observed that the average shear stress increased at day 1 of culture, when the agitation was increased and the culture is performed in half the final volume, and decreased at day 3 (by values similar to the beginning) when the culture was performed in the final volume. Nevertheless, in every case, the average value of maximum shear stress was maintained low. However, when examining the maximum that occurs in a small volume near the impeller, considerable values of maximum shear stress are achieved (> 3 dyn/cm^2 for the 250 mL spinner flask at 40 rpm). Of notice, the maximum values for shear stress and minimum microscale eddies' size is seen for the 250 mL spinner flask in comparison with the spinner of 100 mL working volume. The minimum value of Kolmogorov-scale turbulent eddies (121 μm and 135 μm for the 250 and 100 mL spinner flask, respectively) is within microcarrier diameter range (90-150 μm). The average size is also within that range when spinners are operated at maximum agitation used in half the final volume. For the other culture parameters, these values were maintained higher than bead sizes.

Metabolic characterization of the spinner flask cultures was also performed. The trends of specific glucose consumption rate (**Figure IV-4**) and specific lactate production rate (**Figure IV-5**) had a maximum peak in the beginning of culture corresponding to the beginning of the exponential growth (day 3-4). Following this peak, lower consumption and production values were obtained for the remaining culture time. In addition, the

average apparent yields of lactate from glucose were lower than the theoretical value of 2.

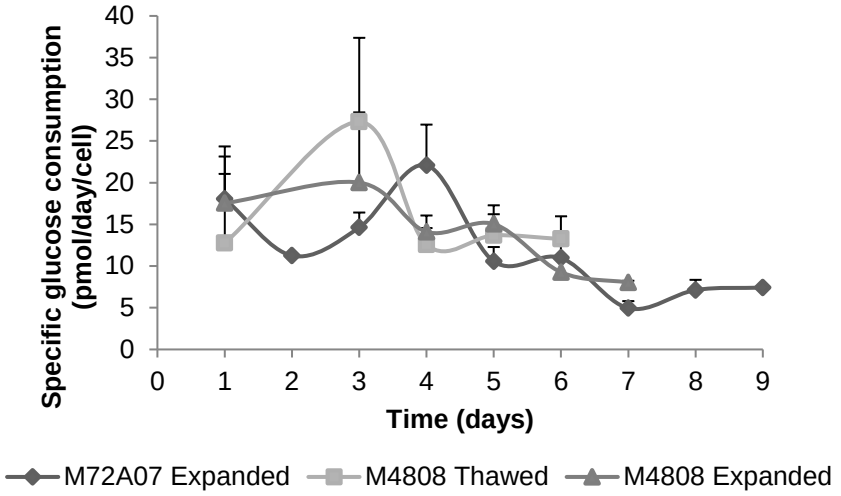


***Figure IV-4**. Glucose metabolism of BM-MSC cultures under dynamic conditions. Specific consumption rate of glucose throughout time in culture of BM MSC, M72A07 Expanded (black), M48A08 Thawed (light grey) and M48A08 Expanded (dark grey) calculated for BM MSC expanded in a XF stirred culture system with 50% medium addition at day 3 and 25% daily renewal from day 4 onwards. Values are represented as mean±SEM (n=3-8).*

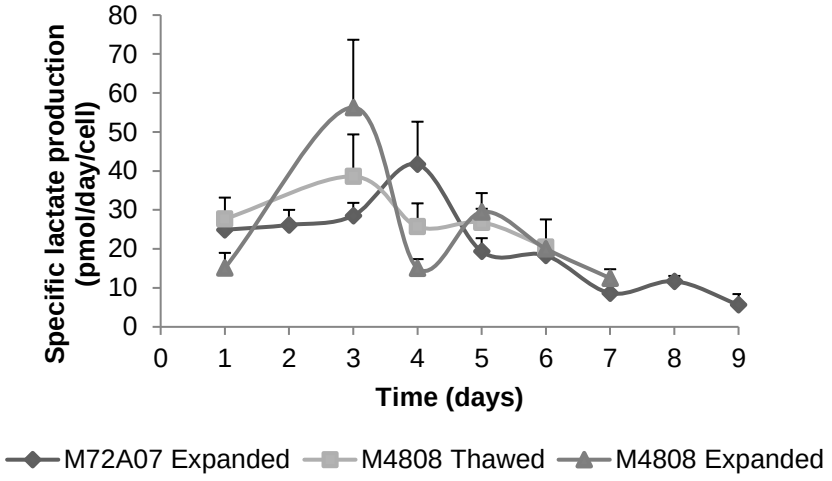


***Figure IV-5**. Lactate metabolism of BM MSC cultures under dynamic conditions. Specific production rate of lactate throughout time in culture of BM MSC, M72A07 Expanded (black), M48A08 Thawed (light grey) and M48A08 Expanded (dark grey) calculated for BM MSC expanded in a XF stirred culture system with 50% medium addition at day 3 and 25% daily renewal from day 4 onwards. Values are represented as mean±SEM (n=3-8).*

After expansion, cells were characterized by flow cytometry using specific monoclonal antibodies according to criteria defined in the literature (Dominici et al., 2006). Over 95% of the population should express CD73, CD90 and CD105 and not express (less than 2%) CD14, CD19, CD31, CD34, CD45, CD80 and HLA-DR (**Figure IV-6**).

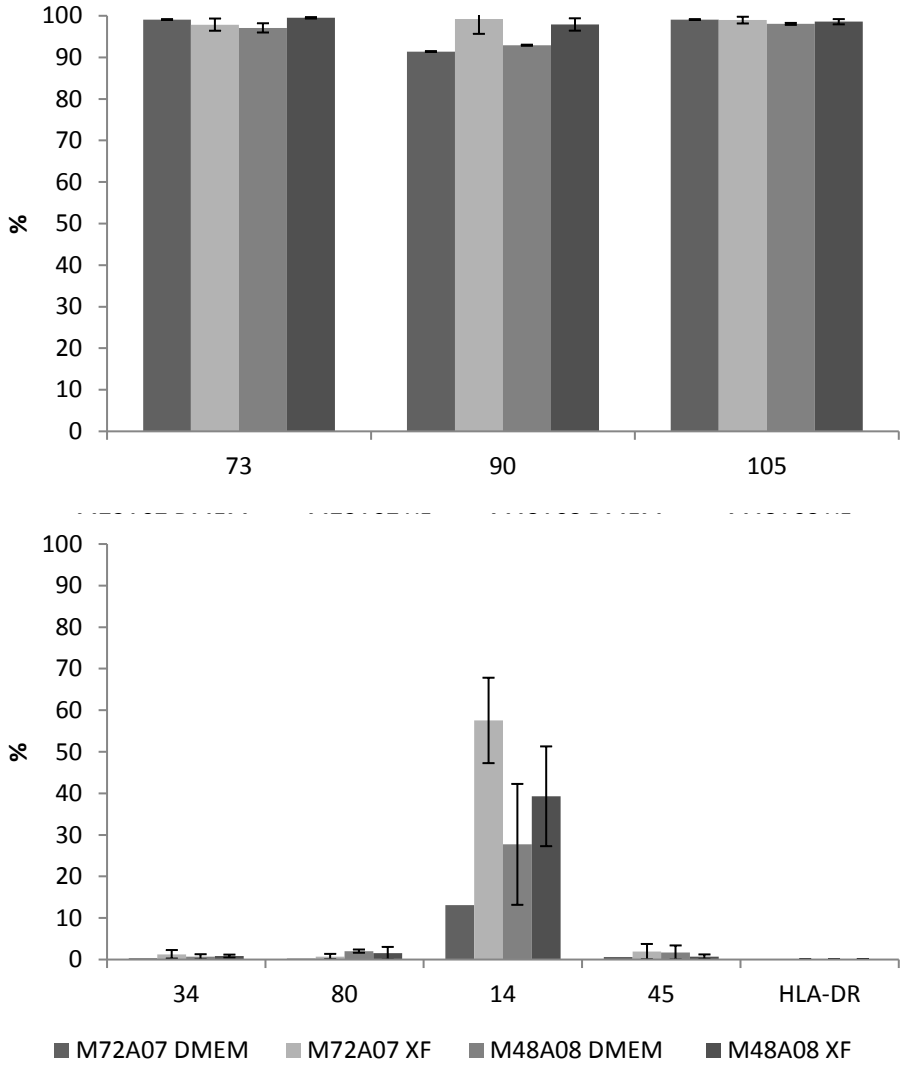


***Figure IV-6**. Immunophenotypic characterization of BM-MSC expanded using both ''conventional'' (DMEM) and ''innovative'' (XF) protocols for the two donors studied (M72A07 and M48A08). The expression (%) of each marker is displayed as mean±SEM (n=3).*

Cell size is a particularly important aspect of the cell product this is a parameter that can have impact on cell administration (*e.g.* intracoronary, intravenously). In this context, the

size of the cells produced by both ‘’conventional’’ and ‘’innovative’’ expansion protocols were assessed (***Table IV-3***) by using *ImageJ* software.

Table IV-3. *Cell diameter measurements (upon cell harvesting, i.e. in suspension) for expanded BM MSC of the two donors produced through the ‘’conventional’’ (DMEM) and ‘’innovative’’ (XF) method. Three photos from random fields were taken and at least 20 measurements were performed in each field.*

	M72A07		**M48A08**	
	Average (µm)	**SEM**	**Average (µm)**	**SEM**
DMEM	16	0.5	16	0.4
XF	12	0.2	12	0.3

As it can be seen by the values displayed on the table, the cells expanded through the ‘’innovative’’ method presented lower dimensions in comparison with the ones expanded through the ‘’conventional’’ method ($p<0.05$). The size of the cells is indeed an important factor in this study since the cells were intended to be infused through IC delivery in an animal model of AMI and we ought to understand if the differences in the size of both expanded populations (innovative *vs.* conventional) altered the index of microcirculation resistance (IMR) (Annex ***Table IV-4***), as the increase of this index is related with the obstruction of microvessels.

IV.4 DISCUSSION AND CONCLUSIONS

Upon several years of research on cell-based therapies for cardiovascular regeneration, the best cell type as well as administration route to promote angiogenesis is yet to be established. In this context, MSCs have been widely studied and implemented in several pre-clinical trials, which have been shown to engraft and reduce infarct size in rodent and large animal models (Kanelidis et al., 2017). Nonetheless, to be able to achieve clinically relevant cell numbers, expansion of these cells *ex vivo* is mandatory. In the phase 1 of this work, it was ought to evaluate the microcirculatory disruption and safety upon expanded MSC administration by real-time IMR measurement in a large pig model, resembling anatomically and physiologically the human system, as the size of these cells are larger in size than heart capillaries. Indeed, the safety of cultured BM-MSC intravenous administration and endomyocardial injection into patients with AMI have been reported (Hare et al., 2009; Rodrigo et al., 2013). However, a direct comparison between IC and intravenous route showed an increased cell engraftment for the IC route on a porcine model, whereas the direct comparison between IC and endomyocardial injection remains more controversial (Freyman et al., 2006; Kanelidis et al., 2017). Despite the IC route offering more advantages, as it is based on daily basis coronary procedures, the possibility of microvascular obstruction or myocardial microinfarctions due to cell size offers apprehension, with some disparities on preclinical data, although with some showing safety and regenerative potential of administered MSC through this route (Houtgraaf et al., 2013; Lee et al., 2014; Wang et al., 2011).

This study is, to the best knowledge, the first one to address the acute microcirculatory effects of IC MSC delivery using IMR measurements in a controlled and blind research design. The number of cells needed for infusion were successfully achieved by expansion under tissue culture flasks in medium containing FBS and their identity characterized by morphology and immunophenotyping, under the International Society of Cellular Therapies (ISCT) definition (Dominici et al., 2006). It was seen an increase of

IMR values upon MSC administration, showing the potential of microcirculatory obstruction. Hence, this shows the potentiality of this technique to experimentally and clinically support *in vivo* real-time monitoring and safety assessment upon MSC administration.

Nevertheless, assessment of this technique in a heart disease context and correlating IMR values after IC delivery of MSCs with data about cell fate and distribution, histological analysis and long-term clinical benefit are important issues that should be addressed. Importantly, although many studies, including clinical trials, report the MSC expansion under FBS containing medium, this has been criticized by the regulatory agencies due to the presence of xenogenic components due to risk of viral and prion diseases transmission, possible immunological reaction induction and batch variability. Another factor is the time consuming and labor intensive of the traditional 2D expansion flasks that adds to low robustness and high costs. Hence, considering the high cell number to meet clinical demand and reduction of cost-of-goods as well as moving towards a good manufacturing practice (GMP) platform, alternative expansion methods have been developed, by using 3-D systems (*i.e.* microcarriers) under dynamic culture conditions (*e.g.* stirred bioreactors), with several studies reporting the production of MSCs using xenogeneic/serum-free medium formulations using scalable culture technologies (Carmelo et al., 2015; de Soure et al., 2016; Dos Santos et al., 2014).

The combination of the dynamic conditions in the spinner flask culture systems with the use of xeno-free media presented in the second part of this chapter represents a feasible and faster alternative to 2D static cell expansion technologies for a clinical-scale MSC production. It was ought to evaluate the safety of MSC infusion and evaluate the effect of different cell doses by measurement of the IMR and ultimately to assess the therapeutic effect of both populations (conventional and innovative) in a swine AMI model. Cell numbers needed for the study were achieved for both ‘’conventional’’ and ‘’innovative’’ platforms. On the other hand, the number of initial cells (inoculum) needed

to reach the final target was considerably higher for the first one. This is corroborated by the total fold increases obtained, with an average of 2.3±1.0 for the ‘’conventional’’ and 4.9±0.4 for the ‘’innovative’’. Importantly, the 2D expansion offered a much more labor intensive and time-consuming method aiding to the benefits of the 3D dynamic system.

Dynamic expansion was carried as described by our previous data (Carmelo et al., 2015). However, a difference is seen for the seeding efficiency between both donors used (61 ± 8.4 % and 92 ± 4.5 % for M72A07 and M48A08, respectively). This may be explained by experimental error of the microcarrier coating (i.e. with Cellstart) that was performed in the same concentration used for 2D culture (2x more diluted). Moreover, usually before starting a dynamic culture, cells are seeded into 2D flasks (seed strain) in order not only to achieve the cell number needed for the seeding, but as well to give cells the opportunity to recover from the post-thaw stress (Schmidt-Mende et al., 2000). Nonetheless, due to time constraints, there was experiments where cells were directly thawed into spinner flasks. As expected, in this situation, the seeding efficiency was lower (37.7±7%) than for the cultures with a seed train, although reaching similar cell densities at day 6 (1.56 x 10^5 ± 0.36 cells/mL). Importantly, successful cell expansion was attained for different sized spinner flasks (100mL and 250mL). Cell seeding density and feeding regimen were proportionally adapted when working with different volumes. Nonetheless, agitation speed was the same, which translated in different average and maximum shear stresses (τ_{max}) and Kolmogorov-scale turbulent eddies sizes (λ_K). As expected, average shear stress decreased and eddie size increased with the increment in volume while maintaining the same agitation speed. Additionally, it is important to note that on days 1 and 2 cell culture was performed in half of the final volume with the higher agitation speed implemented, leading to double the energy dissipation in the vessel and thus increasing the maximum shear stress experienced by the cells. In this case, a maximum of shear stress of 2.49 dyn/cm^2 was observed for the spinner with lower final volume (80 mL). According to these estimations, these culture conditions can generate

microscale eddies of a minimum size of 135 µm, 143 µm and 146 µm, for a final volume of 80 mL, 100 mL and 250 mL, respectively. When eddies are smaller than the microcarriers, they dissipate their kinetic energy against the cells, which can possibly cause cell damage (Cherry and Papoutsakis, 1988; Nienow, 2006). In the present case, for all 3 different final volumes systems, the average microscale eddie size falls within the microcarrier size range (90-150 µm) in that period, which means that these culture conditions may impose cell damage. Importantly, maximum shear stress and minimum eddie size occurs in a small volume near the impeller area with values above 3 dyn/cm^2 for shear stress and minimum eddie sizes of 121 µm. Of notice, these extreme values are achieved for the 250 mL spinner flask, and hence, one needs to be careful when translating a culture system into different sized vessels. In this context, some cell damage might have occurred, especially when operating at maximum agitation rates in half the final volume and in the impeller area. Nonetheless, it has been reported for fibroblasts, that cell damage would only be significant when eddies' size reached two-thirds of the microcarriers' size (Croughan et al., 1987). Additionally, this critera can be translated for human MSC cultures, as reported by Hewitt and colleagues (Hewitt et al., 2011). Hence, microscale eddies of 100 µm or lower would employ significant cell damage. In this case, no such lower estimated values are achieved and therefore the dynamic system presented herein is not theoretically affected by significant cell damage through these factors. Additionally, Yi and colleagues have reported a change on the proteomic profile of cultured MSC being exposed to 3 dyn/cm^2 (maximum value on the present study). More specifically, 13 proteins were found to be up- or down-regulated by over twofold. Such proteins are known to be involved in different cellular processes, which include heat shock cell membrane proteins (e.g. Annexin A2), proteins involved in protein synthesis, degradative proteins, chaperones, metabolic enzymes, and nuclear and chromatin proteins (Yi et al., 2010). Nonetheless, this extrapolation should be done carefully, as here $Re<10^4$ and hence the flow is in the transition region of Reynolds number, not being fully turbulent. Therefore, a more reasonable expression of

‘’moderately turbulent’’ may be applied (Venkat et al., 1996). In this context, several difficulties may arise while addressing this problem via turbulent theory. However, Croughan and co-workers (Croughan et al., 1987) have developed a turbulence-based model for damage potential based on Nagata (Nagata, 1975) suggestions, where the flow becomes turbulent at $Re>10^3$, which is within the values calculated for this work. Hence, as no alternative method has arisen, this methodology was applied regarding cell damage on the stirred system. In addition, it is to note that the most extreme conditions regarding shear stress and eddies’ size occurs, as mentioned before, in a small volume near the impeller region, and therefore, the probability of the microcarriers and cells encountering those factors is lower.

The metabolic profile of the dynamic cultures was also evaluated. Specific glucose consumption and lactate production present a similar trend between donors, which is characterized by an increasing rate on the first days, with a peak on consumption/production correspondent to the initial of the exponential growth phase. After that, levels decreased to lower levels until the end of the exponential growth, being maintained roughly consistent until the end of cell culture. Importantly, the values for glucose consumptions (5 – 30 pmol/day/cell) and lactate production (10 – 60 pmol/day/cell) are within the ranges previously reported by our lab (Carmelo et al., 2015). However, in that study, higher rates were achieved in comparison with the results presented herein, which may be explained by the lower cell adhesion efficiencies that were seen here (due to experimental error / cells directly thawed into the spinners), as well as the different size and volumes used in this work that may affect culture performance. Indeed, results published present higher cell densities for the same timepoints. Interestingly, the peak for higher value of glucose consumption seen for the cells thawed directly into the spinner flasks is not accompanied by the highest value of lactate production, that is observed for cells of donor M48A08. This may be explained by

presumably metabolic and cell cycle adaptation that these cells need in order to recover from post-thaw stress in the dynamic environment (Galipeau, 2013; Santos et al., 2011).

In addition, lactate levels did not reach inhibitory values (maximum of 12 mM). The inhibitory levels have experimentally determined for hMSCs as greater than around 35 and 2 mM for lactate and ammonium, respectively (Schop et al., 2009).

Expanded cells were also immunophenotypically characterized according to the ISCT acceptance criteria. All markers are within the accepted values on what a MSC should or sould not express, apart from CD14. Interestingly, the analyzed cells showed an inconsistent high expression of CD14 by BM MSC together with the almost null expression of HLA-DR. These phenomena may be due to the expression of CD14 cross-reactive epitopes and hence, this may be a false positive (Pilz et al., 2011).

Finally, one of the cell aspects and rationale for the comparison of the populations expanded under the ‘’conventional’’ and ‘’innovative’’ systems would be the cell size, as there is some reluctance on the intracoronary infusion of these cells for regenerative medicine since these cells present higher diameters than heart capillaries. Cells expanded under the conventional protocol presented a diameter of 16 ± 0.45 µm, consistent with results reported on the literature for adult MSCs (15.5 ± 4.1 µm) (Ng et al., 2014). Indeed, cells expanded under the ‘’innovative’’ way, by the protocol developed in our lab (Dos Santos et al., 2014), presented significantly smaller size in diameter than the ones expanded under conventional 2D culture (12 µm vs 16 µm, respectively, $p<0.05$), presenting a 25% reduction in size. Of notice, there are no previous studies focusing on the evaluation of the different culture methodologies impact on BM MSC features, namely size and its effects in coronary microcirculation, as well as their regenerative potential (e.g. secretion of paracrine factors), and hence being a matter of further research.

However, besides the smaller size of the ‘’innovative’’ cells, no relevant IMR differences in comparison with the ‘’conventional’’ group were attained. This may be explained by the fact that this technique is not sensitive enough to see differences between the range of cell sizes in this study for the two populations. Nevertheless, IMR allowed for the differentiation of changes in coronary microcirculation driven by intracoronary delivery of BM-MSC in the absence of changes in other clinical parameters in the coronary circulation at real time. IMR changes are progressive and enabled the evaluation of the effect / dose. Thus, this technique can be considered as a microcirculation disruption subclinical marker, whose real-time assessment may be useful during intracoronary BM MSC delivery and could help to define safety thresholds. Moreover, no difference was shown regarding the state of myocardial damage at the end of the study, although there are several pre-clinical studies reporting reduction of the infarct size upon MSC administration (Makkar et al., 2005; Shake et al., 2002). Moreover, no evidence of engraftment was seen from histologic evaluation after 4 weeks. Other studies had similar results in mouse, sheep and swine MI models, showing the widespread death occurrence from the donor-derived BM-MSC and failure to detect these cells in the days or weeks after a successful transplantation (Dixon et al., 2009; Hashemi et al., 2008; van der Bogt et al., 2009). Of notice, these divergences in results may be due to different employed MSC expansion methodologies, handling, administration method and effect measurement (such as hemodynamic parameters and microcirculatory obstruction). In the future, the evaluation of other variables of ventricular function could add further insights to the study, where the potential regenerative effects of the BM-MSCs could be depicted, as well as the comparison of the two expanded cell populations.

Although majority of the studies use BM derived MSC, these cells can also be obtained from other more attractive sources (as adipose tissue or umbilical cord) due to their ease of access and ready availability. Nonetheless, although they are considered as the same cell type, they may present different characteristics (Hass et al., 2011a; Pill et al., 2015),

and further research in order to depict their regenerative potential in an AMI setting should be pursued. Some efforts have been made in that direction recently with some clinical trials, showing administration safety and, in some cases, clinical improvement (Bartolucci et al., 2017; Gao et al., 2015; Musialek et al., 2015; Perin et al., 2014).

Importantly, the mechanism through which these cells exert their regenerative potential should be addressed to, not only determine which would be to consider as release factors, but also to determine the best via of administration as well as the relevant cell doses. Although differentiation mechanism into myogenic cells have been suggested (Pittenger and Martin, 2004), this mechanism for cardiac therapy with MSCs has been recently criticized (Tao et al., 2016), specially due to poor survival and engraftment in the ischemic tissue. Hence, exploration of the therapeutic effect through paracrine action has gained more attention, with studies showing this mechanism *in vivo* (Cai et al., 2016; Yao et al., 2015). However, there is still a lack of consensus regarding the existence of myocardial regeneration or effective clinical benefit on the MSC-based therapy for MI (Behbahan et al., 2015; Leri and Anversa, 2013).

Overall, this work presents important insights on the combination of the bioengineering field and pre-clinical studies of the application of advanced cell-based therapies for cardiac disorders, giving cues on real time microcirculation disruption measurement that can be further applied to safety assessment on dosage of MSC administration. To our best knowledge, this is the first report where a comparison on cells expanded under different culture platforms was performed.

IV.5 REFERENCES

Alaiti, M.A., Ishikawa, M., and Costa, M.A. (2010). Bone marrow and circulating stem/progenitor cells for regenerative cardiovascular therapy. Translational research : the journal of laboratory and clinical medicine *156*, 112-129.

Bartolucci, J., Verdugo, F.J., Gonzalez, P.L., Larrea, R.E., Abarzua, E., Goset, C., Rojo, P., Palma, I., Lamich, R., Pedreros, P.A.*, et al.* (2017). Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial (RIMECARD Trial [Randomized Clinical Trial of Intravenous Infusion Umbilical Cord Mesenchymal Stem Cells on Cardiopathy]). Circulation research *121*, 1192-1204.

Behbahan, I.S., Keating, A., and Gale, R.P. (2015). Bone Marrow Therapies for Chronic Heart Disease. Stem cells *33*, 3212-3227.

Cai, M., Shen, R., Song, L., Lu, M., Wang, J., Zhao, S., Tang, Y., Meng, X., Li, Z., and He, Z.X. (2016). Bone Marrow Mesenchymal Stem Cells (BM-MSCs) Improve Heart Function in Swine Myocardial Infarction Model through Paracrine Effects. Scientific reports *6*, 28250.

Carmelo, J.G., Fernandes-Platzgummer, A.M., Diogo, M.M., Silva, C.L.d., and Cabral, J.M.S. (2015). A Xeno-free Microcarrier-based Stirred Culture System for the Scalable Expansion of Human Mesenchymal Stem/Stromal Cells isolated from Bone Marrow and Adipose Tissue. Biotechnology Journal *10*, 1235-1247.

Chavakis, E., Koyanagi, M., and Dimmeler, S. (2010). Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back. Circulation *121*, 325-335.

Cherry, R.S., and Papoutsakis, E.T. (1988). Physical mechanisms of cell damage in microcarrier cell culture bioreactors. *32*, 1001-1014.

Croughan, M.S., Hamel, J.F., and Wang, D.I.C. (1987). Hydrodynamic effects on animal cells grown in microcarrier cultures. Biotechnology and bioengineering *29*, 130-141.

de Soure, A.M., Fernandes-Platzgummer, A., da Silva, C.L., and Cabral, J.M. (2016). Scalable microcarrier-based manufacturing of mesenchymal stem/stromal cells. Journal of biotechnology *236*, 88-109.

Dib, N., Khawaja, H., Varner, S., McCarthy, M., and Campbell, A. (2011). Cell therapy for cardiovascular disease: a comparison of methods of delivery. Journal of cardiovascular translational research *4*, 177-181.

Dixon, J.A., Gorman, R.C., Stroud, R.E., Bouges, S., Hirotsugu, H., Gorman, J.H., Martens, T.P., Itescu, S., Schuster, M.D., Plappert, T.*, et al.* (2009). Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. Circulation Journal *120*, 220-229.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315-317.

Dos Santos, F., Campbell, A., Fernandes-Platzgummer, A., Andrade, P.Z., Gimble, J.M., Wen, Y., Boucher, S., Vemuri, M.C., da Silva, C.L., and Cabral, J.M. (2014). A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. Biotechnology and bioengineering *111*, 1116-1127.

Freyman, T., Polin, G., Osman, H., Crary, J., Lu, M., Cheng, L., Palasis, M., and Wilensky, R.L. (2006). A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. European heart journal *27*, 1114-1122.

Galipeau, J. (2013). Concerns arising from MSC retrieval from cryostorage and effect on immune suppressive function and pharmaceutical usage in clinical trials. ISBT Science Series *8*, 100-101.

Gao, L.R., Chen, Y., Zhang, N.K., Yang, X.L., Liu, H.L., Wang, Z.G., Yan, X.Y., Wang, Y., Zhu, Z.M., Li, T.C.*, et al.* (2015). Intracoronary infusion of Wharton's jelly-derived mesenchymal stem cells in acute myocardial infarction: double-blind, randomized controlled trial. BMC medicine *13*, 162.

Hare, J.M., Traverse, J.H., Henry, T.D., Dib, N., Strumpf, R.K., Schulman, S.P., Gerstenblith, G., DeMaria, A.N., Denktas, A.E., Gammon, R.S.*, et al.* (2009). A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. Journal of the American College of Cardiology *54*, 2277-2286.

Hashemi, S.M., Ghods, S., Kolodgie, F.D., Parcham-Azad, K., Keane, M., Hamamdzic, D., Young, R., Rippy, M.K., Virmani, R., Litt, H.*, et al.* (2008). A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. European heart journal *29*, 251-259.

Hass, R., Kasper, C., Bohm, S., and Jacobs, R. (2011). Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. Cell communication and signaling : CCS *9*, 12.

Hewitt, C.J., Lee, K., Nienow, A.W., Thomas, R.J., Smith, M., and Thomas, C.R. (2011). Expansion of human mesenchymal stem cells on microcarriers. Biotechnology letters *33*, 2325-2335.

Houtgraaf, J.H., de Jong, R., Kazemi, K., de Groot, D., van der Spoel, T.I., Arslan, F., Hoefer, I., Pasterkamp, G., Itescu, S., Zijlstra, F.*, et al.* (2013). Intracoronary infusion of allogeneic mesenchymal precursor cells directly after experimental acute myocardial infarction reduces infarct size, abrogates adverse remodeling, and improves cardiac function. Circulation research *113*, 153-166.

Kanelidis, A.J., Premer, C., Lopez, J., Balkan, W., and Hare, J.M. (2017). Route of Delivery Modulates the Efficacy of Mesenchymal Stem Cell Therapy for Myocardial Infarction: A Meta-Analysis of Preclinical Studies and Clinical Trials. Circulation research *120*, 1139-1150.

Lee, J.W., Lee, S.H., Youn, Y.J., Ahn, M.S., Kim, J.Y., Yoo, B.S., Yoon, J., Kwon, W., Hong, I.S., Lee, K.*, et al.* (2014). A randomized, open-label, multicenter trial for the safety and efficacy of adult mesenchymal stem cells after acute myocardial infarction. Journal of Korean medical science *29*, 23-31.

Leri, A., and Anversa, P. (2013). Stem cells: Bone-marrow-derived cells and heart failure—the debate goes on. Nat Rev Cardiol *10*, 372-373.

Makkar, R.R., Price, M.J., Lill, M., Frantzen, M., Takizawa, K., Kleisli, T., Zheng, J., Kar, S., McClelan, R., Miyamota, T.*, et al.* (2005). Intramyocardial injection of allogeneic bone marrow-derived mesenchymal stem cells without immunosuppression preserves cardiac function in a porcine model of myocardial infarction. Journal of cardiovascular pharmacology and therapeutics *10*, 225-233.

Musialek, P., Mazurek, A., Jarocha, D., Tekieli, L., Szot, W., Kostkiewicz, M., Banys, R.P., Urbanczyk, M., Kadzielski, A., Trystula, M*., et al.* (2015). Myocardial regeneration strategy using Wharton's jelly mesenchymal stem cells as an off-the-shelf 'unlimited' therapeutic agent: results from the Acute Myocardial Infarction First-in-Man Study. Postepy w kardiologii interwencyjnej = Advances in interventional cardiology *11*, 100-107.

Nagata, S. (1975). Mixing: principles and applications. Kodansha,Tokyo.

Ng, C.P., Sharif, A.R., Heath, D.E., Chow, J.W., Zhang, C.B., Chan-Park, M.B., Hammond, P.T., Chan, J.K., and Griffith, L.G. (2014). Enhanced ex vivo expansion of adult mesenchymal stem cells by fetal mesenchymal stem cell ECM. Biomaterials *35*, 4046-4057.

Nienow, A.W. (2006). Reactor engineering in large scale animal cell culture. Cytotechnology *50*, 9-33.

Parsa, H., Ronaldson, K., and Vunjak-Novakovic, G. (2015). Bioengineering methods for myocardial regeneration. Advanced drug delivery reviews.

Perin, E.C., Sanz-Ruiz, R., Sanchez, P.L., Lasso, J., Perez-Cano, R., Alonso-Farto, J.C., Perez-David, E., Fernandez-Santos, M.E., Serruys, P.W., Duckers, H.J*., et al.* (2014). Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial. American heart journal *168*, 88-95 e82.

Perin, E.C., and Silva, G.V. (2011). Cell-based therapy for chronic ischemic heart disease--a clinical perspective. Cardiovascular therapeutics *29*, 211-217.

Pill, K., Hofmann, S., Redl, H., and Holnthoner, W. (2015). Vascularization mediated by mesenchymal stem cells from bone marrow and adipose tissue: a comparison. Cell regeneration *4*, 8.

Pilz, G.A., Braun, J., Ulrich, C., Felka, T., Warstat, K., Ruh, M., Schewe, B., Abele, H., Larbi, A., and Aicher, W.K. (2011). Human mesenchymal stromal cells express CD14 cross-reactive epitopes. Cytometry Part A : the journal of the International Society for Analytical Cytology *79*, 635-645.

Pittenger, M.F., and Martin, B.J. (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. Circulation research *95*, 9-20.

Reddy, K., Khaliq, A., and Henning, R.J. (2015). Recent advances in the diagnosis and treatment of acute myocardial infarction. World journal of cardiology *7*, 243-276.

Rodrigo, S.F., van Ramshorst, J., Hoogslag, G.E., Boden, H., Velders, M.A., Cannegieter, S.C., Roelofs, H., Al Younis, I., Dibbets-Schneider, P., Fibbe, W.E*., et al.* (2013). Intramyocardial injection of autologous bone marrow-derived ex vivo expanded mesenchymal stem cells in acute myocardial infarction patients is feasible and safe up to 5 years of follow-up. Journal of cardiovascular translational research *6*, 816-825.

Samsonraj, R.M., Raghunath, M., Nurcombe, V., Hui, J.H., van Wijnen, A.J., and Cool, S.M. (2017). Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine. Stem cells translational medicine *6*, 2173-2185.

Sanganalmath, S.K., and Bolli, R. (2013). Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions. Circulation research *113*, 810-834.

Santos, F., Andrade, P.Z., Abecasis, M.M., Gimble, J.M., Chase, L.G., Campbell, A.M., Boucher, S., Vemuri, M.C., Silva, C.L., and Cabral, J.M. (2011). Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-

based culture system under xeno-free conditions. Tissue engineering Part C, Methods *17*, 1201-1210.

Schmidt-Mende, J., Hellström-Lindberg, E., Joseph, B., and Zhivotovsky, B. (2000). Freezing induces artificial cleavage of apoptosis-related proteins in human bone marrow cells. Journal of immunological methods *245*, 91-94.

Schop, D., Janssen, F.W., van Rijn, L.D.S., Fernandes, H., Bloem, R.M., de Bruijn, J.D., and van Dijkhuizen-Radersma, R. (2009). Growth, Metabolism, and Growth Inhibitors of Mesenchymal Stem Cells. Tissue Eng Part A *15*, 1877-1886.

Shake, J.G., Gruber, P.J., Baumgartner, W.A., Senechal, G., Meyers, J., Redmond, J.M., Pittenger, M.F., and Martin, B.J. (2002). Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg *73*, 1919-1925.

Squillaro, T., Peluso, G., and Galderisi, U. (2016). Clinical Trials With Mesenchymal Stem Cells: An Update. Cell transplantation *25*, 829-848.

Tao, H., Han, Z., Han, Z.C., and Li, Z. (2016). Proangiogenic Features of Mesenchymal Stem Cells and Their Therapeutic Applications. Stem cells international *2016*, 1314709.

van der Bogt, K.E., Schrepfer, S., Yu, J., Sheikh, A.Y., Hoyt, G., Govaert, J.A., Velotta, J.B., Contag, C.H., Robbins, R.C., and Wu, J.C. (2009). Comparison of transplantation of adipose tissue- and bone marrow-derived mesenchymal stem cells in the infarcted heart. Transplantation *87*, 642-652.

Venkat, R.V., Stock, L.R., and Chalmers, J.J. (1996). Study of hydrodynamics in microcarrier culture spinner vessels: A particle tracking velocimetry approach. Biotechnology and bioengineering *49*, 456-466.

Wang, D., Jin, Y., Ding, C., Zhang, F., Chen, M., Yang, B., Shan, Q., Zou, J., and Cao, K. (2011). Intracoronary delivery of mesenchymal stem cells reduces proarrhythmogenic risks in swine with myocardial infarction. Irish journal of medical science *180*, 379-385.

Watt, S.M., Gullo, F., van der Garde, M., Markeson, D., Camicia, R., Khoo, C.P., and Zwaginga, J.J. (2013). The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. British medical bulletin *108*, 25-53.

Yao, Y., Huang, J., Geng, Y., Qian, H., Wang, F., Liu, X., Shang, M., Nie, S., Liu, N., Du, X.*, et al.* (2015). Paracrine action of mesenchymal stem cells revealed by single cell gene profiling in infarcted murine hearts. PloS one *10*, e0129164.

Yi, W., Sun, Y., Wei, X., Gu, C., Dong, X., Kang, X., Guo, S., and Dou, K. (2010). Proteomic profiling of human bone marrow mesenchymal stem cells under shear stress. Molecular and cellular biochemistry *341*, 9-16.

Yoo, S.Y., and Kwon, S.M. (2013). Angiogenesis and its therapeutic opportunities. Mediators of inflammation *2013*, 127170.

IV.6 ANNEX I- CHAPTER IV

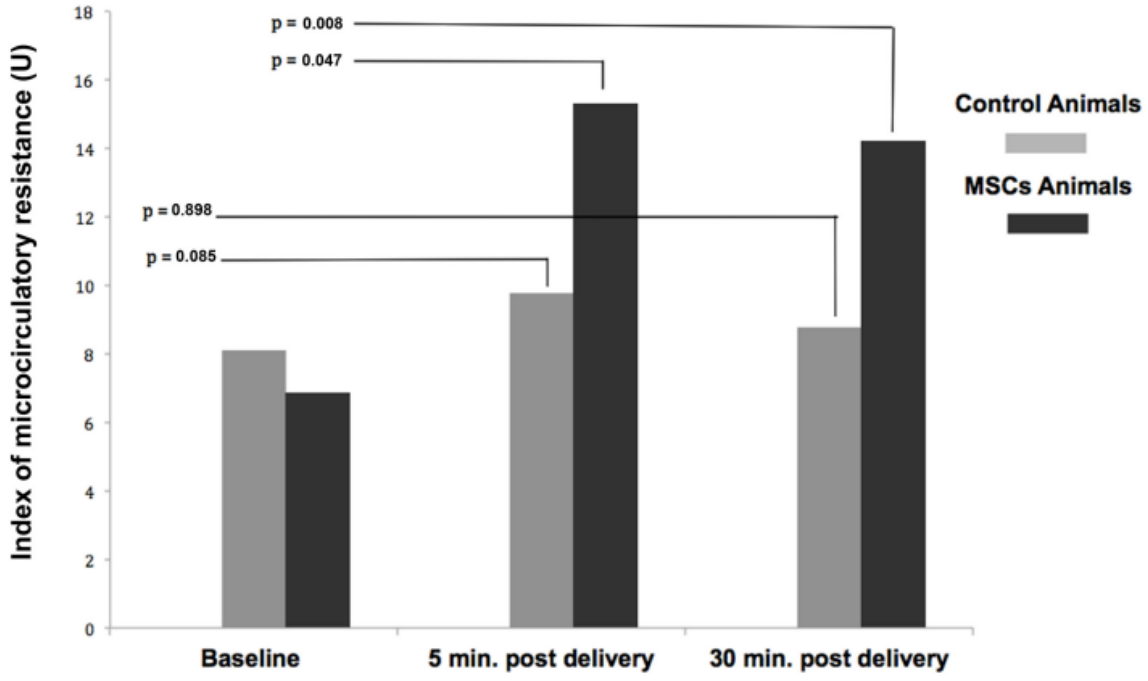


Figure IV-7 *– Index of microcirculatory resistance at baseline, 5 and 30 minutes post MSC delivery. Baseline, 5 and 30 minutes post-delivery IMR values are shown for Control and MSC group (n = 9). Differences from baseline were assessed with repeated measures ANOVA and P-values are adjusted for multiple comparisons (Bonferroni).*

Table IV-4 *- Values of the index of microcirculation resistance (IMR) for baseline (D0), 7 days post-MI (D7 post-MI), post intracoronary infusion (Post-intracoronary) and 4 weeks after intracoronary infusion (D28 post-intracoronary) of the control, "conventional" and "innovative" groups. Results presented as mean ± SD*

IMR	CONTROL (n=6)	CONVENTIONAL (n=6)	INNOVATIVE (n=6)	P
D0 (U)	8.4 ± 0.7	7.6 ± 0.8	9.0 ± 0.3	0.182
D7 post-MI (U)	9.6 ± 1.1	11.0 ± 2.2	10.4 ± 1.1	0.778
Post-intracoronary 1st dose (U)	10.9 ± 2.5	14.8 ± 3.1	15.0 ± 3.6	0.548
Post-intracoronary 2nd dose (U)	**10.2 ± 2.5**	**18.9 ± 3.8**	**21.7 ± 2.7**	**0.034**
Post-intracoronary 3rd dose (U)	**7.5 ± 0.4**	**22.1 ± 6.4**	**19.6 ± 5.0**	**0.024**
Post-intracoronary – 30 min. (U)	**7.6 ± 1.4**	**20.7 ± 5.5**	**19.7± 5.0**	**0.049**
D28 post-intracoronary (U)	11.5 ± 2.5	11.9 ± 1.8	9.4 ± 1.7	0.603

CHAPTER V - DIFFERENT SOURCES OF STROMAL FEEDER LAYERS TO SUPPORT THE EXPANSION OF HEMATOPOIETIC STEM/PROGENITOR CELLS FROM THE UMBILICAL CORD BLOOD.

V.1 SUMMARY

It has been shown, in our lab and others, that bone marrow mesenchymal stem/stromal cells (BM-MSCs) can support the *ex vivo* hematopoietic stem/progenitor cells (HSPCs) expansion (Andrade et al., 2010; da Silva et al., 2010a). Nonetheless, other MSC sources are attractive, due to wider availability and ease of collection, compared to BM, such as adipose tissue (AT-MSCs) and umbilical cord matrix (UCM-MSCs). However, it has been shown that MSCs from different sources present distinct characteristics (Hass et al., 2011a).However, only few studies for the comparison between different sources of MSCs have been performed focusing on their hematopoietic supportive capacity *ex-vivo*. Thus, the aim of this work was to assess and compare the hematopoietic supportive capacity of MSCs from different sources, namely BM-MSC (either isolated through plastic adherence (MSC) or by magnetic-activated cell sorting (MACS) for Stro-1 expression (Stro-1 cells)), UCM-MSC, AT-MSC and fetal liver MSC (FL-MSC, isolated by MACS for Stro-1 expression).

All feeder layers were able to support HSPCs expansion ex-vivo, with AT cells giving the highest fold increase in total cells, and FL and *No Stroma* condition (control without feeder cells) with the lowest expansion. It was attained a fold increase in total cells of 18.6±3.0, 15.6±4.5, 17±2.2, 32.7±5.5, 8.2±0.54, 6.5±0.67 for BM, UCM, Stro-1, AT, FL and no stroma control, respectively. However, the higher the expansion in total cell number, the lower the progenitor content ($CD34^+$, $CD34^+/CD90^+$, $CD34^+/CD133^+$) post-culture leading to a similar fold expansion of these progenitors, with FL and *No stroma* conditions presenting again lower values. Although not significant, UCM-MSC appear to be able to maintain higher percentages of the $CD34^+/CD90^+$ populations leading to an apparent higher fold increase in the number of these progenitors (1.2±0.6) in comparison with the other sources. Regarding the fold increase in $CD34^+/CD133^+$ cells, BM and AT-MSC attained apparent higher values (7.1±1.6 and 7.5±1.0, respectively), again not

statistically significant. Moreover, expanded cells demonstrated similar differentiation ability *in vivo* in all conditions, assessed by clonogenic formation assay (CFUs), as well as primitive population content measured by the cobblestone formation assay (CAFCs), with FL and *No stroma* conditions presenting lower fold increase values in both cases.

Overall, no significant differences were observed between the different feeder layers tested, suggesting the possible use of more readily available MSC sources to support HSPC expansion ex-vivo.

V.2 BACKGROUND

Hematopoietic cell transplantation has been performed for more than 50 years to treat malignant and non-malignant hematological disorders. However, the clinical bottleneck resides on the number of cells that can be retrieved from either adult (such as BM) or neonatal (such as UCB) tissues. The latter offers the advantage of ease of access, less restringing HLA matching and higher enrichment in stem/progenitor cells. Nonetheless, due to the low numbers and primitiveness of cells, its transplantation is usually associated with graft failure. One way to overcome this hurdle is by *ex vivo* expansion of the hematopoietic progenitors.

Interactions between vascular/stromal cells and HSPCs are known to be of great importance for their maintenance, thus making stromal cells ideal to mimic their natural BM niche *in vitro* by recapitulating physiological cues of the hematopoietic niche. It has been shown, in our lab and by others, that bone marrow mesenchymal stem/stromal cells (BM-MSC) can support the *ex vivo* HSPC expansion (Andrade et al., 2010; da Silva et al., 2005a). Nonetheless, other sources of MSC are attractive, due to their availability and ease of collection, such as adipose tissue and umbilical cord matrix.

Distinct co-culture systems have been exploited for the expansion of hematopoietic stem/progenitor cells (Hofmeister et al., 2007; Wagner et al., 2008), with the majority reporting the use of BM-MSC as feeder layers. Nonetheless ,some systems report different stromal sources, different isolation protocols of stromal cells (such as through plastic adherence or antigen cell sorting (e.g. Stro-1)), and diverse cytokine combinations, which consequently leads to divergent results and thus, the role of stroma in hematopoietic cell co-cultures remains a controversial issue (da Silva et al., 2010a).

There are several well-defined sources for MSCs in the body besides the BM, such as AT, lung, liver, umbilical cord blood and matrix (*i.e.* Wharton's jelly; UCM) , synovium tissue, amniotic fluid, FL, dental pulp, and skeletal muscle (reviewed in (Klingemann et al., 2008)) . Additionally, MSC can also be isolated by Stro-1 antigen sorting (through MACS or FACS), as this is one of the best well-known BM MSC markers (Gronthos and Simmons, 1995; Gronthos et al., 2003; Kolf et al., 2007). The isolation through antigen sorting presents the advantage of obtaining a more homogeneous population in comparison with the plastic adherence isolation. Importantly, in contrary with the BM, where HSPCs are usually quiescent, hematopoiesis occurs in the FL during the development, where the HSPCs are actively dividing and self-renewing, suggesting that the FL provides an extended HSPC support microenvironment (Mikkola and Orkin, 2006). Nonetheless, several studies suggest that MSCs from different sources present different characteristics (Hass et al., 2011a).

Although some studies have been made to determine different feeder layer potential on HSPC expansion ex-vivo (Magin et al., 2009), to date, no comprehensive comparison between different sources of MSC has been performed focusing on their hematopoietic supportive capacity. Thus, BM cells (either isolated through plastic adherence (MSC) or by MACS for Stro-1 expression (Stro-1 cells)), UCM MSC, AT MSC and FL MSC (isolated by MACS for Stro-1 expression) were tested and compared as hematopoietic feeder layers.

V.3 RESULTS

Our lab has previously established and optimized a UCB derived HSPC expansion in co-culture with BM-MSC, using QBSF-60® medium (Andrade et al., 2010). Here we present the ability of different MSC sources, namely BM isolated by two methods (plastic adherence and Stro-1), AT, UCM, and FL to support UCB derived HSPC expansion *ex vivo*.

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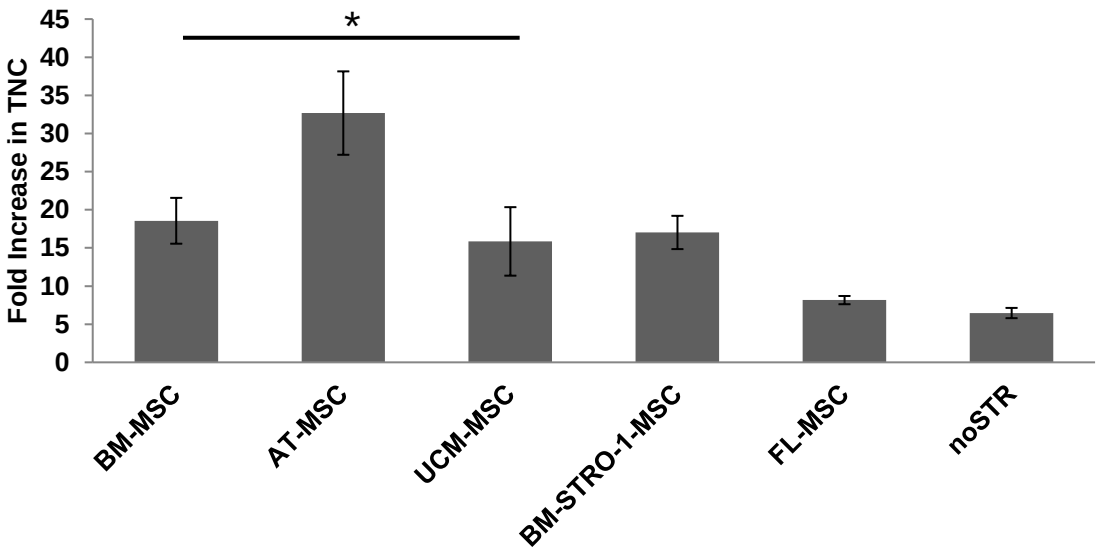


Figure V-1 *– Fold increase in total nucleated cells (TNC) from UCB cells for each MSC-HSC co-culture system: bone marrow (BM), adipose tissue (AT), umbilical cord matrix (UCM), BM Stro-1, fetal liver (FL) Stro-1 and No stroma control (noSTR). Results presented as mean±SEM (n=3-6, *p<0.05).*

Cells from the different sources studied were expanded in 2D cell culture flasks with low glucose DMEM containing 10% FBS (MSC qualified) for at least 2 passages before co-culture. Upon reaching 90-100% confluence, MSC were treated with Mitomycin C (to arrest their growth) and then co-culture with CD34+-enriched cells isolated from UCB

was initiated and maintained for 7 days (asdescribed in Material and Methods). To assess MSC supportive capacity in HSPC *ex vivo* expansion, analysis of fold change in total nucleated cells (TNC) (***Figure V-1***), hematopoietic stem/progenitor phenotypes CD34+, CD34+/CD133+ and CD34+CD90+ (***Figure V-2* A and B**), and for the more committed progenitors from the early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41a+ cells) was performed (***Figure V-2* C**). In addition, the clonogenic potential of both fresh and expanded UCB cells was assessed by BFU-E, CFU-MIX and CFU-GM assays (**Figure V-3**), as well as their ability to form cobblestone areas (CAFC) (***Figure V-4*).**

All feeder layers were able to support *ex vivo* hematopoietic cell expansion, although with FL-MSC derived feeder layer presenting similar TNC fold values to the No stroma control (8.2±0.54 and 6.5±0.67 fold, respectively). AT-MSC gave the higher expansion fold in total cell number, presenting a value of 32.7±5.5. The cell expansion attained on BM, UCM and Stro1-MSC feeder layers was similar, achieving 18.6±3.0, 15.6±4.5, 17±2.2 fold, respectively. Nonetheless, it was seen a statistical significant difference between the BM cells AT cells and UCM cells.

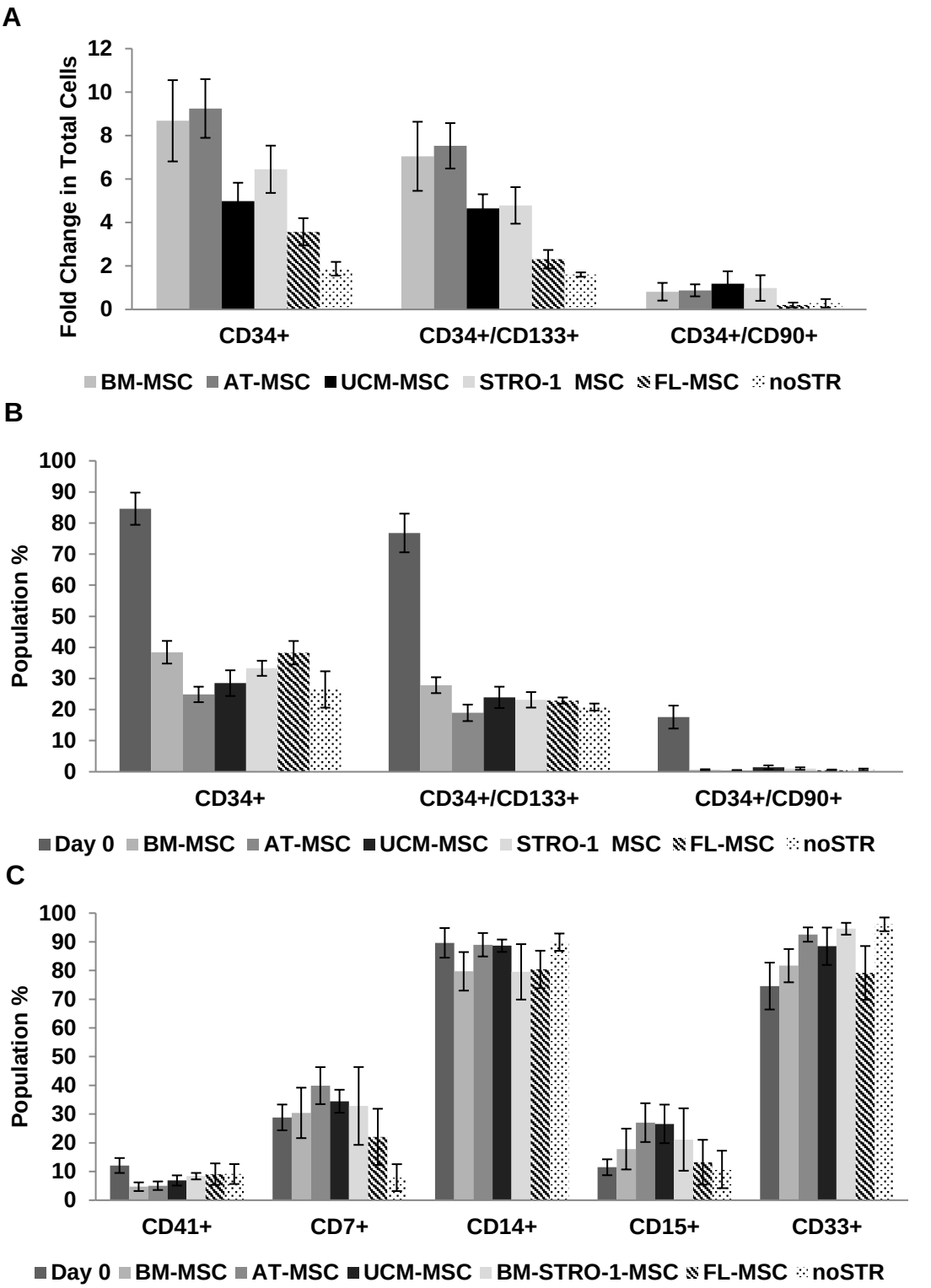


Figure V-2 *– Immunophenotypic characterization of the pre- and post-expanded CD34+ cells. (A) fold change in total hematopoietic stem/progenitor phenotype (CD34+, CD34+/CD133+ and CD34+/CD90+); (B) Content in percentage of the hematopoietic stem/progenitor phenotype; (C) Content in percentage in early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41+). Results presented as mean±SEM (n=3-6)*

Moreover, immunophenotypic analysis of pre- and post-expanded cells was performed. As expected a decrease from 85% to 25-38 % after expansion on the hematopoietic stem/progenitor cells was observed in all conditions (***Figure V-2*** **B**), with BM MSC and FL MSC presenting slightly higher values in $CD34^+$ percentages of 38%, whereas the other conditions had values in the 25-33% range. Regarding CD34+/CD133+ and CD34+/CD90+ cell populations, a decrease from 77% to 19-27% (with BM MSC supporting the higher value) and 18% to 0.5-1.5% (with UCM giving the higher value) was observed. Nonetheless, when observing the fold expansion in total progenitor cells (***Figure V-2*** **A**), BM and AT-MSC feeder layers were however able to attain a higher increase of these progenitors concomitantly, more specifically to a 9 and 7-fold increase in CD34+ and CD34+/CD133+ cells. However, a relatively higher value for CD34+/CD90+ total cells was attained by the UCM-MSC feeder layer (1.2-fold), whereas for the other conditions there was a decrease in these progenitors (0.2-1-fold).

It was also analyzed the differentiative potential of the UCB cells expanded in these co-culture systems, by testing for early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41a+ cells) (***Figure V-2*** **C**). All conditions were able to successfully maintain/expand these progenitors, in agreement with previous results from our lab (Andrade et al., 2013a; Andrade et al., 2010).

In terms of the clonogenic potential (**Figure V-3**), the trend is consistent with the TNC fold expansion, where it is seen a higher fold increase in BFU-E and CFU-GM for UCB cells generated with the support of the AT-MSC feeder layer (13.4 and 13.5-fold, respectively), although not statistically significant. Cells expanded over BM-MSC and Stro-1 BM MSC presented similar clonogenic potential. Moreover, UCB cells co-cultured with UCM-MSC attained a lower fold in the total clonogenic potential, reaching similar

values to FL-MSC and *No Stroma* conditions specifically for BFU-E (3.2, 3.3 and 2.4-fold respectively).

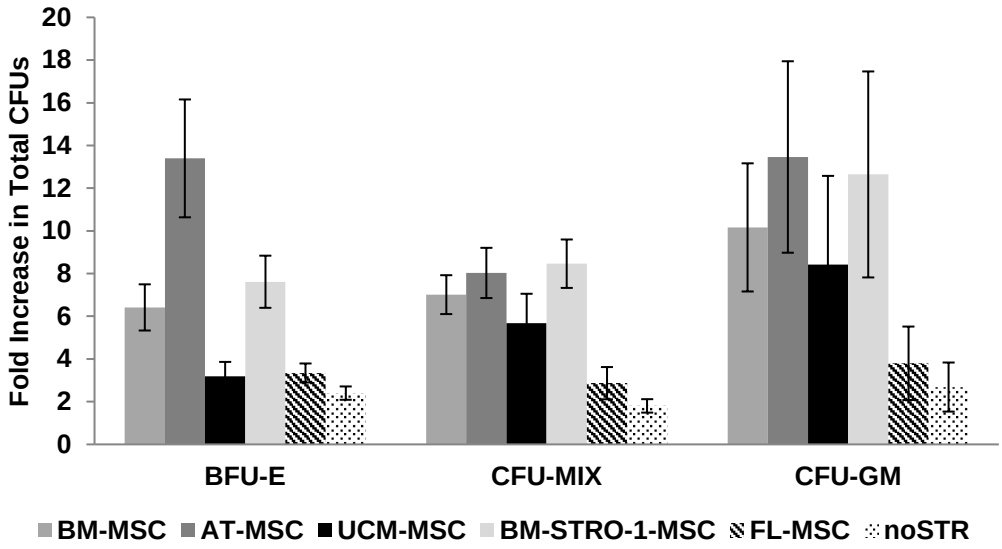


Figure V-3 - *Clonogenic potential of the expanded cells. Fold increase in BFU-E, CFU-MIX and CFU-GM for each condition. Results presented as mean±SEM (n=3-6).*

Finally, the expansion level in CAFCs was also measured (***Figure V-4***), which is described as a predictor of in vivo repopulating ability (Ploemacher et al., 1989). Once again, it was seen a lower expansion for UCB cells cultured over FL-MSC and under *No stroma* conditions, whereas all other conditions led to a roughly similar higher value, in the 14-16-fold range.

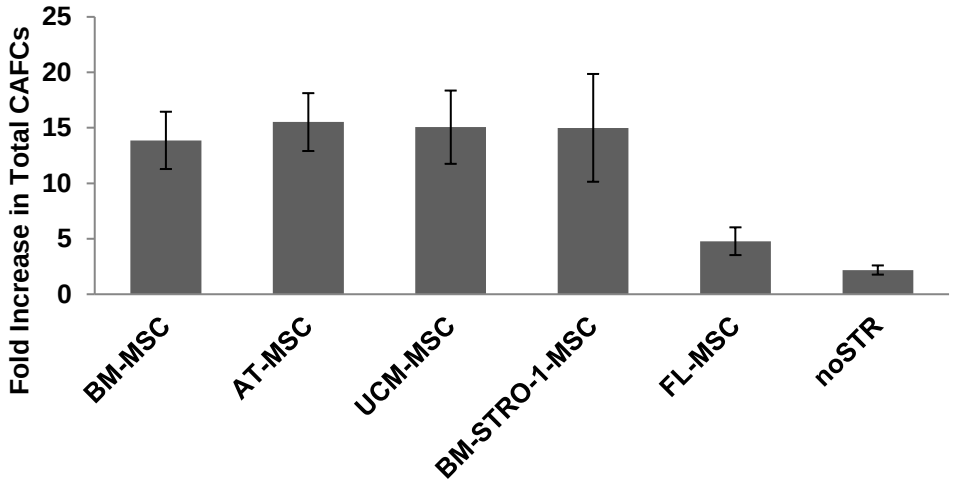


Figure V-4 *- Fold increase in cobblestone area-forming cells (CAFCs) for HSPC expanded with the different MSC feeder layers. Results presented as mean±SEM (n=3-6).*

V.4 DISCUSSION AND CONCLUSIONS

Hematopoietic stem cells are able to give rise to all blood cell types (Doulatov et al., 2012) and its transplantation has been widely used for the treatment of malignant and non-malignant hematopoietic diseases. Of the sources available, UCB presents attractive advantages as its easy non-invasive accessibility, higher cell primitiveness and less restringing HLA matching, increasing the availability of this treatment to a wider population. However, its use in adults has been restricted by the low cell numbers that can be retrieved. In order to overcome this hurdle, an effort on the development of several *ex vivo* culture systems have been reported. Nonetheless, the *ex-vivo* expansion of the HSPC population is difficult to achieve as they tend to differentiate during proliferation in culture (Hofmeister et al., 2007). This may be presumably caused by a lack of appropriate cues that are provided *in vivo* by the microenvironment. In vivo, HSPC are located mainly in the bone marrow where there are specific interactions within a specific and complex microenvironment called the stem cell niche, which regulates their fate in terms of quiescence, self-renewal and differentiation, by an orchestra of signals mediated by soluble factors and/or cell-to-cell contact (Schofield, 1978; Zhang et al., 2003). Interestingly, MSC are an important component of this niche. Hence, in order to mimic *in vivo* conditions, it has been shown the ability of MSC to support stem/progenitor cell maintenance and expansion through co-culture systems (Wagner et al., 2008).

It has been widely reported the use of BM-MSC as support for HSPC culture (Andrade et al., 2010; da Silva et al., 2010a; Jing et al., 2010b). However, other MSC sources may present attractive advantages as higher availability and ease and non-invasive collection, such as AT-MSC and UCM-MSC. Some studies have reported the use of these alternative sources as stromal feeder layers, as AT-MSC (Corre et al., 2006; Nishiwaki et al., 2012), UCM-MSC (Bakhshi et al., 2008; Friedman et al., 2007; Tipnis and Viswanathan, 2010), and BM stromal cells isolated based on Stro-1 expression (rather than plastic adherence) MSC (Dennis et al., 2002; Goncalves et al., 2006).

Nonetheless, results have been in some cases contradictory, which may be due to the different isolation methods and culture formulations used in the studies. Hence, it was aimed herein to perform a comprehensive and systematic comparison on the ability of the different MSC feeder sources on HSPC maintenance and expansion.

Here, it was shown that all feeder layers were able to support UCB cell expansion with exception of FL-MSC that led to similar values as the *No Stroma* control. Moreover, UCB cells expanded over AT-MSC presented the higher fold expansion in total cells. Indeed, Nakao and colleagues have shown that mice derived AT-MSC were able to attain higher fold expansion in total and hematopoietic progenitors derived UCB than BM-MSC and to facilitate hematopoiesis more effectively in mice (Nakao et al., 2010). Later, the authors also reported the same effect when using human derived cells (Nishiwaki et al., 2012). In contrary, although AT-MSC co-culture led to the highest expansion in total cells, it appears that it also promoted cell differentiation corroborated by the immunophenotypic analysis, where it was seen a sharper decrease on the hematopoietic stem/progenitor content (CD34+, CD34+/CD13+ and CD34+/CD90+ cells), whilst being able to maintain or even expand early lymphoid (CD7+) and myeloid (CD14+, CD15+ and CD33+) progenitors, as well as to demonstrate higher clonogenic capacity in comparison with the other feeder layers. These findings are in agreement, however, with the work reported by Corre and colleagues, where it is compared the supportive capacity of BM-MSC and AT-MSC on the maintenance and expansion of UCB derived HSPCs and it is suggested that the latter does not support HSPC self-renewal, but differentiation towards lymphoid and myeloid lineages (Corre et al., 2006). This effect could be explained by their observations on the secretion level of certain cytokines by the adipose derived cells with contrasting effects upon immature hematopoietic cells (i.e. MIP-1a inhibiting the proliferation of stem cells, IL-6, G-CSF and GM-CSF promoting the differentiation of committed progenitors).

Interestingly, in a work recently published by Mattiucci and colleagues, it is suggested that BM derived adipocytes (BM-A) are able to support HSPC survival in a co-culture system by secretion of cytokines directly involved in hematopoiesis regulation. They also shown, that although these BM-A are morphologically similar to adipocytes derived from AT (AT-A), the latter was not able to support HSPC survival *ex vivo*. Indeed, it was observed a distinct gene expression signature compared with AT-A, suggesting that BM-A are involved in BM niche homeostasis processes (Mattiucci et al., 2018).

Regarding the UCM-MSC co-culture, lower fold expansions in TNC as well in total hematopoietic stem/progenitor cells and concomitant clonogenic potential were attained in comparison specifically with BM-MSC, AT-MSC and Stro-1 BM-MSC conditions. These results are in contradiction from what has been published by Tipnis and Viswanathan, where it is shown a higher ability of UCM-MSC derived feeder layer to support and maintain UCB derived HSPC expansion in comparison with a BM-MSC feeder layer. Here, it was also assessed cytokine secretion profile from both stromal layers, with UCM-MSC having an increase on the release of certain factors involved in hematopoietic maintenance and differentiation, such as LIF, HGF, Flt3, G-CSF, GM-CSF, Angiogenin and TPO (Tipnis and Viswanathan, 2010). Moreover, Friedman and colleagues has reported the release of significant concentrations of hematopoietic growth factors in culture by UC-MSCs (UC-MSCs secreted significantly higher concentrations of G-CSF, GM-CSF, HGF, LIF, IL-1a, IL-6, IL-8, and IL-11 compared with BM-MSCs, whereas BM-MSCs was shown to produce more VEGF and SDF-1b than UC-MSCs) and augmentation of hematopoietic colony formation when co-cultured with UCB mononuclear cells, as well as higher engraftment rates on mice upon co-transplantation of CD34+ cells and UCM-MSC (Friedman et al., 2007).

On the other hand, in other study published by Bakhshi and colleagues, where they also compare and test the hematopoietic supportive capacity of BM and UCM-MSC derived feeder layers on long-term culture-initiating cell (LTC-IC) assay, it was observed that

UCM feeder layers performed less or as good as BM (Bakhshi et al., 2008). In another comparative study, Magin and co-workers showed that the tested potential feeder cell types (BM-MSC, Wharton's Jelly cells (UCM) and HUVECS) showed similar potential for UCB CD34+-support in vitro (Magin et al., 2009). These differences encountered with the work present herein and the literature may be explained by the different MSC isolation and expansion methods, as well as the different medium compositions and cytokines used for HSPC cultivation. To note that, in the case of this work, the establishment of UCM-MSC derived feeder layer was accompanied with the inability of cell growth arrest by inactivation with Mitomycin C. Several concentrations of this agent were tested although without success (i.e. 0.5 µg/mL – 5 µg/mL). Thus, co-culture initiation was performed upon reaching 70% confluence and cell growth was observed, even after changing to serum-free hematopoietic cell medium. Hence, the results presented in this work regarding specifically this feeder layer, need to be carefully and critically analyzed, as it may not represent the optimal co-culture conditions and further work needs to be performed.

Stro-1 MSC are derived from BM but isolated based on the expression of the Stro-1 marker by MACS, rather than adherence on plastic and hence, having the advantage of reducing feeder layer donor variability (Goncalves et al., 2006). These cells are believed to be a more primitive and homogeneous MSC population within the BM, however it seen a loss of this marker throughout serum medium *ex vivo* culture (Gronthos and Simmons, 1995). Not surprisingly, this feeder layer was able to support and maintain HSPC expansion and their differentiative potential in similar levels as the BM MSC counterpart. Indeed, Dennis and colleagues have reported that Stro-1 derived feeder layer was able to induce a 10- to 20-fold increase in colony-forming units from expanded BM HSPC, and to sustain cobblestone area colony-forming cells with a distinct time course for the different hematopoietic precursors (Dennis et al., 2002). Later, and in agreement with these results, Gonçalves and colleagues also reported the successful use of Stro-1

feeder layer from the BM, including a side-by-side comparison with cells isolated based solely on plastic adherence (i.e. from the same BM donors), on the expansion and maintenance of human BM derived HSPC cells, whilst retaining their engraftment ability in a preimmune fetal sheep model (Goncalves et al., 2006). Importantly, it was shown that the differentiative potential was shifted towards the myeloid lineage, in agreement with the results reported in this work, where it is shown a maintenance/expansion of the myeloid related markers (CD14+, CD15+ and CD33+ cells), as well as to maintain/expand the lymphocytic differentiative potential (CD7+).

By contrast with the BM niche, where HSPCs are normally quiescent, within the human fetal liver (FL), there is a higher frequency of cycling HSPCs undergoing self-renewal (Mikkola and Orkin, 2006), which suggests that the FL provides a microenvironment that is more conducive in supporting HSPCs. Thus, it was hypothesized that a FL-MSC feeder layer would provide either same or even better HSPC support in comparison with the other feeder layers tested. However, the opposite was seen, with the Stro-1 FL-MSC stromal layer studied herein presenting similar results as the *No stroma* control (although presenting always slightly higher values), giving the poorest support to HSPC maintenance/expansion relatively to the other feeder layers. Nonetheless, this might be due to storage of the cells for a long period of time as well as the medium used in this experiment was different from the one used for the previous isolation and expansion of these cells, and hence, this might have affected the quality of the cells. In the work developed by Campagnoli and colleagues, they have shown the ability of fetal blood derived MSC to support UCB derived HSPC proliferation and differentiation in long term culture in the same extent of the control (M210B4 stromal line from mice) (Campagnoli et al., 2001). Furthermore, Young and co-workers have reported a population of $CD34^{lo}CD133^{lo}$ from fetal liver that is able to differentiate into hepatocyte and mesenchymal lineage, and to support both UCB and FL HSPC proliferation, due to the expression of growth factors involved in HSPC expansion, as well as maintenance of

engraftment potential (Yong et al., 2016). Although the results presented herein seem to be contradictory with the aforementioned reports, it is important to note the differences on the cell population used as stromal layer, as well as to be aware that the fetal liver hematopoietic niche is composed by several types of cells which constructs a complex microenvironment not replicable *in vitro*. Interestingly, Chou and colleagues reported the ability of hepatic progenitors to support *ex* vivo expansion of mice BM-HSPC in co-culture, stating to demonstrate a direct proof that hepatic progenitors (DLK^+ cells) are the principle supportive cells for the expansion of hematopoietic stem and progenitors in the fetal liver (Chou et al., 2013). This adds to the possibility, in the case of the fetal liver, that MSC are not the major responsible for HSPC proliferation and maintenance and thus, may not represent the best choice as a supportive *ex vivo* feeder layer.

In conclusion, this work provides a systematic comparison between MSCs from different sources as feeder layers to support and expand UCB derived HSPC. All feeder layers tested were able to support HSPC expansion, with AT giving higher total numbers but, at the same time, producing more differentiated cells. Nonetheless, UCB cells expanded over BM MSC (including the one isolated based on Strom-1 expression) and AT MSC attained similar stem/progenitor fold expansion, as well as differentiative potential, whereas for the co-cultures with UCM MSC lower values of expansion were attained, which could be due to methodological hurdles on the establishment of this feeder layer. Moreover, FL-MSC performed the least, with values similar to the control. However, the use of that stromal layer would have anyhow significant ethical drawbacks in comparison with the other sources. Taking all together, AT-MSC seems to be a promising candidate for future clinical applications were co-culture strategies are used for UCB HSPC expansion, as it led to similar amount of total progenitor cells compared to the BM feeder layers studied, and presents the most available MSC source.

However, efforts towards the use of human origin, clinical-grade MSC prepared under good manufacturing practice (GMP) -compliant conditions need to be addressed, to

overcome possible contamination risks from compounds of animal origin such as FBS. Hence the next step would be to repeat this study with MSC expanded under xeno-free conditions.

Overall, this work generated scientific knowledge on the differential supportive capacity of different MSC sources for HSPC *ex vivo* expansion, which has been quite difficult to address with the studies made so far, due to the variety of isolation, expansion and medium formulation (including cytokine cocktails) used. Ultimately, this study might also provide important insights on the potential use of these cells as adjuvants in a co-transplantation scenario.

V.5 REFERENCES

Andrade, P.Z., de Soure, A.M., Dos Santos, F., Paiva, A., Cabral, J.M., and da Silva, C.L. (2013). Ex vivo expansion of cord blood haematopoietic stem/progenitor cells under physiological oxygen tensions: clear-cut effects on cell proliferation, differentiation and metabolism. Journal of tissue engineering and regenerative medicine.

Andrade, P.Z., dos Santos, F., Almeida-Porada, G., da Silva, C.L., and JM, S.C. (2010). Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells. Molecular bioSystems *6*, 1207-1215.

Bakhshi, T., Zabriskie, R.C., Bodie, S., Kidd, S., Ramin, S., Paganessi, L.A., Gregory, S.A., Fung, H.C., and Christopherson, K.W., 2nd (2008). Mesenchymal stem cells from the Wharton's jelly of umbilical cord segments provide stromal support for the maintenance of cord blood hematopoietic stem cells during long-term ex vivo culture. Transfusion *48*, 2638-2644.

Campagnoli, C., Roberts, I.A., Kumar, S., Bennett, P.R., Bellantuono, I., and Fisk, N.M. (2001). Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood *98*, 2396-2402.

Chou, S., Flygare, J., and Lodish, H.F. (2013). Fetal hepatic progenitors support long-term expansion of hematopoietic stem cells. Experimental hematology *41*, 479-490 e474.

Corre, J., Barreau, C., Cousin, B., Chavoin, J.P., Caton, D., Fournial, G., Penicaud, L., Casteilla, L., and Laharrague, P. (2006). Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors. Journal of cellular physiology *208*, 282-288.

da Silva, C.L., Gonçalves, R., Crapnell, K.B., Cabral, J.M., Zanjani, E.D., and Almeida-Porada, G. (2005). A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Experimental hematology *33*, 828-835.

da Silva, C.L., Goncalves, R., dos Santos, F., Andrade, P.Z., Almeida-Porada, G., and Cabral, J.M. (2010). Dynamic cell-cell interactions between cord blood haematopoietic progenitors and the cellular niche are essential for the expansion of CD34+, CD34+CD38- and early lymphoid CD7+ cells. Journal of tissue engineering and regenerative medicine *4*, 149-158.

Dennis, J.E., Carbillet, J.-P., Caplan, A., and Charbord, P. (2002). The STRO-1+ Marrow Cell Population Is Multipotential. Cell Tissues Organs *170*, 73-82.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell stem cell *10*, 120-136.

Friedman, R., Betancur, M., Boissel, L., Tuncer, H., Cetrulo, C., and Klingemann, H. (2007). Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation *13*, 1477-1486.

Goncalves, R., Lobato da Silva, C., Cabral, J.M., Zanjani, E.D., and Almeida-Porada, G. (2006). A Stro-1(+) human universal stromal feeder layer to expand/maintain human bone marrow hematopoietic stem/progenitor cells in a serum-free culture system. Experimental hematology *34*, 1353-1359.

Gronthos, S., and Simmons, P.J. (1995). The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood *85*, 929-940.

Gronthos, S., Zannettino, A.C., Hay, S.J., Shi, S., Graves, S.E., Kortesidis, A., and Simmons, P.J. (2003). Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci *116*, 1827-1835.

Hass, R., Kasper, C., Bohm, S., and Jacobs, R. (2011). Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. Cell communication and signaling : CCS *9*, 12.

Hofmeister, C.C., Zhang, J., Knight, K.L., Le, P., and Stiff, P.J. (2007). Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone marrow transplantation *39*, 11-23.

Jing, D., Fonseca, A.V., Alakel, N., Fierro, F.A., Muller, K., Bornhauser, M., Ehninger, G., Corbeil, D., and Ordemann, R. (2010). Hematopoietic stem cells in co-culture with mesenchymal stromal cells--modeling the niche compartments in vitro. Haematologica *95*, 542-550.

Klingemann, H., Matzilevich, D., and Marchand, J. (2008). Mesenchymal Stem Cells - Sources and Clinical Applications. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie *35*, 272-277.

Kolf, C.M., Cho, E., and Tuan, R.S. (2007). Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis research & therapy *9*, 204.

Magin, A.S., Korfer, N.R., Partenheimer, H., Lange, C., Zander, A., and Noll, T. (2009). Primary cells as feeder cells for coculture expansion of human hematopoietic stem cells from umbilical cord blood--a comparative study. Stem cells and development *18*, 173-186.

Mattiucci, D., Maurizi, G., Izzi, V., Cenci, L., Ciarlantini, M., Mancini, S., Mensa, E., Pascarella, R., Vivarelli, M., Olivieri, A.*, et al.* (2018). Bone marrow adipocytes support hematopoietic stem cell survival. Journal of cellular physiology *233*, 1500-1511.

Mikkola, H.K., and Orkin, S.H. (2006). The journey of developing hematopoietic stem cells. Development *133*, 3733-3744.

Nakao, N., Nakayama, T., Yahata, T., Muguruma, Y., Saito, S., Miyata, Y., Yamamoto, K., and Naoe, T. (2010). Adipose tissue-derived mesenchymal stem cells facilitate hematopoiesis in vitro and in vivo: advantages over bone marrow-derived mesenchymal stem cells. The American journal of pathology *177*, 547-554.

Nishiwaki, S., Nakayama, T., Saito, S., Mizuno, H., Ozaki, T., Takahashi, Y., Maruyama, S., Nishida, T., Murata, M., Kojima, S.*, et al.* (2012). Efficacy and safety of human adipose tissue-derived mesenchymal stem cells for supporting hematopoiesis. International journal of hematology *96*, 295-300.

Ploemacher, R.E., van der Sluijs, J.P., Voerman, J.S., and Brons, N.H. (1989). An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood *74*, 2755-2763.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

Tipnis, S., and Viswanathan, C. (2010). Umbilical cord matrix derived mesenchymal stem cells can change the cord blood transplant scenario. Int J Stem Cells *3*, 103-118.

Wagner, W., Saffrich, R., and Ho, A.D. (2008). The Stromal Activity of Mesenchymal Stromal Cells. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie *35*, 185-193.

Yong, K., Keng, C.T., Tan, S., Loh, E., Chang, K., Tan, T., Hong, W., and Chen, Q. (2016). Human CD34loCD133lo fetal liver cells support the expansion of human CD34hiCD133hi hematopoietic stem cells. Cellular & molecular immunology *13*, 605-614.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W., Ross, J., Haug, J., Johnson, T., Feng, J.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

CHAPTER VI - EX-VIVO EXPANSION OF CIRCULATING ANGIOGENIC CD34+ PROGENITORS FROM THE UMBILICAL CORD BLOOD (UCB) UNDER DYNAMIC CONDITIONS

VI.1 SUMMARY

Several culture systems have been studied to improve the number and angiogenic potency of $CD34^+$ cells. Nevertheless, a scalable and GMP compliant system is yet to be achieved in order to obtain clinically meaningful numbers and required potency. Firstly, a comparison between two serum-free different media, SCGM (Cellgenix), that was used in Chapter III, and StemSpan (StemCell Technologies), a medium developed for culture and expansion of hematopoietic cells, was performed. Both culture media were supplemented with SCF (100ng/mL), Flt-3 ligand (100ng/mL), TPO (20ng/mL), IL-6 (20ng/mL) and VEGF (50ng/mL).The results have shown that the StemSpan medium was able to support a higher fold increase in total cell number (57.5±3.6 at day 7 and 116.17±9.4 at day 10) with concomitant higher fold increase in number of progenitors (20.2±10.3 and 55.9±12.7 for $CD34^+$; 20.1±1.2 and 39.2±11.1 for $CD34^+/CD133^+$ cells at day 7 and day 10 respectively), in comparison with the SCGM medium (fold increases of 19.8±7.4, 4.5±2.7 at day 7; 54.6±17, 8.7±4.3 at day 10, in total cell number, $CD34^+$ and $CD34^+/CD133^+$, respectively). Interestingly, markers associated with angiogenic and endothelial populations (CD31, CD105, CD14, CD18 and CD16) were either maintained or even increased in content when cultured with the StemSpan medium.

Moreover, it was performed a study for the scale-up of the expansion of $CD34^+$ cells from UCB into spinner flasks both under atmospheric conditions (normoxia (21% O_2)) and hypoxia (5% O_2) under serum-free conditions, together with the *in vitro* characterization of the final product. A successful dynamic scalable process was achieved, where an improvement in terms of quantity (total cell number fold increase of 32.1±0.9 and 45.6±6.8 at day 7; 83.9±7.9 and 101.3±25.7 at day 10, normoxia and hypoxia respectively) was shown. The angiogenic profile was assessed by flow cytometry and tube formation assay, in comparison with uncultured cells. In addition, the hypoxia condition revealed to enhance the angiogenic properties of the final cell product in both quantity and quality.

VI.2 BACKGROUND

The therapeutic properties of $CD34^+$ cells (which is an enriched population for endothelial progenitor cells -EPCs) have been demonstrated in several pre-clinical and clinical studies, namely for the treatment of ischemic and cardiovascular diseases. However, the therapeutic application of these cells has been hampered by the difficulty in isolating enough number of circulating $CD34^+$ cells from patient's blood while maintaining their quality for clinical use. To overcome this limitation, different suspension culture systems have been established for the *ex vivo* expansion of these cells, which demonstrated efficiency in promoting neovascularization in ischemic animal models upon transplantation. Also, considering the source, umbilical cord blood (UCB) is a promising candidate as its collection is non-invasive and the availability is immediate. Of notice, our group had previously established a suspension culture system for human EPCs, with increased quantity and quality (QQc) of the cell product (Alaiti et al., 2012) , and its scale-up into cell culture bags (Mata et al., 2015) (Chapter III), in order to move a step forward to meet GMP standards and clinically meaningful cell doses.

Several studies have been made where the impact of oxygen tension and fluid shear stress forces are investigated in EPC culture. Nevertheless, a fully controlled system, able to comply with GMP, where these factors are explored is yet to be achieved.

Hence, the aim of this chapter was to establish a scalable system for the manufacturing of these cells while studying the effect of shear stress and oxygen tension in order to enhance the angiogenic potential of the expanded cells.

VI.3 RESULTS

VI.3.1 MEDIUM COMPARISON UNDER STATIC CULTURE CONDITIONS

The aim of this work was to assess the differences between two different media, SCGM and StemSpan SFEM II (SS). Also, it was tested if adding VEGF only at day 7 (SCGM+7VEGF and SS+7VEGF) would promote firstly the expansion and lastly the commitment towards a more endothelial lineage. In the next figures are the results from fold increase (FI) in total nucleated cells (TNC) (**Figure VI-1**. A), FI in total CD34+ cells (**Figure VI-1**. B) and in CD34+/CD133+ cells (**Figure VI-1**. C) and the percentage of endothelial related markers pre- and post-expansion (**Figure VI-2**).

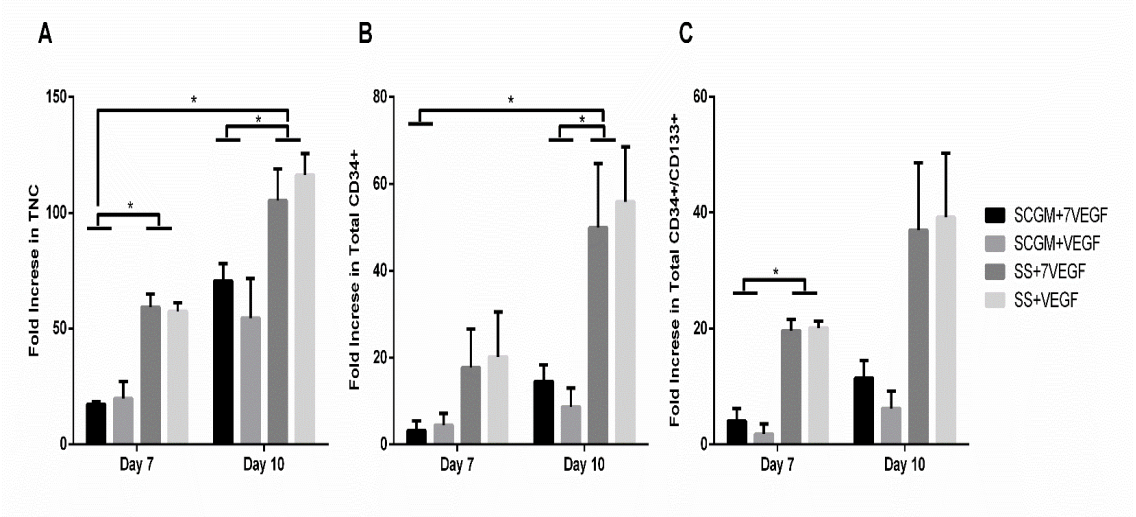


Figure VI-1 *- Fold increase in total nucleated cells (A), in total CD34+ cells (B) and in total CD34+/CD133+ cells (C). Values are represented as mean±SEM (n=3, *p<0.05).*

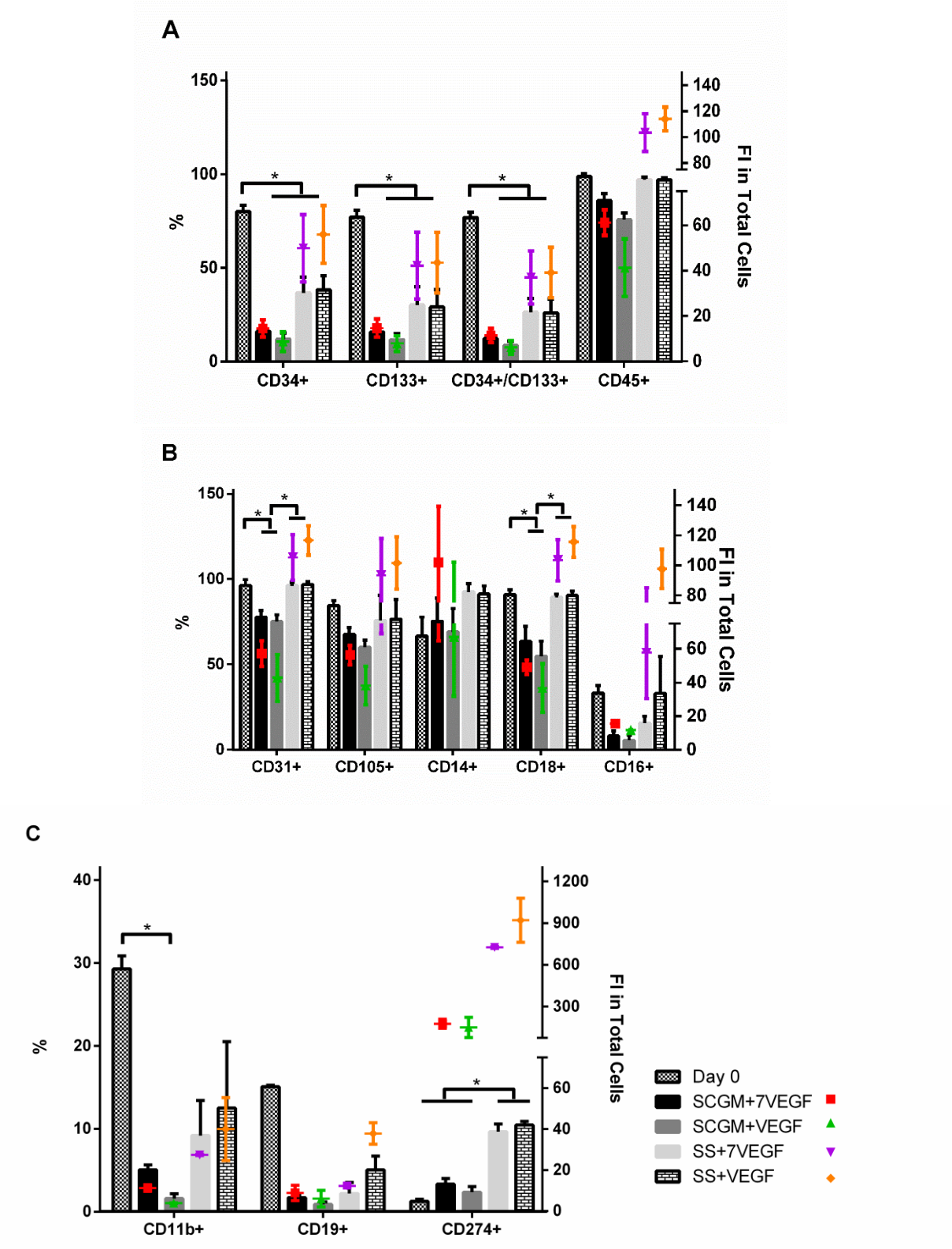


Figure VI-2 *- Immunophenotypic analysis of pre- and post-expanded cells regarding endothelial related markers. Bars represent the % values and dots represent the fold increase (FI) in total number of cells. Values are represented as mean±SEM (n=3, *p<0.05).*

The results obtained herein showed a higher capacity of the StemSpan medium to expand the total number of cells, as well as to increase the numbers of the more primitive subpopulation (CD34+ and CD34+/CD133+). Moreover, it was also seen a higher percentage of endothelial related markers on the post-expanded cells with StemSpan in comparison with the SCGM medium. The expression of other hematopoietic markers (CD11b, CD16 and CD19), namely from lymphoid lineage, was maintained at low levels and did not change significantly or even tended to decrease. Interestingly, there was an upregulation of the marker CD274 with the expansion especially noticed when using StemSpan.

Regarding the addition of VEGF in the beginning of the culture (day 0) or only at day 7, no significant differences were seen in the two media used.

Overall, it was shown that StemSpan medium was able to support the *ex vivo* expansion as well as to prime the final cell populations towards an endothelial lineage in a more efficient way that SCGM medium, hence being chosen to pursue the following dynamic studies.

VI.3.2 EXPANSION AND ANGIOGENIC PRIMING OF UCB CD34+ ENRICHED CELLS IN SPINNER FLAKS

The aim of this work was to develop a dynamic and scalable system for the expansion and priming of the CD34+ cells derived from UCB towards a higher angiogenic profile. Factors known to enhance the angiogenic properties of the cells, such as shear stress and oxygen tension, were also assessed.

CD34+ cells were cultured under static (well plates) and dynamic (spinner flask, 30mL StemSpan®) conditions, according to Materials and Methods.

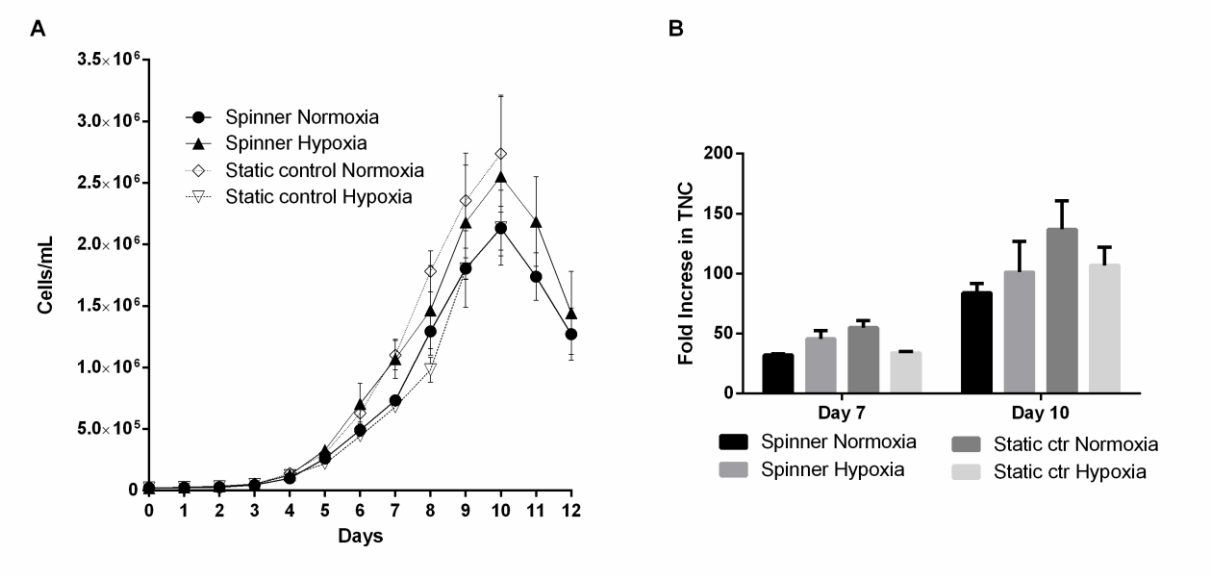


Figure VI-3 *- Growth curves of UCB cell expansion under static and dynamic conditions (A); Fold increase of total nucleated cells (FI TNC) at day 7 and day 10. Values are represented as mean±SEM (n=5 for normoxia and n=3 for hypoxia).*

In this preliminary study, the aim was to see for how long the expansion could be maintained without medium renewal (i.e. removal/dilution of culture medium enriched in autocrine factors and addition of fresh medium) and what proliferation in total and progenitor cells was obtained in these systems.

The expansion was successful in all conditions, with the start of the exponential phase at day 4 and a maximum of expansion at day 10 (**Figure VI-3**. A). Concomitantly, the fold increase in total cells were higher at day 10 for all conditions, with no significant differences between them with a FI in total cells for the dynamic systems of 32±0.90 and 46±6.8 at day 7; 84±7.9 and 101±25.7 at day 10, normoxia and hypoxia respectively. Nonetheless, the opposite tendency was observed for the static control counterparts, where a higher total cell FI was seen for the normoxia condition, with 55±5.9 and 34±0.9 at day 7; 137±23.8 and 107±15.2 at day 10, normoxia and hypoxia respectively. As expected, the percentage of the primitive population decreased with expansion, with an average of 28% and 23% for CD34+ cells and 25% and 19% for CD34+/CD133+ cells, at day 7 and day 10, respectively (**Figure VI-4** A). Nonetheless, it was observed an increase of total primitive subpopulation (**Figure VI-4** A), specifically an average of 15-

fold and 31-fold for CD34+ and 15-fold and 27-fold for CD34+/CD133+, at day 7 and day 10 respectively. Moreover, regarding the expression of endothelial related markers, it is seen either maintenance (CD31+ average of 95% and average FI of 42 and 95-fold, at day 7 and day 10 respectively; CD18+ average of 80% and average FI of 53 and 112-fold, at day 7 and day 10 respectively) or an increase (CD105 average of 67% at day 0 increasing to an average of 95% and 56-fold at day 7 and 87% and 111-fold at day 10; CD14 average of 84% at day 0 increasing to an average of 98% in both timepoints, with an average of 53-fold at day 7 and 119-fold at day 10) with the expansion (B). Consequently, it was again seen a decrease on the percentage of the other hematopoietic markers (CD11b from an average of 19% at day 0 to an average of 3% at both timepoints post-expansion; CD19 from an average of 10% at day 0 to an average of 0.5% post-expansion) of the post-expanded cells (**Figure VI-4** C). Interestingly, there is an increase of the CD274 percentage on the final population, specially seen for the dynamic systems, starting with an average of 0.5% at day 0 and increasing to an average of 10% and 19% for the dynamic systems and 5% and 11% for the static controls, at day 7 and day 10, respectively. Still, no significant differences were observed between the different conditions tested.

Interestingly, although no significant differences were achieved, there are two points that makes this study pertinent to pursue: 1) the same tendency within different experiments is the same regarding higher TNC fold expansion with concomitant higher number of progenitors of the cells expanded under dynamic hypoxia system in comparison with the dynamic normoxia counterpart; 2) the opposite is observed for the static controls, which may indicate that both factors, shear stress and oxygen tension, play a complementary role. One should take into consideration the correlation of both effects when engineering the expansion system for these cells. Also, the results of static hypoxia at day 10 are for n=1.

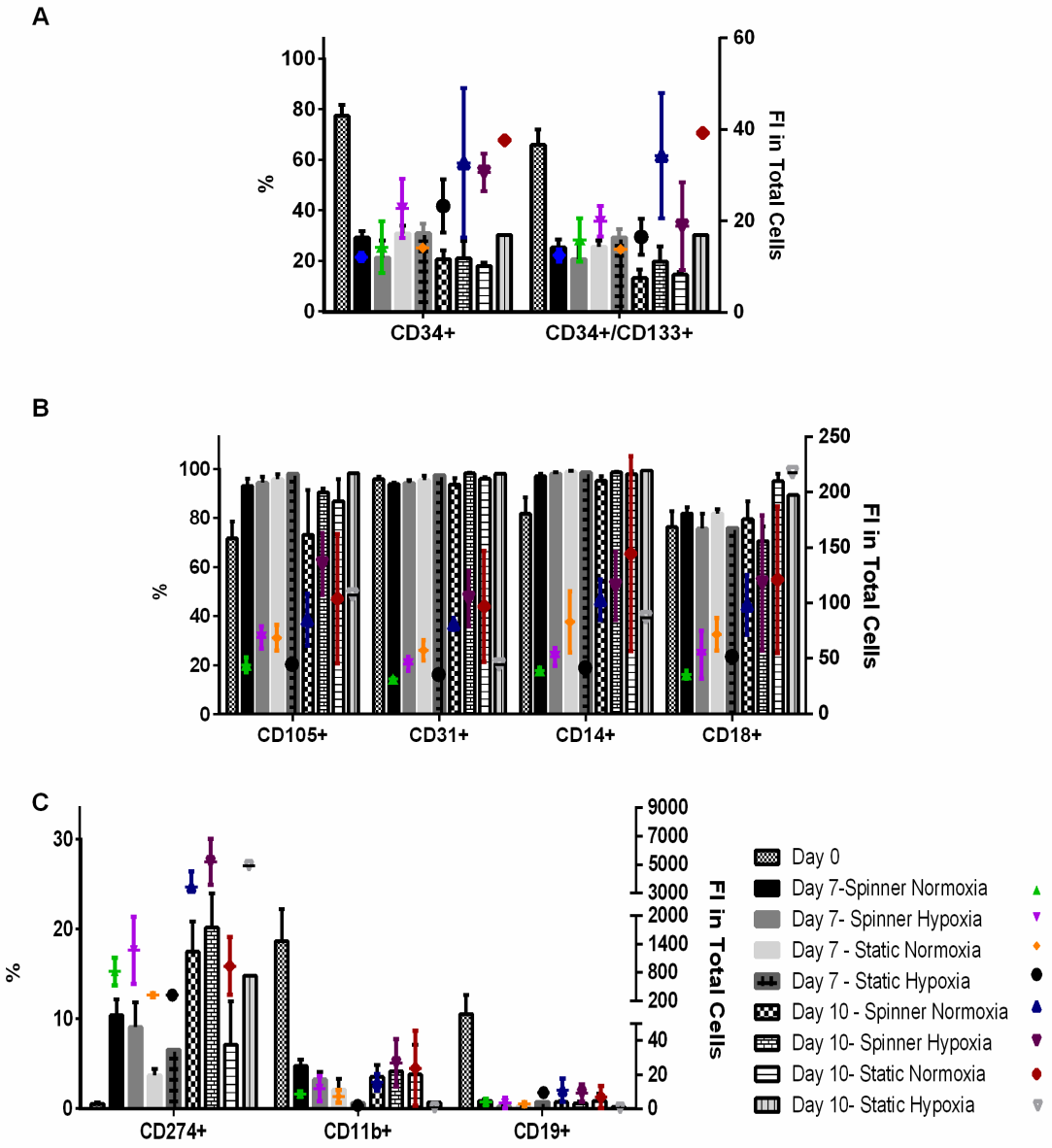


Figure VI-4 *- Immunophenotypic characterization of pre- and post- expanded cells. Fold increase in total primitive cells (CD34+; CD34+/CD133+) (A); Percentage of endothelial related markers (B and C) and other hematopoietic markers (C). Bars represent the % values and dots represent the fold increase (FI) in total number of cells. Values are represented as mean±SEM (n=5 for normoxia, n=3 for hypoxia, n=1 for D10 static hypoxia).*

A preliminary analysis of the concentration profile of nutrients and metabolites during the expansion under dynamic conditions was done (n=1), for both normoxia and hypoxia systems (**Figure VI-5**). Moreover, to assess the metabolic patterns of these cultures, the specific consumption and production rates were calculated (**Figure VI-6**).

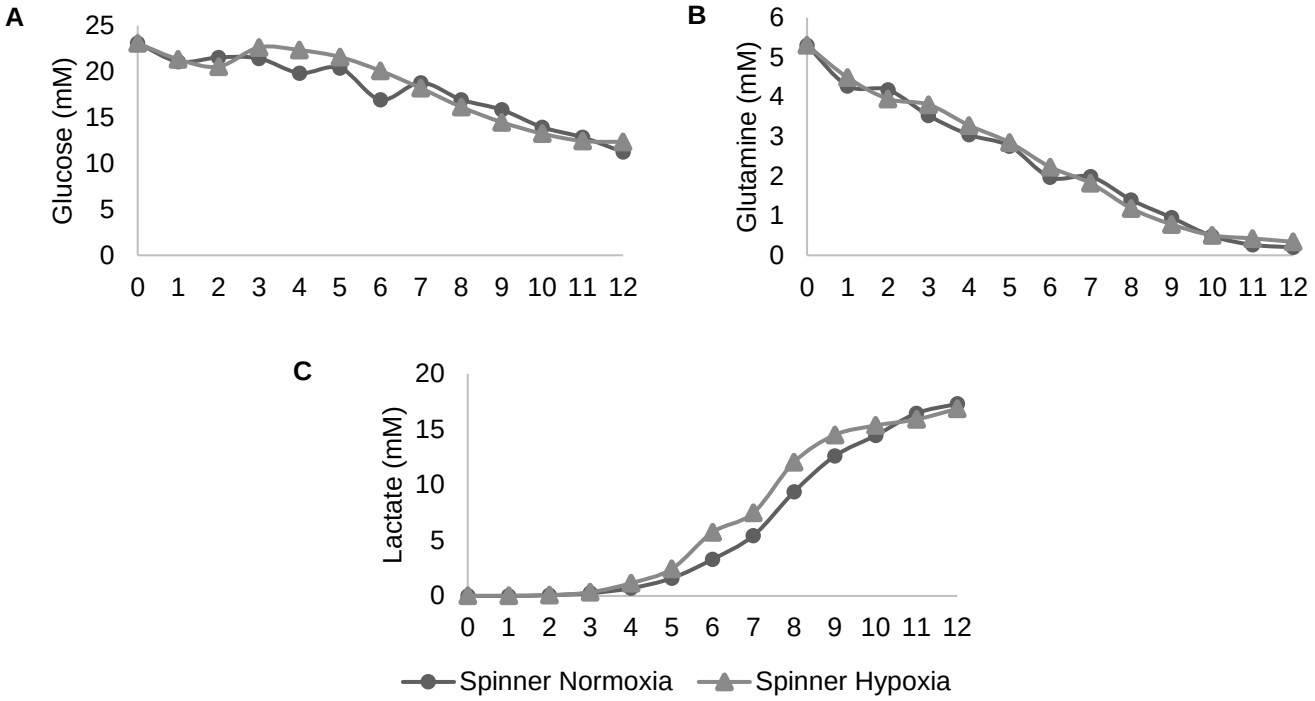


Figure VI-5 *– Concentration profiles of glucose, glutamine and lactate during CAPCs in spinner flasks in both normoxia and hypoxia (5% O_2). n=1.*

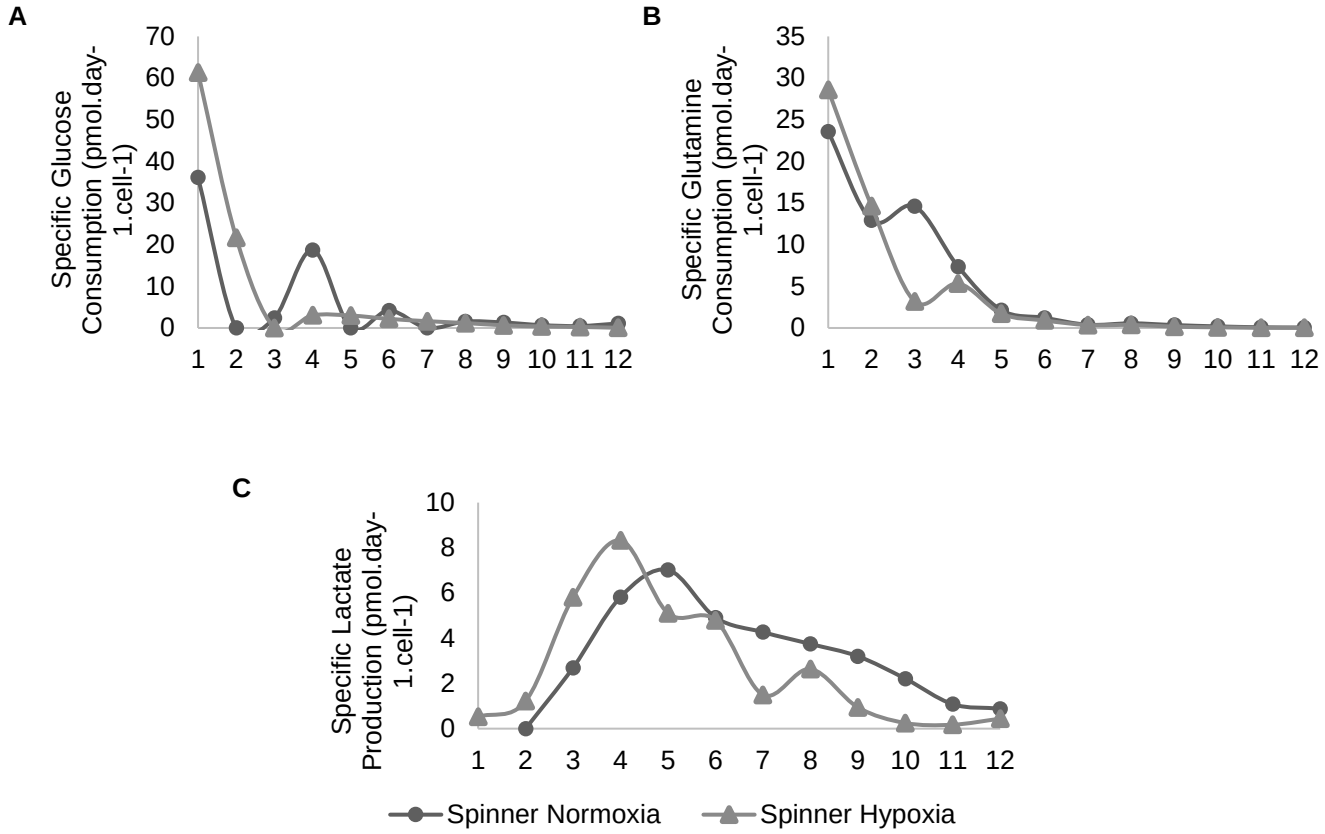


Figure VI-6 – *Specific production/consumption rates of glucose, glutamine and lactate during CAPCs in spinner flasks in both normoxia and hypoxia (5% O_2). n=1*

No significant differences were observed for the concentration profiles between both systems. However, it appears that the expansion under hypoxia produced a slightly higher lactate concentration from day 5 onwards. Interestingly, the levels of glucose did not reach a limitative value, whereas the glutamine value did (0.5 mM at day 10). However, glutamine degrades with time at 37ºC, and hence a control for this degradation was performed by measure of a mock system containing only medium throughout time. The value obtained for glutamine degradation was 0.11 mM/day, which was taken into account when presenting the results of this profile. Glucose and lactate concentrations were maintained high/low in the beginning of the expansion, until day 4, where a sharper decrease/increase is seen. This is coherent with the initial of the cell exponential growth phase, where higher cell numbers are obtained. Glucose levels reached a minimum of around 11.5 mM and lactate levels reached a maximum of around 17 mM, both described as non-limiting for hematopoietic cells (Patel et al., 2000a). Hence, glutamine depletion

might be one of the factors that caused the expansion end (and resultant cell death) after day 10.

Regarding the metabolic specific consumption/ production rates **Figure VI-6**, shows similar trends in all cases, which are characterized by a decrease in glucose consumption rates in the first days of culture with arrest of the consumption at day 3 and a consequent increase at day 4, corresponding to the initial of the exponential growth phase. After that, the rates decrease again to lower levels near to 0. Moreover, the glucose consumption rate appears to be higher for the normoxia condition. In terms of the glutamine specific consumption, once again it is seen a high consumption in the first days with a subsequent decrease until day 6, where after the rates are maintained low (near 0) until the end of the expansion. Once again, it is noted that the consumption rate of glutamine is higher for the normoxic condition. Lastly, the lactate consumption rates profile is similar between both conditions, where there is an increase of production with a peak at day 4 for hypoxia and day 5 for normoxia, and a decrease thereafter to lower values until culture end. Interestingly, the hypoxia condition had higher maximum production rate (at day 4) in comparison with the normoxia system (day 5), but it decreased to lower values from day 6 onwards.

The *in vitro* tube formation assay showed higher number of branch points when using post-expanded cells in comparison with the pre-expanded and control (HUVECs only). Specifically, it is seen a statistical significant difference at day 7 for the normoxia conditions, with static and dynamic in comparison with the pre-expanded cells (116±4.6; 123±5.1; 112±4.6, respectively) and higher angiogenic potential presented by the dynamic system. Interestingly, although without statistical significance with exception for the static controls at day 10, hypoxia conditions (144±13.2, 141±24.3 for dynamic hypoxia and 122±5.0; 125±7.2 for static hypoxia, at day 7 and day 10, respectively) presented higher percentage of branch points in comparison with the normoxia counterpart (123±5.1, 135±11.7 for dynamic normoxia and 116±4.6; 120±6.8 for static

normoxia, at day 7 and day 10, respectively). These results suggest that the dynamic condition promoted higher angiogenic potential in comparison with the static, being the highest promoted by the dynamic expansion under hypoxia (**Figure VI-7**)

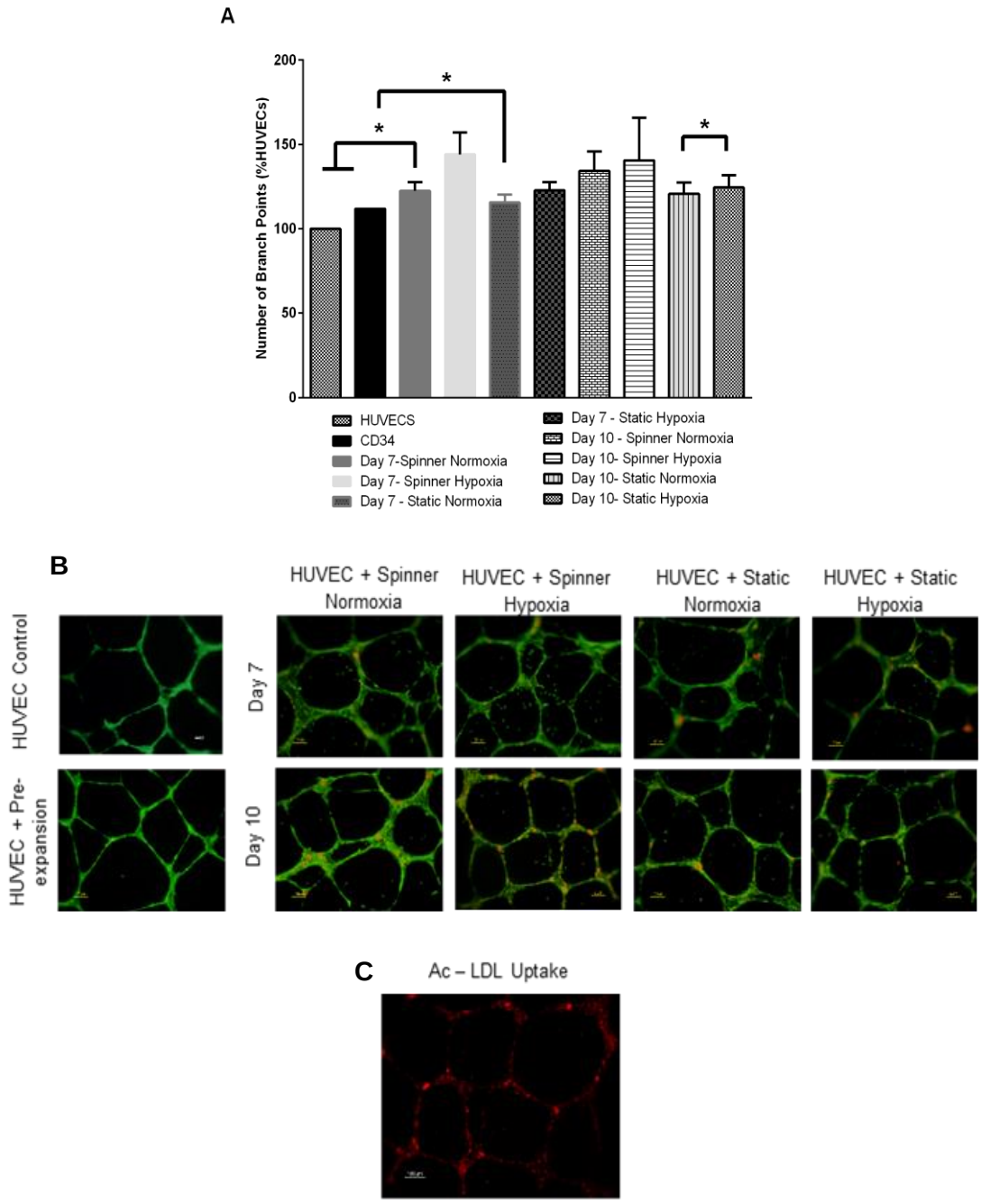


Figure VI-7 *- Assessment of the angiogenic potential* in vitro *through tube formation assay by the co-culture of pre- and post-expanded cells (1x10^3) with HUVEC (1.5x10^4) (n=5 Normoxia; n=3 Hypoxia).* ***(A)*** *Percentage of the number of branch points relative to HUVEC (100%)* ***(B)*** *Representative photomicrograph obtained in the assay for the control (HUVEC only) and co-culture of HUVEC + pre-expanded cells and HUVEC + post-expanded cells after 18 hours of incubation.* ***(C)*** *Ac LDL uptake. Results are given as mean ± SEM, *p<0.05.*

VI.4 DISCUSSION AND CONCLUSIONS

Physiological factors such as shear stress and oxygen tension are known to have an influence on the quality of the culture of angiogenic progenitor cells. This may rely on the natural environment where these can be found, as well as the triggered response to such factors. In the natural environment, circulating angiogenic progenitor cells are subjected to vascular shear stress of blood flow, which depending on the vessels, it can be from 1-6 dyn/cm^2 to 10-70 dyn/cm^2 for veins and arterial normal values, respectively (Papaioannou and Stefanadis, 2005). Moreover, it is also known that the natural niche of CD34+ cells within UCB is at hypoxic oxygen tensions (around 5% O_2). For instance, it has been shown that shear stress not only promotes the differentiation of the tissue EPCs, as well as the circulating phenotype (Obi et al., 2012; Obi et al., 2009; Yamamoto et al., 2003). As mentioned previously, these cells have the potential to be applied on the treatment of ischemic diseases (such as limb ischemia and myocardial infraction). Nonetheless, for the achievement of that goal, a robust, scalable and GMP-compliant system needs to be achieved. This will allow not only the process control but will also reduce *cost-of-goods* (CoGs) in comparison to the lab-scale and manual systems due to the reduction of labor and optimization of the process. To this end, this work aimed at the establishment of a scalable stirred system together with the assessment of the effect of the shear stress and oxygen tension on expansion and quality of the final cell product.

One of the factors that can drastically influence the expansion performance is the culture medium chosen. One should consider which culture medium to use based on (but not only), on the following aspects, when possible: i) the medium is xeno-/serum- free; ii) is able to comply with GMP standards; iii) it is easy and reliable to be obtained (assess supply chain); iv) is economically viable; v) performs the best for the final product to be achieved. Although SCGM (Cellgenix) was used in the Chapter III, due to recent literature showing better results using StemSpan medium (Masuda et al., 2012), it was aimed to assess and compare both media for the expansion of angiogenic circulating

cells ($CD34^+$) derived from UCB. For the experimental conditions tested, StemSpan medium showed not only to be able to expand the total number of cells at a higher extent, but remarkably to keep a higher content of the more primitive population (CD34+, CD34+/CD133+) and maintain, or even increase the content on endothelial related markers (CD105+, CD31+, CD14+, CD18+). Hence, this medium was chosen to pursue to test the capability of developing a dynamic system for the expansion of circulating angiogenic progenitor cells.

Here, the focus was on developing a scalable system, but also to assess the effect of shear stress and oxygen tension, factors that are known to have a biological influence on the biological mechanism of angiogenesis, on the quality of the obtained final product. Overall, a scalable system in spinner flasks was established, although with no significant differences seen between normoxia and hypoxia in terms of cell expansion. However, when observing the results within the same experiment, the same tendency of higher FI for the hypoxia system was maintained in all experiments. Indeed, it has been shown for UCB-CD34+ expansion an increase in cell proliferation under hypoxic (5% O_2) conditions in comparison with normoxia (Andrade et al., 2013a). Interestingly, the opposite tendency was observed for the static control counterparts, where a higher total cell FI was seen for the normoxia condition. As it can be seen, a higher FI of the static controls is achieved at day 10. This can be explained by the fact that the shear stress may have a detrimental effect on more sensitive cells by causing mechanical damage, and hence higher cell death occurs in the beginning of cell culture (Andrade-Zaldivar et al., 2008).

Regarding the characterization of the achieved cell population, it is observed an expected overall decrease of progenitor cells in comparison with the pre-expanded cells (CD34+, CD34+/CD133+), since assumedly stem/progenitor cells divide through culture asymmetrically producing one of the same kind (stem/progenitor) and other already more committed (differentiation) (Beckmann et al., 2007; Bullock et al., 2007). However, no significant difference was seen between the conditions tested, with a slightly decrease

between day 7 and day 10, roughly around 30% and 20%, respectively. In terms of the endothelial markers, it was seen an increase of the percentage of CD105+ and CD14+ markers, and a maintenance of high percentage of CD31+ and CD18+, again with no difference between the conditions tested and similar on both time points. Interestingly, for the CD105 marker, a slightly higher expression was observed on day 7 in comparison with day 10. This may be due to the start of cell growth arrest at day 10. Indeed, it has been reported that endoglin/CD105 is highly detectable in cultured endothelial cells showing high levels of RNA, DNA, and proteins consistent with cellular proliferation and activation and correlated with the rate of cellular proliferation and density in cultures of human umbilical vein endothelial cells (HUVEC) (Fonsatti et al., 2010).

In the context of this chapter, the aim of ex-vivo expansion is not only to increase cell numbers but to prime the cells towards a more endothelial lineage, and hence a decrease in the hematopoietic population. As demonstrated by the results of **Figure VI-4 C** a decrease of these markers is obtained post expansion (CD11b and CD19). Importantly, there was an increase of the CD274+ percentage on the final population. Indeed, it was also observed that conditions in dynamic culture were more prone to increase this population than their respective static controls, with higher values at day 10. The interaction of this ligand with its receptor is known to inhibit T-cell activation and cytokine production, which may have the potential to address the limitations imposed by immune rejection and graft-versus-host disease (Rodig et al., 2003; Wilcox et al., 2009), and therefore, being an important factor for the final cell product.

A preliminary study on the metabolic profile of these cells cultured under dynamic system was performed by measuring the concentration of glucose, glutamine and lactate on the media throughout expansion. Although this was done for only one experiment, the profiles obtained herein are interesting. Firstly, it is seen a slightly decrease of glucose concentration and a sharper decrease on glutamine concentration, where its degradation has been considered. Contrarily to glucose, glutamine concentration reached a limiting

value (near 0) at day 10, what it seems to be the end of the exponential growth phase. Hence, this may suggest that glutamine depletion to be one of the factors responsible for cell growth arrest. Indeed, glucose and glutamine metabolism are interrelated: glutamine transport is the rate limiting step in the activation of mammalian target of rapamycin (mTOR), and mTOR upregulates the Glut1 glucose transporter and glucose transport (Nicklin et al., 2009; Sundrud et al., 2009). Although the metabolism patterns of these specific cells is yet to be studied deeply, some work already performed with hematopoietic stem/progenitor cells and their differentiation can give some hints on the how the metabolism affects cell proliferation and differentiation. For instance, Oburoglu and colleagues (Oburoglu et al., 2014) have shown that these cells require the ASCT2 glutamine transporter and active glutamine metabolism for erythroid specification, where mechanistically, erythroid specification requires glutamine-dependent de novo nucleotide biosynthesis. Nonetheless, while glucose and glutamine are both precursors in the tricarboxylic acid (TCA) cycle, and precursors in lipids, nucleotides, and amino acids production, their relative contributions to metabolic pathways driving HSPC lineage commitment remain to be defined. However, linear pathways can increase the flow-through for high metabolic demand adaptation, and hence, conversion of the TCA cycle to such pathways, termed glutaminolysis, are a considerable advantage for rapidly dividing cells, such as stem cells and cancer cells (Newsholme; et al., 1985; Wise and Thompson, 2010). Glutamine can either be metabolized as substrate for protein biosynthesis or as source of energy. Inside the cell, glutamine is converted to glutamate and then to α-ketoglutarate, yielding ammonia (NH4+). α-ketoglutarate is converted to pyruvate which can either be converted to lactate or alanine or be completely broken down in the TCA cycle, being an energy-efficient catabolism, resulting in 27 moles of ATP and 2 moles of ammonia. Inefficient catabolism can yield 1 mole of lactate or alanine, 1 mole of ammonia and 9 moles of ATP. Glutamine consumption and ammonia production on cell cultures can indicate whether glutamine is being used for either efficient or inefficient energy production or for protein biosynthesis only (Schop et al.,

2009). Hence, it would be important for this study to also measure the concentration of ammonia that would probably give some clues on what way is glutamine being processed. Interestingly, some studies have shown that glutamine administration in ischemic or diabetic animal models promotes the mobilization of circulating EPCs by increasing the release of CXCL-12, VEGF, and NO and contributing for a higher vasculogenic potential *in vivo* (Pai et al., 2016; Su et al., 2017). Hence, putting together these evidences, glutamine may play a key role on the proliferation and differentiation of EPCs.

Nonetheless, other components, such as cytokines or other inhibitory molecules (Csaszar et al., 2012), will also need to be addressed for cell growth arrest. Indeed, the half-life time of cytokines is known to be quite short, some even being stable only for a couple of days at 37ºC, and they are known to be essential for both maintenance and growth of CD34+ derived cells (Banu et al., 2001; Gothot A et al., 1998; Spence SE et al., 1998). Thus, a more thorough study of all these factors (nutrients and cytokines by ELISA, for example) needs to be performed to achieve an optimized feeding strategy yielding the maximum number of cells with the higher angiogenic potential possible for this system. The lactate levels increased throughout time, although not reaching a limiting value either (18 mM *vs* inhibiting value of 20 mM) (Patel et al., 2000b). When observing the specific consumption rates, it is seen that the normoxia condition has higher values for both glucose and glutamine. Interestingly, these consumptions start at a maximum value, have a slight increase at the beginning of exponential growth phase and decrease to almost 0 with time. This high consumption rate in the beginning of culture could be explained by the adaptation of the cells to the culture system which usually is associated with higher stress and metabolic adaptation. Unexpectedly, when observing the lactate specific consumption, is noted that the peak occurs earlier for hypoxia than for normoxia (day 4 and day 5, respectively), although the peak corresponding to the initial of exponential growth for the specific glucose consumption is

at day 4 for both. This may be due to experimental error, as this was a preliminary experiment (n=1) and more replicates needs to be done in order to be able to take solid conclusions.

To characterize the angiogenic potential *in vitro* of the expanded cells, a tube formation assay with HUVEC co-culture was performed. All post expansion conditions showed a higher tube formation potential in comparison with the pre-expansion cells, measured by the percentage of branch points in comparison with HUVEC control. Overall, the dynamic cultures presented an increased ability on tube formation than their static counterparts. Higher difference appears to be at day 7 between the dynamic systems, although the potential does not change significantly between both time points. Of notice, the effect of shear stress and oxygen tension has been extensively described throughout the literature to being part of the biological aspects of the angiogenesis mechanism and to even enhance the angiogenic ability and proliferation of the cells when exposed to these (Obi et al., 2012; Obi et al., 2009; Papaioannou and Stefanadis, 2005; Yamamoto et al., 2003). This may suggest that the system tested herein (spinner flask under 5% O_2) might be pertinent to be further explored to optimize the *ex vivo* production of angiogenic progenitor cells where both factors can be applied (in an attempt to mimic *in vivo* conditions) to enhance their angiogenic properties. However, the results presented herein are not consistent with some other studies in the literature in terms of cell proliferation between static and shear exposed systems (flow), that has been shown to be greater for the latter. These differences may be explained by the different type of shear stress that was applied that in the majority of those studies, is through tangential flow, mimicking vasculature traffic. Hence this is another matter of further research: if different types of flow and shear stress can cause the same effect on cell proliferation.

Efforts on this direction have already been made by Abaci and colleagues *(*Abaci *et al.,* 2012*)*, where they developed a microbioreactor with independent control of shear stress and oxygen tensions. Although that system might be useful to study the differentiation

and maturation into endothelial cells, the system presented here has the advantage of ease of scalability, which can be further adapted into a fully controlled stirred bioreactor, moving towards a GMP compliant process required for cell manufacturing.

In addition, as a characteristic of endothelial like lineage, it was also shown the ability of the cells to take up AC-LDL, which further contributes for the statement of priming through culture (Asahara et al., 1997; Rehman, 2003).

Overall, it was demonstrated the scale-up from 2D culture to dynamic and scalable spinner flasks, while maintaining expansion of the total number of cells as well as the final population produced assessed by surface markers and their angiogenic potential measured *in vitro*. Although no significant difference was observed between normoxia and hypoxia systems, it was seen the same tendency within the same experiment, pointing the latter system to have more positive features in some of the results (total number of cells and tube formation assay for example). Nonetheless, more experiments need to be performed in order to define that. Of notice, this is, to the author's best knowledge, the first report aiming to bioengineer angiogenic UCB CD34+ cell culture to explore shear stress and oxygen tension in a scalable manufacturing system to improve quantity and angiogenic quality of the final product.

In the future, it would be interesting to also address the effect of higher shear stress, since *in vivo* these cells are subjected to high shear in blood vessels, and in the experiment herein the shear stress was considerably low (0.8 dyn/cm^2). Moreover, qPCR and ELISA assays to access their paracrine activity should be performed and finally, to address the regenerative potential *in vivo* in an ischemic animal model.

VI.5 REFERENCES

Abaci, H.E., Devendra, R., Soman, R., Drazer, G., and Gerecht, S. (2012). Microbioreactors to manipulate oxygen tension and shear stress in the microenvironment of vascular stem and progenitor cells. Biotechnology and applied biochemistry *59*, 97-105.

Alaiti, M.A., Ishikawa, M., Masuda, H., Simon, D.I., Jain, M.K., Asahara, T., and Costa, M.A. (2012). Up-regulation of miR-210 by vascular endothelial growth factor in ex vivo expanded CD34+ cells enhances cell-mediated angiogenesis. Journal of cellular and molecular medicine *16*, 2413-2421.

Andrade-Zaldivar, H., Santos, L., and De Leon Rodriguez, A. (2008). Expansion of human hematopoietic stem cells for transplantation: trends and perspectives. Cytotechnology *56*, 151-160.

Andrade, P.Z., de Soure, A.M., Dos Santos, F., Paiva, A., Cabral, J.M., and da Silva, C.L. (2013). Ex vivo expansion of cord blood haematopoietic stem/progenitor cells under physiological oxygen tensions: clear-cut effects on cell proliferation, differentiation and metabolism. Journal of tissue engineering and regenerative medicine.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Banu, N., Rosenzweig, M., Kim, H., Bagley, J., and Pykett, M. (2001). Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix. Cytokine *13*, 349-358.

Beckmann, J., Scheitza, S., Wernet, P., Fischer, J.C., and Giebel, B. (2007). Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. Blood *109*, 5494-5501.

Bullock, T.E., Wen, B., Marley, S.B., and Gordon, M.Y. (2007). Potential of CD34 in the regulation of symmetrical and asymmetrical divisions by hematopoietic progenitor cells. Stem cells *25*, 844-851.

Csaszar, E., Kirouac, D.C., Yu, M., Wang, W., Qiao, W., Cooke, M.P., Boitano, A.E., Ito, C., and Zandstra, P.W. (2012). Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell stem cell *10*, 218-229.

Deborah Schop, Frank W. Janssen, Linda D.S. van Rijn, Hugo Fernandes, Rolf M. Bloem, Joost D. de Bruijn, and Dijkhuizen-Radersma, R.v. (2009). Growth, Metabolism, and Growth Inhibitors of Mesenchymal Stem Cells. Tissue Eng Part A *15*, 1877-1886.

Gothot A, Pyatt R, McMahel J, Rice S, and EF, S. (1998). Assessment of proliferative and colony-forming capacity after successive in vitro divisions of single human CD34+ cells initially isolated in G0. Experimental hematology *26*, 562-570.

Masuda, H., Iwasaki, H., Kawamoto, A., Akimaru, H., Ishikawa, M., Ii, M., Shizuno, T., Sato, A., Ito, R., Horii, M.*, et al.* (2012). Development of serum-free quality and quantity control culture of colony-forming endothelial progenitor cell for vasculogenesis. Stem cells translational medicine *1*, 160-171.

Mata, M.F., Lopes, J.P., Ishikawa, M., Alaiti, M.A., Cabral, J.M., da Silva, C.L., and Costa, M.A. (2015). Scaling up the ex vivo expansion of human circulating CD34(+)progenitor cells with upregulation of angiogenic and anti-inflammatory potential. Cytotherapy *17*, 1777-1784.

Newsholme;, E.A., Crabtree;, B., Salleh;, M., and Ardawi, M. (1985). Glutamine metabolism in lymphocytes its biochemical, physiological and clinical importance. Quarterly Journal of Experimental Physiology *70*, 473-489.

Nicklin, P., Bergman, P., Zhang, B., Triantafellow, E., Wang, H., Nyfeler, B., Yang, H., Hild, M., Kung, C., Wilson, C.*, et al.* (2009). Bidirectional transport of amino acids regulates mTOR and autophagy. Cell *136*, 521-534.

Obi, S., Masuda, H., Shizuno, T., Sato, A., Yamamoto, K., Ando, J., Abe, Y., and Asahara, T. (2012). Fluid shear stress induces differentiation of circulating phenotype endothelial progenitor cells. J Appl Physiol *303*, 595-606.

Obi, S., Yamamoto, K., Shimizu, N., Kumagaya, S., Masumura, T., Sokabe, T., Asahara, T., and Ando, J. (2009). Fluid shear stress induces arterial differentiation of endothelial progenitor cells. J Appl Physiol *106*, 203-211.

Oburoglu, L., Tardito, S., Fritz, V., de Barros, S.C., Merida, P., Craveiro, M., Mamede, J., Cretenet, G., Mongellaz, C., An, X.*, et al.* (2014). Glucose and glutamine metabolism regulate human hematopoietic stem cell lineage specification. Cell stem cell *15*, 169-184.

Pai, M.H., Shih, Y.M., Shih, J.M., and Yeh, C.L. (2016). Glutamine Administration Modulates Endothelial Progenitor Cell and Lung Injury in Septic Mice. Shock *46*, 587-592.

Papaioannou, T.G., and Stefanadis, C. (2005). Vascular Wall Shear Stress: Basic Principles and Methods. Hellenic Journal of Cardiology *46*, 9-15.

Patel, S.D., Papoutsakis, E.T., Winter, J.N., and Miller, W.M. (2000). The lactate issue revisited: novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures. Biotechnol Prog *16*, 885-892.

Rehman, J. (2003). Peripheral Blood "Endothelial Progenitor Cells" Are Derived From Monocyte/Macrophages and Secrete Angiogenic Growth Factors. Circulation *107*, 1164-1169.

Rodig, N., Ryan, T., Allen, J.A., Pang, H., Grabie, N., Chernova, T., Greenfield, E.A., Liang, S.C., Sharpe, A.H., Lichtman, A.H.*, et al.* (2003). Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. European journal of immunology *33*, 3117-3126.

Spence SE, Keller JR, Ruscetti FW, McCauslin CS, Gooya JM, Funakoshi S, Longo DL, and WJ., M. (1998). Engraftment of ex vivo expanded and cycling human cord blood hematopoietic progenitor cells in SCID mice. Experimental hematology *26*, 507-514.

Su, S.T., Yeh, C.L., Hou, Y.C., Pai, M.H., and Yeh, S.L. (2017). Dietary glutamine supplementation enhances endothelial progenitor cell mobilization in streptozotocin-induced diabetic mice subjected to limb ischemia. The Journal of nutritional biochemistry *40*, 86-94.

Sundrud, M.S., Koralov, S.B., Feuerer, M., Calado, D.P., Kozhaya, A.E., Rhule-Smith, A., Lefebvre, R.E., Unutmaz, D., Mazitschek, R., Waldner, H.*, et al.* (2009). Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. Science *324*, 1334-1338.

Wilcox, R.A., Feldman, A.L., Wada, D.A., Yang, Z.Z., Comfere, N.I., Dong, H., Kwon, E.D., Novak, A.J., Markovic, S.N., Pittelkow, M.R.*, et al.* (2009). B7-H1 (PD-L1, CD274) suppresses host immunity in T-cell lymphoproliferative disorders. Blood *114*, 2149-2158.

Wise, D.R., and Thompson, C.B. (2010). Glutamine addiction: a new therapeutic target in cancer. Trends in biochemical sciences *35*, 427-433.

Yamamoto, K., Takahashi, T., Asahara, T., Ohura, N., Sokabe, T., Kamiya, A., and Ando, J. (2003). Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. J Appl Physiol *95*, 2081-2088.

CHAPTER VII - ALPHA TESTING OF A NEW PROTOTYPE MEDIUM FOR HEMATOPOIETIC STEM/PROGENITOR CELL EXPANSION

VII.1 SUMMARY

Umbilical cord blood (UCB) hematopoietic stem/progenitor cell (HSPC) expansion is mandatory to meet the clinical demands for non-pediatric transplantation. However, in order to move towards the clinic, several aspects need to be taken into account, more specifically, the manufacturing needs to be performed under good manufacturing practices (GMP), where medium formulation and components need to be carefully chosen in order to attain high expansion yield with progenitor maintenance and usually be free of xeno(geneic)/serum components. Thus, several companies have understood this need and they have been developing media suited for HSPC *ex-vivo* expansion within these parameters. Hence, Company X have been developing a new clinical grade xenofree medium, SP, together with an optimized cytokine cocktail for HSPC expansion. As collaborators, our lab participated in the alpha testing of this not yet commercially available medium. A comparative study with the QBSF-60 medium used in our lab, together with the previously optimized cocktail (Z9) (Andrade et al., 2010) was performed, as well as the use of bone marrow mesenchymal stem/stromal cells (BM-MSCs) derived feeder layers (Str), which we have previously shown to enhance and support UCB CD34+ enriched cell proliferation. It was shown that the SP medium, supplemented with Soluble SCF (100 ng/mL), Flt3-L (100 ng/mL), IL-3 (20 ng/mL) and IL-6 (20 ng/mL), in combination with our stromal feeder layer technology (SP+Str) led to the highest fold expansion in total cell (TNC) numbers (41±13) and progenitors (50±12 CD34+, 26±9.9 CD34+/CD133+ and 268.9±201.5 CD34+/CD90+ cells), as well as maintenance/expansion of megakaryocyte (CD41+), early lymphoid (CD7+) and myeloid (CD14+, CD15+ and CD33+) lineages. Concomitantly with the higher TNC and progenitor expansion, the total fold of colony forming units (CFU) and cobblestone area forming cells (CAFCs) was significantly higher for UCB cells expanded under this condition.

Furthermore, a preliminary study on the dynamic culture using SP medium, with and without stroma (in this case, BM MSCs that have previously expanded adherent to microcarriers), was assessed in spinner flasks. Here it is shown a successful scale-up of the co-culture system into spinner flasks, with 7.6-fold expansion in TNCs. However, the static controls (with and without stroma) produced similar values of expansion for UCB CD34+ enriched cells. Interestingly, static condition without stroma was able to maintain higher levels of the progenitor population compared with Z9 culture system with stroma (17.6±7.6 vs 16.8±5.4 for CD34+; 11.6±4.6 vs 10.5±4.2 for CD34+/CD133+; 38.2±24.4 vs 8.1±5.4 for CD34+/CD90+).

Finally, it was ought to assess the engraftment ability of pre- and post SP expanded cells in static culture in the presence of stroma. It was observed that non-expanded UCB cells upon transplantation gave rise to T lymphocyte lineages, whereas the expanded grafts did not. Nonetheless, it was seen a BM long-term engraftment (16 weeks) in both cases, however in a lower extent for the expanded population.

VII.2 BACKGROUND

Umbilical cord blood (UCB) represents an attractive and readily accessible source of stem cells for Regenerative Medicine. In particular, UCB represents a unique and rich source of hematopoietic stem/progenitors cells (HSPCs) and its transplantation have been performed worldwide to treat malignant and nonmalignant disorders in pediatric and adult patients (Ballen et al., 2013). Nonetheless, the bottleneck resides on the number of progenitors that can be retrieved from this source, which not reach clinical demand for the treatment of an adult patient. Therefore, the development of efficient and cost-effective strategies to generate large numbers of functional stem cells, while maintaining the progenitor cells populations would be of major importance. Since the breakthrough work of Dexter and colleagues on the *ex-vivo* culture of hematopoietic cells using stromal cells as a feeder layer (Dexter et al., 1973; Dexter et al., 1984), several methods for human hematopoietic cell cultivation have been developed. Those include serum-containing or serum-free culture systems, using pre-established xenogeneic or human feeder layers, including human MSCs with (or without) the addition of exogenous cytokine cocktails, while others include the addition of cytokines in liquid culture in the absence of stroma (reviewed in (Andrade et al., 2015; Dahlberg et al., 2011a; Pineault and Abu-Khader, 2015)). However, in order to be *good manufacturing practice* (GMP)-compliant, HSPC *ex-vivo* expansion processes require clinical-grade medium/supplements formulations, as well as effective standards and methodologies for preclinical safety and efficacy evaluation, product characterization, and process validation and control (Kirouac and Zandstra, 2008). Companies understand this market demand, and efforts are being made towards the development of completely defined xeno/serum-free media for HSPC expansion, usually combined with an optimized cytokine cocktail.

Alpha testing relies on the product quality improvement , while ensuring beta readiness. It is performed near the end of the development process, thus variation on the

formulation is likely to occur. This process is usually a time-consuming, consisting in 3-5x the length of the next test phase, as it requires many iterations. Contrarily to beta-testing – which usually involves the whole product team (i.e. product marketing, support, quality testing and engineering) and is tested by product customers –, alpha testing focuses on emulating ~80% of the customers, and is normally performed by test engineers and by collaborations with laboratories specialized in the area of focus, such as our lab. Thus, Alpha testing allows for the most critical issues to be fixed, and it gives strong indications of how a product performs, whether it meets the market criteria. After this fine tuning of the product, one can then proceed to the last testing phase (beta), which explores the limits of a product, and provides a decent notion of what customers think about the product, and what they are likely to experience when they purchase it.

Of importance, static conditions in traditional culture flasks have some limitations as in the cell numbers generated, their non-homogeneous nature, monitoring difficulty, and extensive handling requirement for feeding/harvesting procedures (Cabrita et al., 2003; dos Santos et al., 2011). In this context, dynamic cultures have been developed as alternatives to standard flask cultures for *in vitro* culture.

Therefore, in this work in collaboration with Company X, it was aimed to test their new non-commercially available SP on UCB derived HSPC expansion and maintenance in both static and dynamic cultures, testing cultures with and without stromal layer (BM-MSC). Moreover, a preliminary *in vivo* engraftment study of the produced cells was performed on NOD scid gamma (NSG) mice, in collaboration with Instituto de Medicina Molecular (IMM).

VII.3 RESULTS

VII.3.1 SP MEDIUM PERFORMANCE UNDER STATIC CULTURE

Evaluation of the performance of the new SP medium supplemented with the recommended cytokine cocktail - Soluble SCF (100 ng/mL), Flt3-L (100 ng/mL), IL-3 (20 ng/mL) and IL-6 (20 ng/mL) - under static culture was performed and compared against serum-free culture (QBSF-60) system using human BM-MSC-derived feeder layers (Str) and supplemented with cytokines (Z9 comprising Soluble SCF (60 ng/mL), Flt-3L (55 ng/mL), TPO (50ng/mL) and bFGF (5 ng/mL)) that was previously established at iBB-IST (Andrade et al., 2010). Moreover, it was ought to assess SP performance in conjugation with our lab technology (MSC-HSPC co-culture – SP+Str). *No stroma* cultures were also performed.

To assess the impact of different medium and experimental conditions on UCB HSPC *ex vivo* expansion, analysis of fold change in total nucleated cells (TNC) (**Figure VII-1**) the hematopoietic stem/progenitor phenotypes CD34+, CD34+/CD133+ and CD34+CD90+ (

***Figure* VII-2 A and B**), and for the more committed progenitors from the early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41+ cells) was performed (

Figure* VII-2 C**). In addition, the clonogenic potential of both fresh and expanded cells was assessed by BFU-E, CFU-MIX and CFU-GM assays (Figure VII-3***), as well as their ability to form cobblestone areas (CAFC) (***Figure VII-4***).

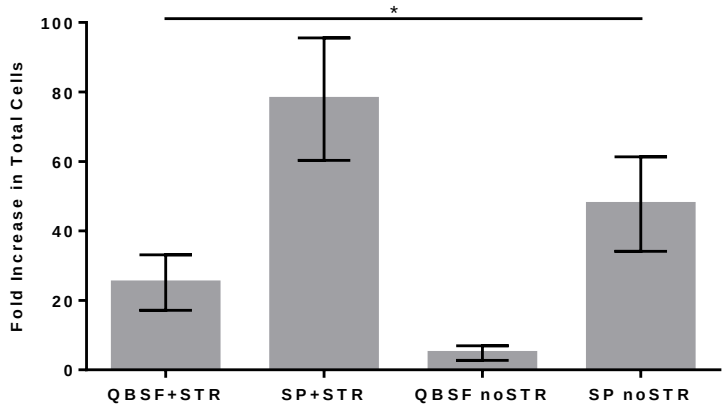


Figure VII-1 *- Fold increase in total nucleated cells (TNC) for each medium (QBSF and SP) with (Str) and without (noStr) BM-MSC stromal feeder layer. QBSF supplemented with Soluble SCF (60 ng/mL), Flt-3L (55 ng/mL), TPO (50ng/mL) and bFGF (5 ng/mL); SP supplemented with Soluble SCF (100 ng/mL), Flt3-L (100 ng/mL), IL-3 (20 ng/mL) and IL-6 (20 ng/mL). Results presented as mean±SEM (n=5, *p<0.05).*

The new medium developed by Company X, in the absence of feeder layer (SP noStr) together with their recommended cocktail was shown to promote higher fold expansion in TNC in comparison with our routinely used culture conditions comprising QBSF medium with a BM-MSC feeder layer (stroma) (QBSF+Str), with 48±14 and 25±7.9 – fold, respectively ($p<0.05$). Interestingly, the highest TNC fold expansion was attained upon the combination of SP medium with our stroma co-culture system (SP+Str) presenting 77±18 – fold ($p<0.05$). As expected, and as previously reported for QBSF medium (Andrade et al., 2010), the condition without stroma (QBSF noStr) led to the lowest fold expansion (4.8±2.1,$p<0.05$).

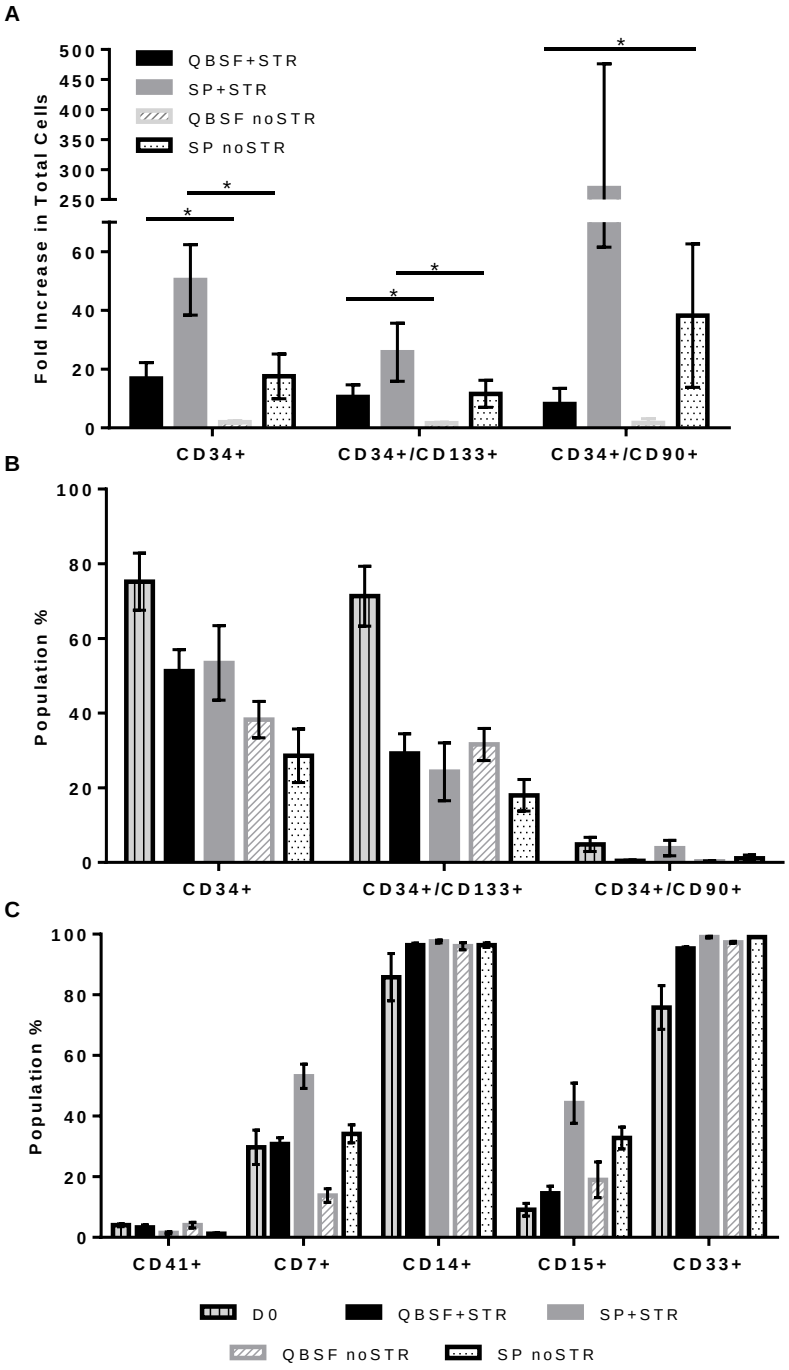


Figure VII-2 *- Immunophenotypic characterization of the pre- and post-expanded CD34+ cells. (A) fold change in total hematopoietic stem/progenitor phenotype (CD34+, CD34+/CD133+ and CD34+/CD90+); (B) Content in percentage of the hematopoietic stem/progenitor phenotype; (C) Content in percentage in early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41+). Results presented as mean±SEM (*p<0.05, n=5).*

Moreover, immunophenotypic analysis of pre- and post-expanded cells was performed by flow cytometry. As expected, a decrease from 75% to roughly similar values after expansion on the hematopoietic stem/progenitor cells was observed in all conditions (

Figure* VII-2 B**), with feeder layer cultures, SP+Str and QBSF+Str presenting slightly higher values in CD34+ percentage of 53% and 51%, respectively, whereas the other conditions had a decrease to 28 and 38%, for SP noStr and QBSF noStr, respectively. Regarding CD34+/CD133+ and CD34+/CD90+ populations, a decrease from 71% to 18-32% (with QBSF noStr giving the highest value) and 5% to 0.3-4% (with SP+Str giving the higher value) was observed, which may suggest a higher ability of the combination SP+Str to maintain a hematopoietic stem/progenitor population. Indeed, when observing the fold expansion in total progenitor cells (Figure V-2* A**), SP+Str was able to attain a higher increase of these progenitors concomitantly, more specifically to a 50.4±11.9, 25.8±9.8 and 269±208 - fold increase ($p<0.05$) in CD34+, CD34+/CD133+ and CD34+/CD90+ cells, respectively. In addition, SP medium (without stroma) led to a slightly higher stem/progenitor expansion than the previous optimized QBSF+Str system in our lab (17.6±7.6 vs 16.8±5.4 for CD34+; 11.6±4.6 vs 10.5±4.2 for CD34+/CD133+; 38.2±24.4 vs 8.1±5.4 for CD34+/CD90+).

The differentiative potential of the cells expanded in these culture systems was also analyzed, by testing for early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41a+ cells) (

***Figure* VII-2 C**). With exception of the QBSF noStr condition, all culture systems were able to maintain/expand these progenitors. Interestingly, it was seen a higher ability of SP+Str to increase CD7+ early lymphoid to 53% and CD15+ myeloid lineages to 44%, in comparison with pre-expanded cells and expanded cells under the other conditions. In addition, QBSF medium maintain a higher CD41+ megakaryocytic population (3% and 4% for Str and noStr conditions, respectively, at day 7?) than SP medium, where it was

seen a decrease in this population (roughly 1% for both Str and noStr). This is in agreement with the results reported previously, where it is a seen a shift towards the myeloid lineage, with the presence of megakaryocytic cells, when using QBSF medium (Andrade et al., 2013a; Andrade et al., 2010).

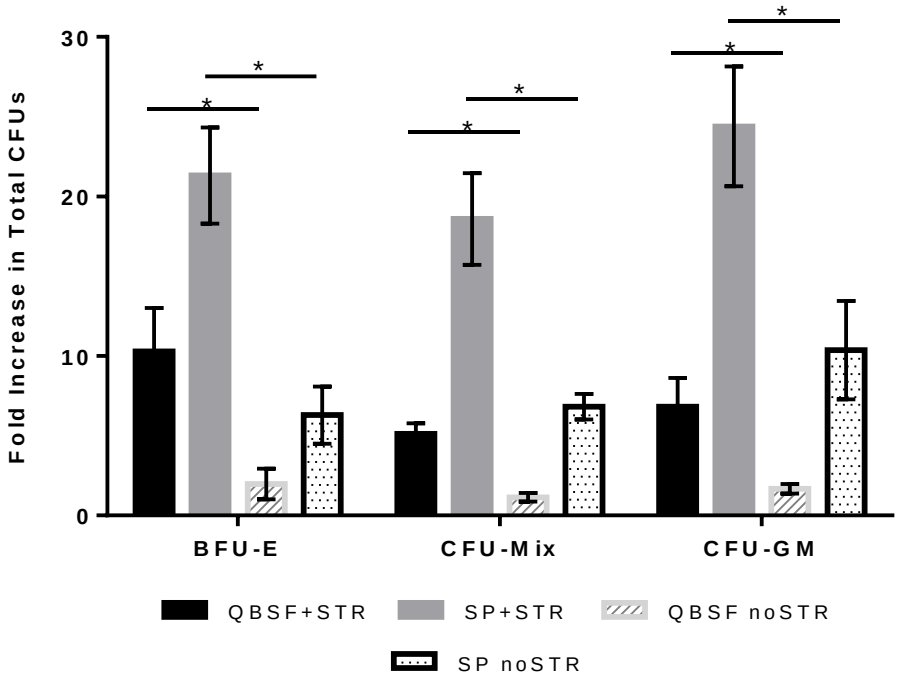


Figure VII-3 *- Clonogenic potential of the expanded cells. Fold increase in BFU-E, CFU-MIX and CFU-GM for each condition. Results presented as mean±SEM (*p<0.05, n=5).*

In terms of the clonogenic potential, the trend is consistent with the TNC and CD34+ fold expansion, where it is seen a significant higher fold increase in BFU-E, CFU-Mix and CFU-GM generated by SP+Str (21.3±3.0, 18.6±2.3 and 24.4±3.8-fold, ($p<0.05$) respectively). QBSF+Str and SP noStr presented similar clonogenic potential, whereas QBSF noStr attained an expected lower clonogenic potential ($p<0.05$), concomitant with lower TNC fold increase.

Finally, the expansion level in CAFCs was also measured (***Figure VII-4***), which is described as a predictor of in vivo repopulating ability (Ploemacher et al., 1989). Once again, it was seen an increased fold for SP+Str system (37.1±9.4 – fold, $p<0.05$), whereas QBSF noStr attained the lowest, with just 1.9±1 – fold ($p<0.05$). Interestingly, SP noStr led to a higher cobblestone formation (17.9±5.1 – fold, $p<0.05$) than the iBB-IST used system, QBSF+Str (12.2±4.0, $p<0.05$). This is in agreement, however, with the higher stem/progenitor cell content seen by immunophenotypic analysis for SP noStr.

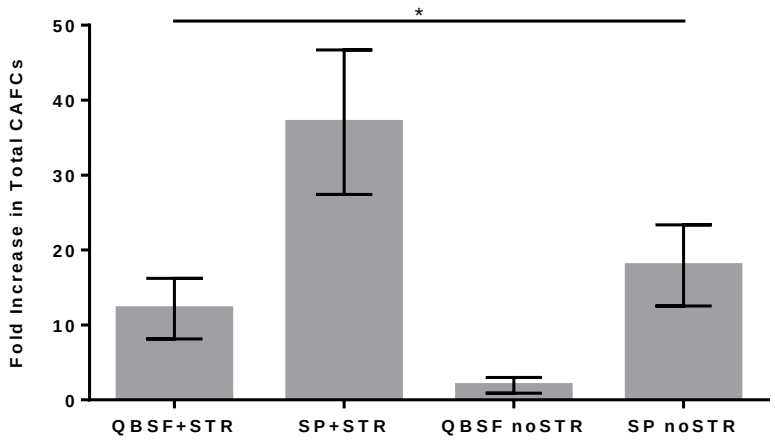


Figure VII-4 *- Fold increase in cobblestone area-forming cells (CAFCs) for HSPC expanded under the different mediums with and without stromal layer. Results presented as mean±SEM (*p<0.05, n=5).*

VII.3.2 SP MEDIUM IN UCB HSPCP EXPANSION UNDER DYNAMIC CONDITIONS

To test the ability of SP to expand UCB derived HSPC in a dynamic system, UCB CD34+ enriched cells were cultured in a spinner-flask (V_T = 15 mL, 5 x 10^4 cells/mL). Co-culture with stromal cells was also evaluated, with BM-MSC previously expanded in adherence to plastic microcarriers under xeno-free media, in a spinner flask, previously established in our lab (Carmelo et al., 2015; Santos et al., 2011) (***Figure VII-5***). Relevant static controls with (Str) and without (noStr) stroma were performed.

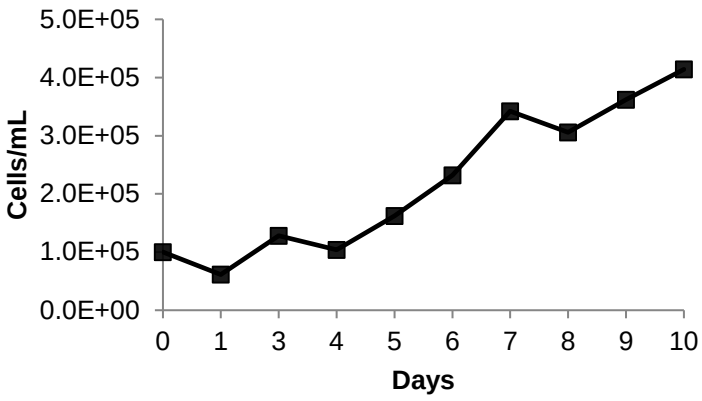


Figure VII-5 *– BM MSC expansion on plastic microcarriers on spinner flask under xenofree conditions. Growth curve (cells/mL) throughout culture time (days) (n=1).*

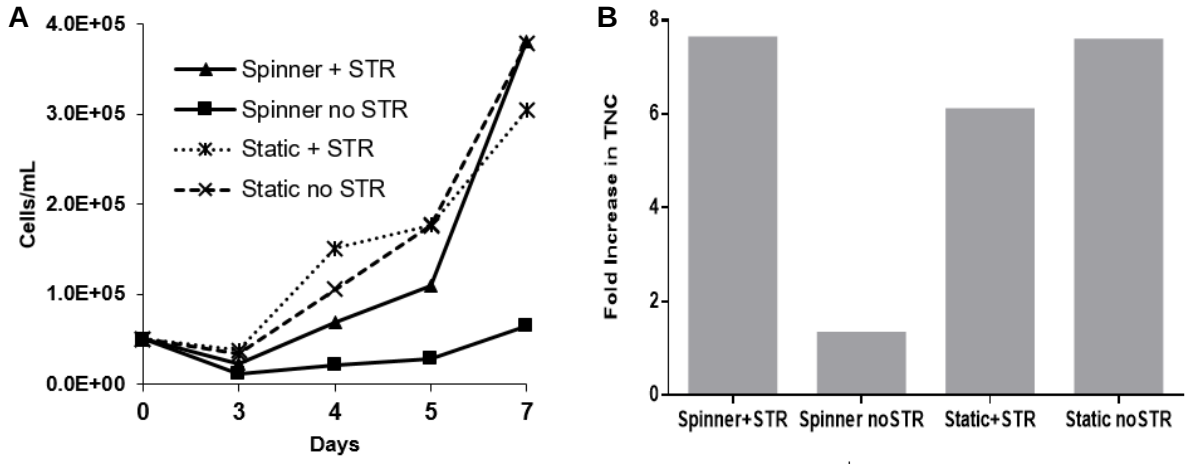


Figure VII-6 *– UCB derived HSPC expansion under dynamic (spinner) and static conditions, with (+ Str) and without (noStr) BM-MSC stromal feeder layer. (A) growth curve (cells/mL) throughout time of expansion (days). (B) Fold increase in total nucleated cells (TNC) (n=1).*

UCB cell expansion was characterized by analysis of cell count throughout time (growth curve) (***Figure VII-6*** **A**), fold increase in TNC (***Figure VII-6*** **B**), hematopoietic stem/progenitor phenotypes CD34+, CD34+/CD133+ and CD34+CD90+ (***Figure VII-7*** **A and B**) , and for the more committed progenitors from the early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41a+ cells) (***Figure VII-7*** **C**).

Conditions under Static noStr and Spinner+Str presented similar growth curves and concomitant TNC expansion fold (7.6-fold), converging roughly to the same cell density ($3.8x10^5$ cells/mL), whilst static+Str attained slightly lower cell densities ($3x10^5$ cells/mL) and fold expansion (6-fold) and Spinner noStr presenting the less efficient system in retrieved cell numbers ($6.5x10^4$ cells/mL) and fold expansion (1.3-fold). To note that regarding the static controls, these values are significantly lower than the ones presented in the previous section focusing on the study of static culture performance of SP medium.

Furthermore, immunophenotypic analysis demonstrate an expected decrease on the hematopoietic stem/progenitor population from 94% to 47-64% in CD34+, from 86% to 17-33% for CD34+/CD133+. It appears that the static+Str condition was able to maintain these primitive populations more efficiently. Indeed, for the CD34+/CD90+ progenitors, it was seen an increase from 0.5% to 1.4% in that condition, whereas a maintenance or drop in that population was observed for the other conditions. In agreement, total stem/progenitor expansion followed the same trend, with similar values for Spinner+Str and static with and without stroma, with exception for CD34+/CD90+ fold expansion for both static conditions that presented higher values, especially for static culture with stroma (16.3-fold).

Finally, the differentiative potential of the UCB expanded in these systems was analyzed, by testing for early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41+ cells) (***Figure VII-7* C**). Firstly, it was seen a decrease on the megakaryocytic lineage population in all conditions. Nonetheless, all culture systems where able to maintain/expand early lymphoid and myeloid lineages. Interestingly, it appears that early lymphoid progenitors (CD7+) are more efficiently expanded in dynamic conditions with stroma. The same occurs for the myeloid CD15+ marker, although with spinner and static cultures giving rise to roughly the same proportion.

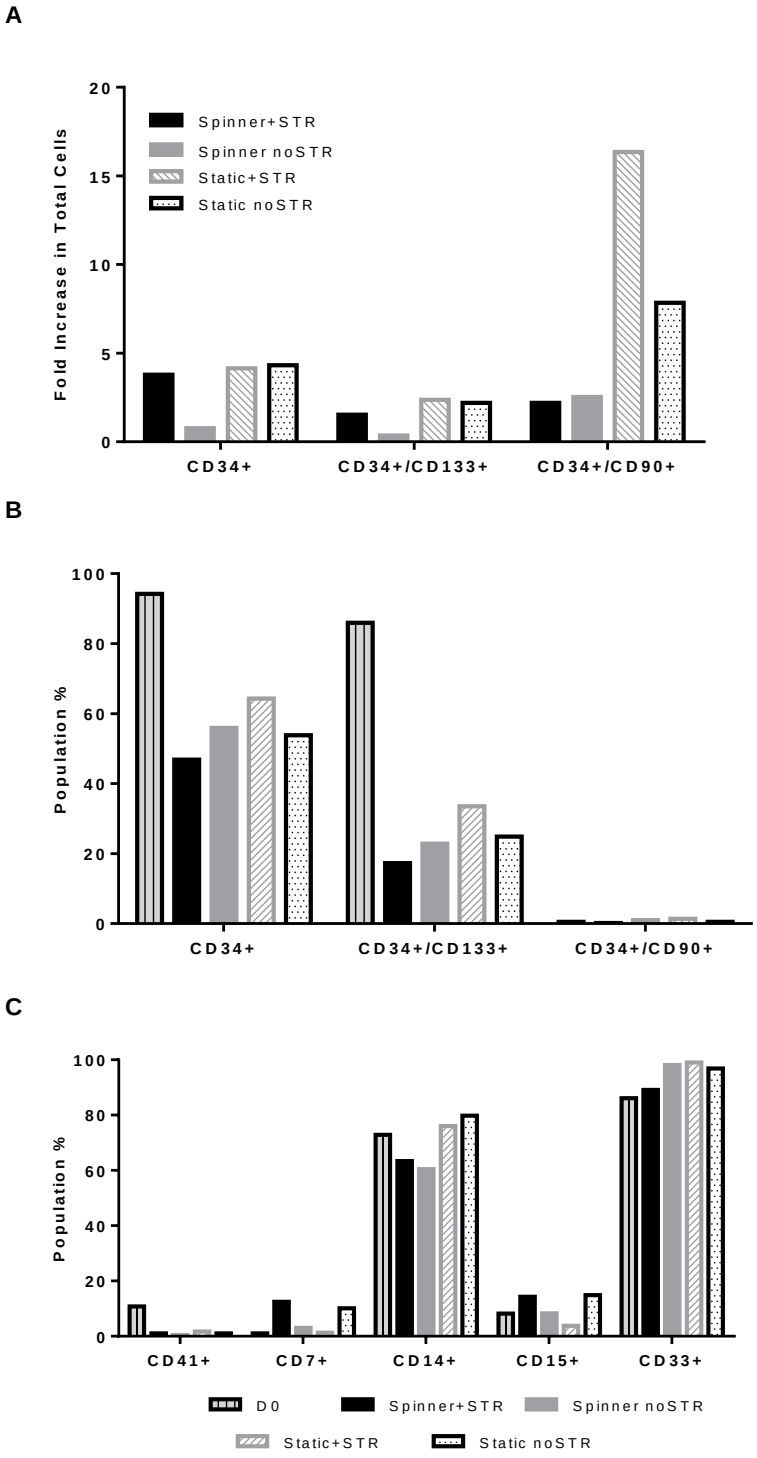


Figure VII-7 *- Immunophenotypic characterization of the pre- and post-expanded CD34+ cells under static and dynamic cultures. (A) fold change in total hematopoietic stem/progenitor phenotype (CD34+, CD34+/CD133+ and CD34+/CD90+); (B) Content in percentage of the hematopoietic stem/progenitor phenotype; (C) Content in percentage in early lymphoid (CD7+), myeloid (CD14+, CD15+ and CD33+) and megakaryocytic lineages (CD41+) (n=1).*

VII.3.3 *IN VIVO* STUDY OF THE REPOPULATING POTENTIAL OF UMBILICAL CORD DERIVED HSPC EXPANDED USING SP MEDIUM IN A NSG MICE MODEL

Ultimately, the objective of expanding UCB HSPC *in vitro* is to transplant these cells to treat hematological malignancies, thus, it is critical that not only the expansion system to achieve a relevant number of hematopoietic stem/progenitor cells, but also that these cells are able to efficiently engraft into the living tissues of the recipient. Hence, a preliminary study of the cells produced under SP in co-culture with BM-MSC feeder layer (best condition tested – see **VII.3.1**) on the homing and engraftment ability was performed in a NSG mice model, in collaboration with iMM. A schematic figure of the experiment layout is presented in ***Figure VII-8*.**

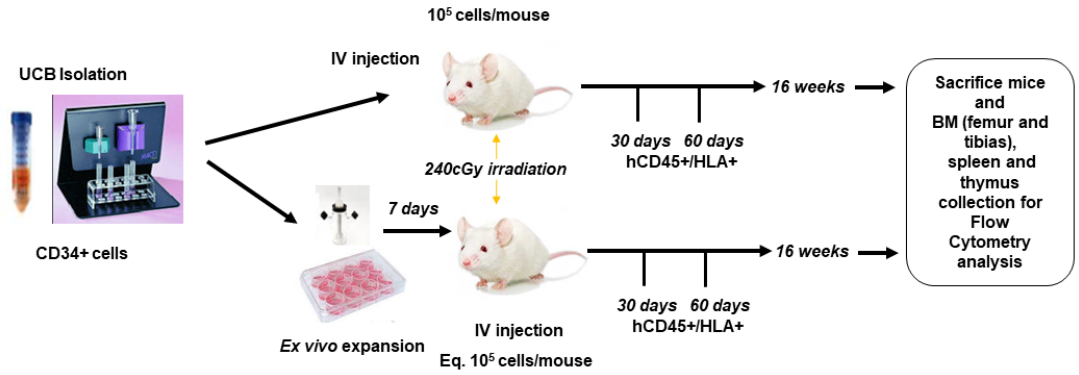


Figure VII-8 *– Schematics of the experiment outline to assess engraftment potential in a NSG mice model. Pre- and post-expanded UCB derived CD34+ cells cultured in SP were intravenously injected in the tail vein and mice kept for 16 weeks. At this endpoint, mice were sacrificed and bone marrow, spleen and thymus collected to analyze for human hematopoietic populations. In addition, bleeds were made at 30 and 60 days post infusion timepoints to look for circulating human hematopoietic cells (hCD45+/HLA+). n=5/condition.*

Hence, 240 cGy-irradiated mice were transplanted via tail vein injection either with fresh (primary) or expanded fractions of CD34+, at IMM mouse facility (***Figure VII-8*,** n/condition = 5; 1x10^5 cells/mouse). Cord blood purification and CD34+ expansion was performed at iBB-IST, for 7 days in cytokine supplemented SP medium, in co-culture with BM-MSCs in static conditions. Fold increase in total nucleated cells (TNC), in

hematopoietic progenitors (CD34+/CD133+) and hematopoietic stem cells (CD34+CD38-CD90+CD45RA-) was measured from day 0 to day 7 (***Figure VII-9***).

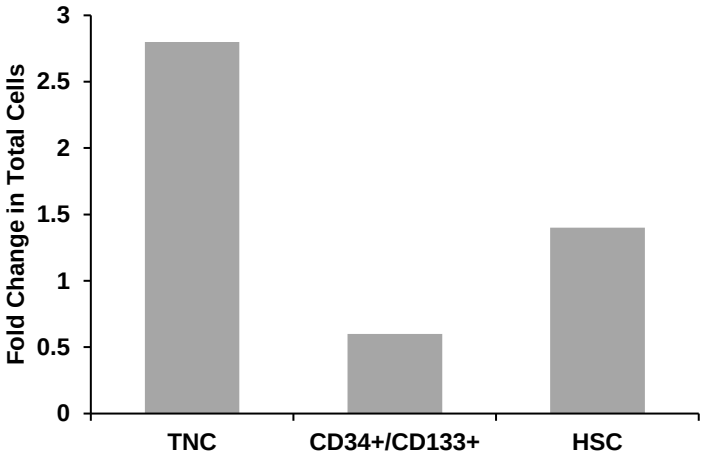


Figure VII-9 *– Fold increase in total cells of the attained hematopoietic populations: Total nucleated cells (TNC), hematopoietic progenitors (CD34+/CD133+) and hematopoietic stem cells (HSPC, CD34+CD38-CD90+CD45RA-).*

A modest fold expansion in TNC and HSPC was attained, by 2.8 and 1.4-fold, respectively. In addition, it was observed a decrease on the hematopoietic progenitor population relatively to the initial starting material (0.6-fold).

Peripheral blood samples were collected 30 and 60 days following transplantation for flow cytometry detection of human surface markers. No human cells were detected in the periphery for day 30. At day 60, one mouse from the primary UCB transplantation group was found dead. Human CD45 surface marker (i.e. type I transmembrane protein that is in various forms present on all differentiated hematopoietic cells) was detected in all of the surviving mice transplanted either with primary (**Supplementary Figure VII-1**) or expanded UCB cells (**Supplementary Figure-VII-2**). Furthermore, $CD45^+/CD19^+$ cells (specific for B lymphocytes) were also found to be in the periphery of all transplanted mice (**Supplementary Figure VII-1,Supplementary Figure-VII-2)**. On the other hand, only mice transplanted with non-expanded UCB cells group were found to have $CD3^+$

cells (present throughout T lymphocyte development) in the periphery (**Supplementary Figure VII-1,Supplementary Figure-VII-2)**. $CD34^{+}$, CD56 (NK cells), and CD235a (erythroid lineage) surface markers were not detected in neither of groups.

Sixteen weeks following transplantation, mice were sacrificed, and samples taken from the bone marrow, thymus, spleen, and peripheral blood, to analyze several surface markers by flow cytometry. Mice were considered to be positively engrafted if human if human CD45+/HLA+ values were detected above 1%.

For the group receiving non-expanded UCB cells, only one mouse survived (1 out 3), while for the group transplanted with expanded UCB cells all mice survived. Engraftment (CD45+/HLA+ > 1%) was detected in all organ samples for the animals receiving non-expanded cells. (***Figure VII-10***).

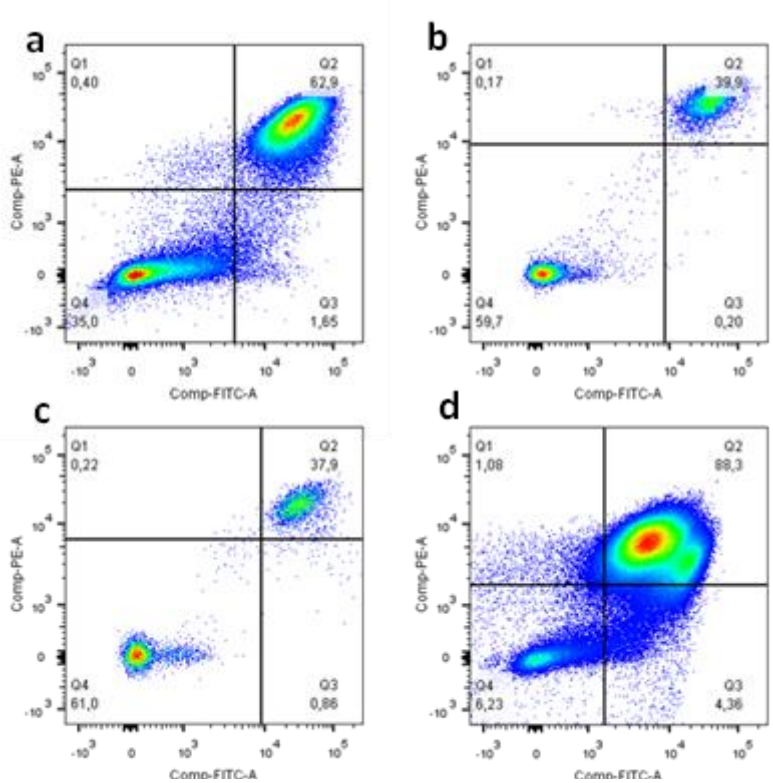


Figure VII-10 *– Immunophenotypic analysis of animals receiving non-expanded cells ***(LGR-00359 mouse) Engraftment in a) Bone Marrow b) Thymus c) spleen d) Peripheral Blood*

On the other hand, for the group of animals receiving expanded cells, cell engraftment was only detected in bone marrow and peripheral blood samples (***Figure VII-11*****, Supplementary Figure VII-3**), and to a lesser extent. The absence of cells in the thymus for this group is consistent with the absence of T lymphocyte cell surface markers at day 60 (**Supplementary Figure-VII-2**).

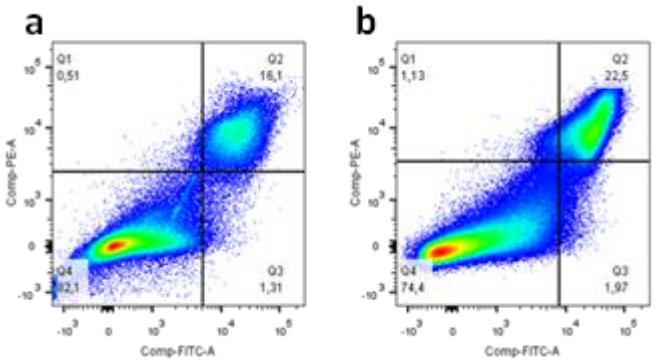


Figure VII-11 *– Immunophenotypic analysis of animals receiving expanded cells *** (LGR-00362 mouse) Engraftment in a) Bone Marrow b) Peripheral Blood*

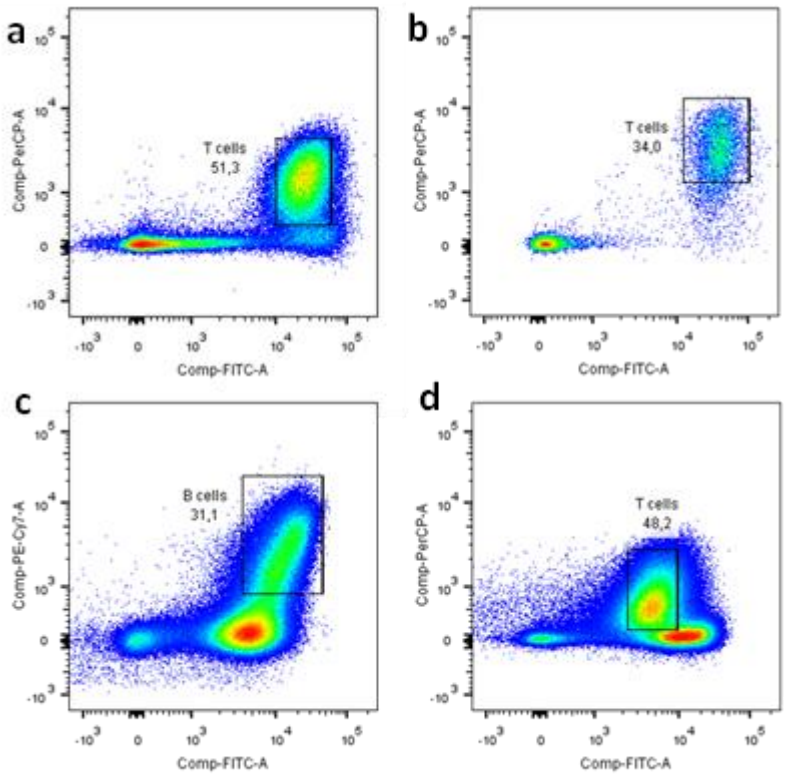


Figure VII-12 *– Immunophenotypic analysis of mice infused with pre-expanded cells (LGR-00359 mouse): a) Bone Marrow, T lymphpcytes; b) b) Thymus, T lymphocytes; c) spleen, B lymphocytes; d) Spleen, T lymphocytes.*

Additionally, the animal receiving non-expanded cells had human T lymphocytes in the bone marrow and thymus, as well as B and T lymphocytes in the Spleen (***Figure VII-12*****),** whereas in the group receiving expanded cells it was only found to have markers specific for B lymphocytes in the bone marrow and spleen (***Figure VII-13*****, Supplementary Figure VII-4**).

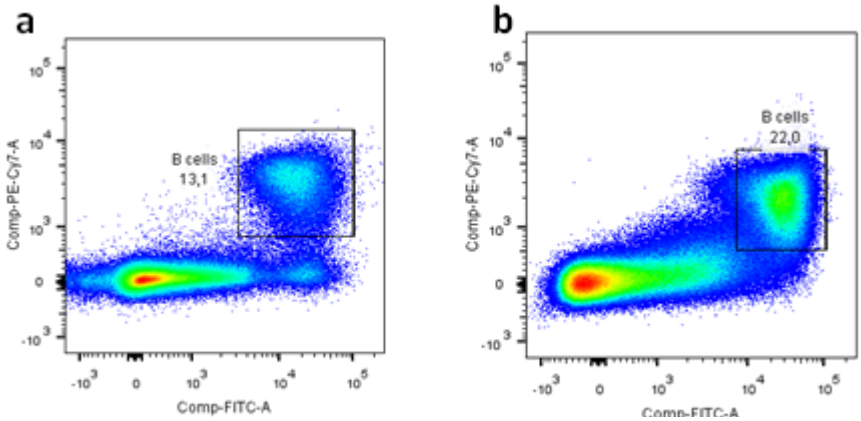


Figure VII-13 *- Immunophenotypic analysis of mice infused with expanded cells (LGR-00362 mouse): a) Bone Marrow, B lymphocytes b) Spleen, B lymphocytes.*

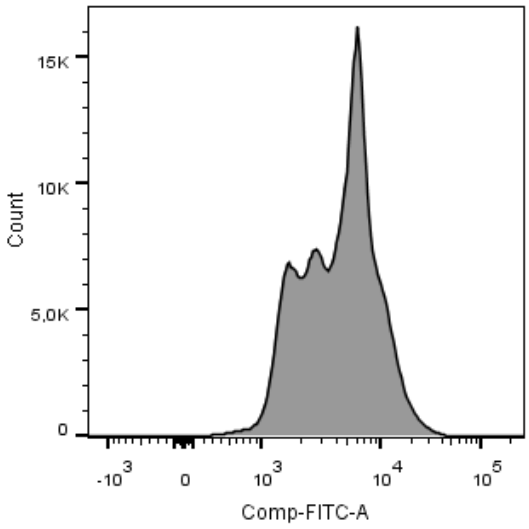


Figure VII-14 *- Immunophenotypic analysis of mice infused with expanded cells (LGR-00362 mouse) – myeloid lineage in the bone marrow.*

No $CD34^+$, $CD33^+$ (myeloid lineage), CD56 (NK cells), and CD235a (erythroid lineage) surface markers were detected in neither of the organs of both groups, with exception of one mouse in the expanded cell transplantation group, which had $CD33^+$ positive cells in the bone marrow (***Figure VII-14***).

VII.4 DISCUSSION AND CONCLUSIONS

The major bottleneck to the widespread use of umbilical cord blood (UCB) in hematopoietic stem/progenitor (HSPC) cell therapy is the low cell dose available. The ability to successfully expand HSPC ex vivo would have a profound impact in HSPC transplantation, but also in terms of tumor cell purging, somatic cell gene therapy and adoptive immunotherapy. However, the major focus in HSPC expansion is the rational establishment of optimal culture conditions to provide an amplification of the primitive HSPC (AurÈlie et al., 2008) namely: cytokine cocktails used (Andrade et al., 2010), enrichment for initial stem/progenitor cells, the presence/absence of feeder layers (da Silva et al., 2005b; da Silva et al., 2010b; da Silva et al., 2009b) or duration of culture (Douay, 2001a), which have a critical impact on the final quality of the expanded cells and on the cost effectiveness of the cell manufacturing process (Kirouac and Zandstra, 2008). Importantly, culture medium used have a major impact on the manufacturing and it needs to be carefully chosen in order to attain not only higher quantity but to assure quality of the final product, and to be compliant with GMP standards (for instance, to be serum/xeno-free). Several companies have realized this market need and Company X in particular aims to enter the competitive market of medium development for HSPC with the development of a new clinical grade xeno-free medium: SP together with the suggested cytokine cocktail. Thus, this project was performed within the collaboration between iBB-IST and Company X to perform the alpha testing of their new developed medium, by combining iBB-IST expertise in HSPC expansion, including co-culture systems with BM MSC, using the reagents developed by Company X currently not

commercially available to the iBB-IST, allowing early stage feasibility testing and optimization of workflow solutions based upon performance in their culture system.

SP performance was tested against the routinely HSPC expansion system used at iBB-IST, in co-culture with BM-MSC (QBSF+Str). In addition, it was also tested the combined effect of our system (BM-MSC feeder layer) with this new medium (citokine-supplemented) (SP+Str). As seen by the data, SP medium (SP noStroma) was able to perform better in terms of fold expansion in TNC in comparison with the system used in our lab (QBSF+Str), whereas giving similar values in stem/progenitor maintenance and expansion compared to QBSF+Str. Interestingly, when combining SP medium with our co-culture technology, the performance in HSPC expansion increased significantly. Importantly, it was seen that by adding the stromal layer, this expansion system was not only able to increase TNC but as well to maintain a higher content in early progenitors (CD34+, CD34+/CD90+) and concomitant fold expansion of these populations, in agreement with previous reports (Jing et al., 2010a; Noort et al., 2002). Of notice, SP medium has a higher maintenance capacity of the most primitive CD34+/CD90+ population, still an effect enhanced by the use of the stromal feeder layer.

Regarding differentiative potential of the expanded UCB cells, early lymphoid and myeloid, including megakaryocytic, lineages were maintained/expanded in all conditions. However, SP medium resulted in higher differentiative potential for early lymphoid (CD7+) and myeloid (CD15+) lineages, whereas QBSF yielded a higher differentiative potential of UCB cells towards megakaryocytic lineages (CD41+), which is in agreement with previous reports (Andrade et al., 2013a; Andrade et al., 2010). Concomitantly, the capacity for CFU and CAFCs formation by the expanded UCB cells was the best when using the SP+Str culture system, consistent with the higher fold expansion in TNC, CD34+ and the more primitive CD34+/CD90+ population. Hence, it is shown that the conjugation of our technology (MSC-HSPC co-culture) with the prototype medium SP showed highly positive results in terms of quantity and quality of the expanded HSPCs.

However, it is mandatory to develop a clinical-grade and cost-effective system. Thus, HSPC expansion in dynamic culture (spinner flasks) with SP medium was tested preliminarily (n=1), as it provides a scalability step towards a fully controlled bioreactor system. Again, cultures with and without stromal layers were tested. All systems expanded HSPCs effectively and at the same extent with exception of spinner flask without stroma (SP noStr). These observations enforce the ability of the MSC-derived stromal layer on supporting *ex vivo* HSPC expansion, even in stirred conditions, potentially more stressful due to agitation shear stress. However, when comparing historical data from our lab using QBSF+Str (Andrade et al., 2011; Andrade et al., 2013a; Andrade et al., 2010) and the results of the static experiment presented in VII.3.1. , it is seen that there was an underperformance in terms of fold expansion in TNC and progenitors. This could be due to the low viability percentage (60%) of cells following CD34+ purification through MACS in this particular experiment. In addition, low values of viability at the start of the culture in combination with potential detrimental sheer stress present in dynamic cell culture might had a severe impact on the cells. Of importance, regarding the static controls, it was not observed a clear benefit of the stromal layer in TNC fold expansion of UCB cells, contrarily to what was attained in the previous experiments in alpha testing process of this product. This may be due to the nature of alpha testing, where medium fine tuning and formulation changes occurs throughout the process. Thus, the lot that was used for the first experiments may be different from the one used for the dynamic study, which may provide an explanation for the differences between the results. On the other hand, an increased maintenance of the most primitive CD34+/CD90+ population was benefited by the stromal layer, in agreement with previous results. Nonetheless, in the spinner flask it was obtained a considerable higher number of cells ($3.8x10^5$ cells/mL) in comparison with the static counterpart ($3.0x10^5$ cells/mL), showing the ability of this system to be scaled-up in order to achieve clinical meaningful numbers.

In these studies, the scale-up of the of UCB hematopoietic progenitors with or without microcarrier immobilized MSC-derived stromal cells was evaluated under stirred conditions, using spinner flasks, using a novel culture medium not commercially available supplemented with cytokines. Efficient, but moderate expansion of UCB CD34+ cells was obtained in a short-time period (7 days), generating significant cell numbers. The system presented herein features process scalability and might be compliant with GMP standards for the production of cellular products, namely through the use of commercially available, highly controlled disposable technologies.

No conclusive results were retrieved from the *in vivo* engraftment of the expanded HSPC in comparison to freshly purified HSPC (non-expanded cells). Only 1 out of 3 mice transplanted with fresh UCB cells survived up to the sixteenth week, while 3 out of 3 mice transplanted with expanded UCB cells survived. However, engraftment in surviving mice occurred to a lesser extent in the latter than in the former. Furthermore, transplantation of non-expanded UCB cells gave rise to T lymphocyte lineages, whereas that was not observed for the expanded cells. In either group, the absence of CD34+ cells in the bone marrow after 16 weeks indicates that long-term reconstitution is unlikely to be maintained. Poor engraftment, lack of lineage diversity and CD34+ may be attributed both to the poor *ex-vivo* expansion of UCB cells, and to the low viability values upon purification (60%).

In conclusion, although the combination of cytokine supplemented SP with our feeder layer technology represent the best system tested, the results of the study under dynamic conditions and the *in vivo* study must be repeated. Moreover, variation in medium composition and methodological problems (low viability after MACS isolation) may have led to these discrepancies in the results.

VII.5 REFERENCES

Andrade, P.Z., de Soure, A.M., Dos Santos, F., Paiva, A., Cabral, J.M., and da Silva, C.L. (2013). Ex vivo expansion of cord blood haematopoietic stem/progenitor cells under physiological oxygen tensions: clear-cut effects on cell proliferation, differentiation and metabolism. Journal of tissue engineering and regenerative medicine.

Andrade, P.Z., dos Santos, F., Almeida-Porada, G., da Silva, C.L., and JM, S.C. (2010). Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells. Molecular bioSystems *6*, 1207-1215.

Andrade, P.Z., dos Santos, F., Cabral, J.M., and da Silva, C.L. (2015). Stem cell bioengineering strategies to widen the therapeutic applications of haematopoietic stem/progenitor cells from umbilical cord blood. Journal of tissue engineering and regenerative medicine *9*, 988-1003.

AurÈlie, C., Christophe, D., Emilie, R., Denis, C., Bernadette, G., Laetitia, B., Annelise, B.-G., Olivier, P.-L., Georges, U., Lucile, A.*, et al.* (2008). A Cross-Talk Between Stromal Cell-Derived Factor-1 and Transforming Growth Factor-beta Controls the Quiescence/Cycling Switch of CD34^{<FONT SIZE='-1'>+</FONT>} Progenitors Through FoxO3 and Mammalian Target of Rapamycin. Stem Cells *26*, 3150-3161.

Ballen, K.K., Gluckman, E., and Broxmeyer, H.E. (2013). Umbilical cord blood transplantation: the first 25 years and beyond. Blood *122*, 491-498.

Cabrita, G.J., Ferreira, B.S., da Silva, C.L., Goncalves, R., Almeida-Porada, G., and Cabral, J.M. (2003). Hematopoietic stem cells: from the bone to the bioreactor. Trends Biotechnol *21*, 233-240.

Carmelo, J.G., Fernandes-Platzgummer, A.M., Diogo, M.M., Silva, C.L.d., and Cabral, J.M.S. (2015). A Xeno-free Microcarrier-based Stirred Culture System for the Scalable Expansion of Human Mesenchymal Stem/Stromal Cells isolated from Bone Marrow and Adipose Tissue. Biotechnology Journal *10*, 1235-1247.

da Silva, C.L., Goncalves, R., Crapnell, K.B., Cabral, J.M.S., Zanjani, E.D., and Almeida-Porada, G. (2005). A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Exp Hematol *33*, 828-835.

da Silva, C.L., Goncalves, R., Dos Santos, F., Andrade, P.Z., Almeida-Porada, G., and Cabral, J.M.S. (2010). Dynamic cell-cell interactions between cord blood haematopoietic progenitors and the cellular niche are essential for the expansion of CD34(+), CD34(+)CD38(-) and early lymphoid CD7(+) cells. J Tissue Eng Regen Med *4*, 149-158.

da Silva, C.L., Gonçalves, R., Porada, C.D., Ascensão, J.L., Zanjani, E.D., Cabral, J.M.S., and Almeida-Porada, G. (2009). Differences amid bone marrow and cord blood hematopoietic stem/progenitor cell division kinetics. Journal of Cellular Physiology *220*, 102-111.

Dahlberg, A., Delaney, C., and Bernstein, I.D. (2011). Ex vivo expansion of human hematopoietic stem and progenitor cells. Blood.

Dexter, T.M., Allen, T.D., Lajtha, L.G., Schofield, R., and Lord, B.I. (1973). Stimulation of differentiation and proliferation of haemopoietic cells in vitro. J Cell Physiol *82*, 461-473.

Dexter, T.M., Spooncer, E., Simmons, P., and Allen, T.D. (1984). Long-term marrow culture: an overview of techniques and experience. Kroc Found Ser *18*, 57-96.

dos Santos, F., Andrade, P.Z., Eibes, G., da Silva, C.L., and Cabral, J.M. (2011). Ex Vivo Expansion of Human Mesenchymal Stem Cells on Microcarriers. Methods Mol Biol *698*, 189-198.

Douay, L. (2001). Experimental culture conditions are critical for ex vivo expansion of hematopoietic cells. J Hematother Stem Cell Res *10*, 341-346.

Jing, D., Fonseca, A.-V., Alakel, N., Fierro, F.A., Muller, K., Bornhauser, M., Ehninger, G., Corbeil, D., and Ordemann, R. (2010). Hematopoietic stem cells in co-culture with mesenchymal stromal cells - modeling the niche compartments in vitro. Haematologica *95*, 542-550.

Kirouac, D.C., and Zandstra, P.W. (2008). The systematic production of cells for cell therapies. Cell Stem Cell *3*, 369-381.

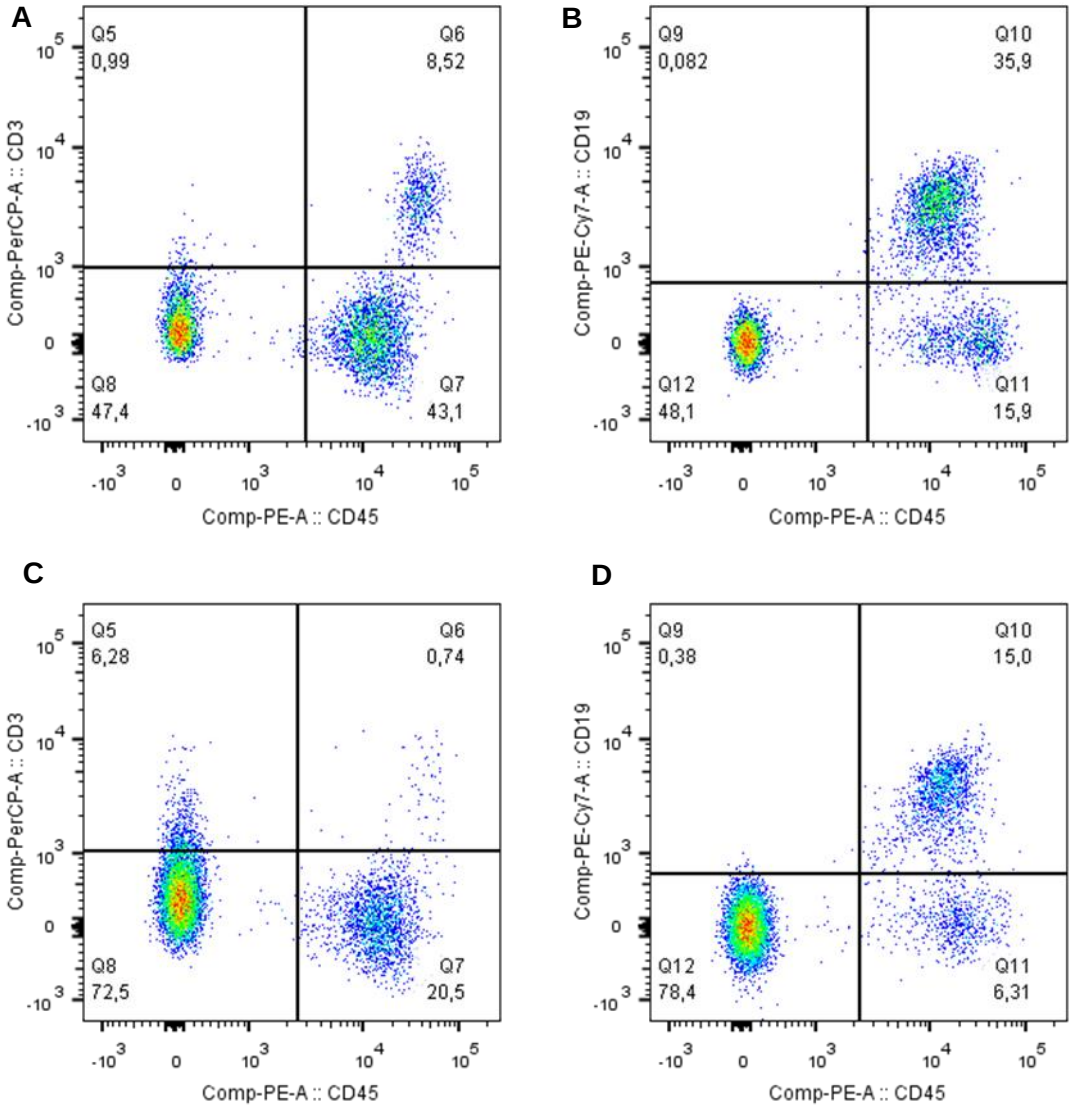
Noort, W.A., Kruisselbrink, A.B., in't Anker, P.S., Kruger, M., van Bezooijen, R.L., de Paus, R.A., Heemskerk, M.H., Lowik, C.W., Falkenburg, J.H., Willemze, R.*, et al.* (2002). Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol *30*, 870-878.

Pineault, N., and Abu-Khader, A. (2015). Advances in umbilical cord blood stem cell expansion and clinical translation. Exp Hematol *43*, 498-513.

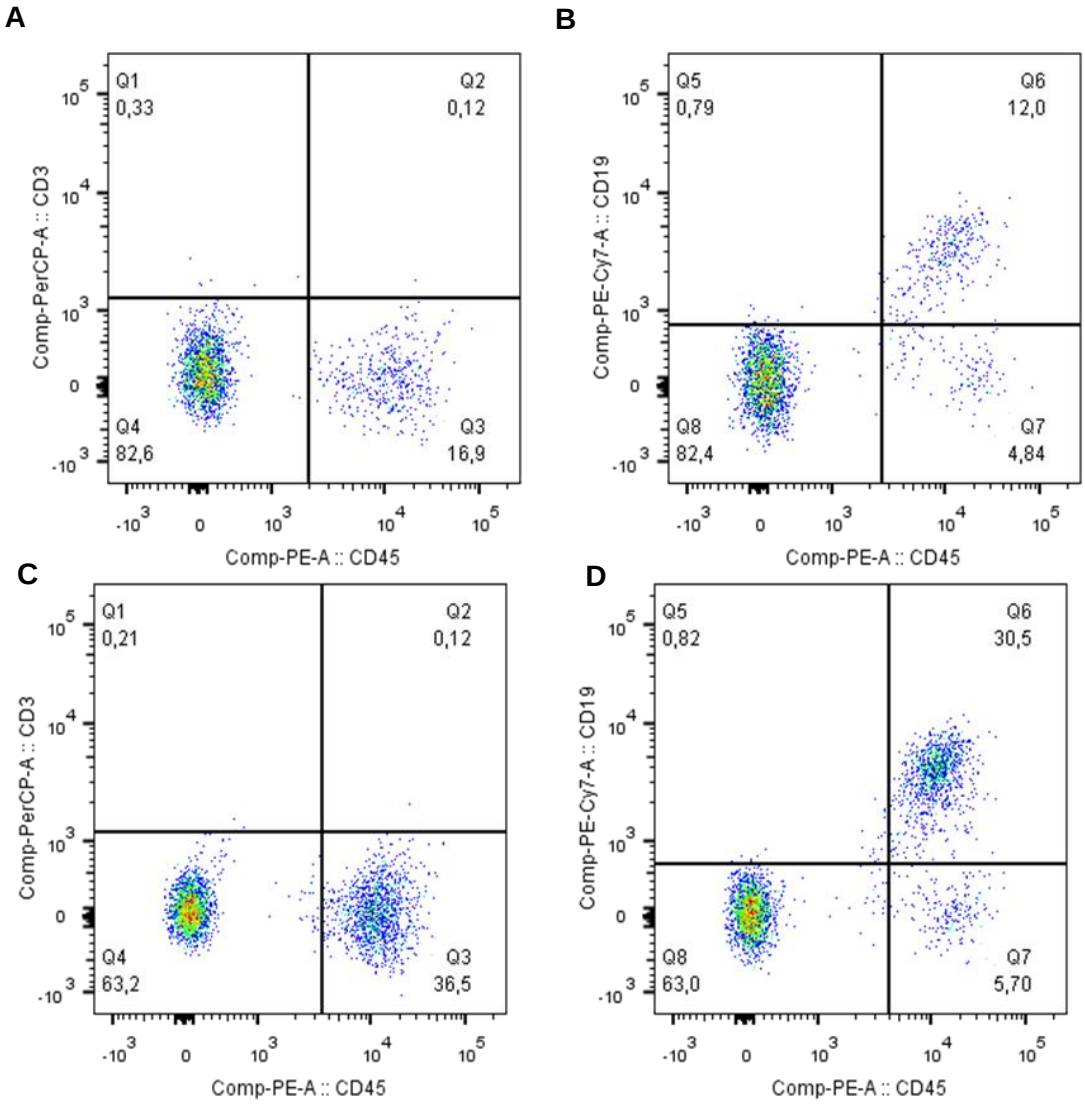
Ploemacher, R.E., van der Sluijs, J.P., Voerman, J.S., and Brons, N.H. (1989). An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood *74*, 2755-2763.

Santos, F., Andrade, P.Z., Abecasis, M.M., Gimble, J.M., Chase, L.G., Campbell, A.M., Boucher, S., Vemuri, M.C., Silva, C.L., and Cabral, J.M. (2011). Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions. Tissue engineering Part C, Methods *17*, 1201-1210.

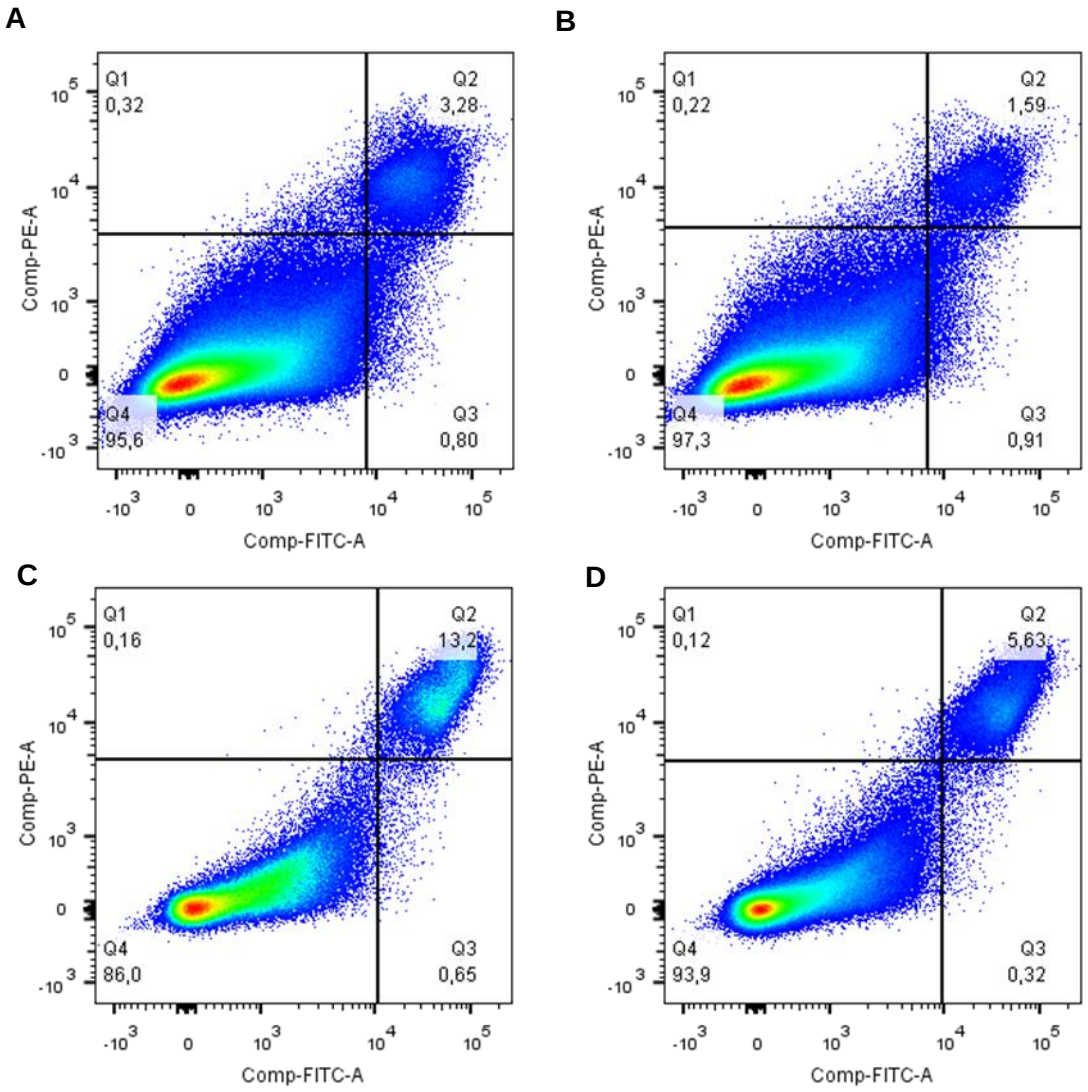
VII.6 SUPPLEMENTARY FIGURES – CHAPTER VII



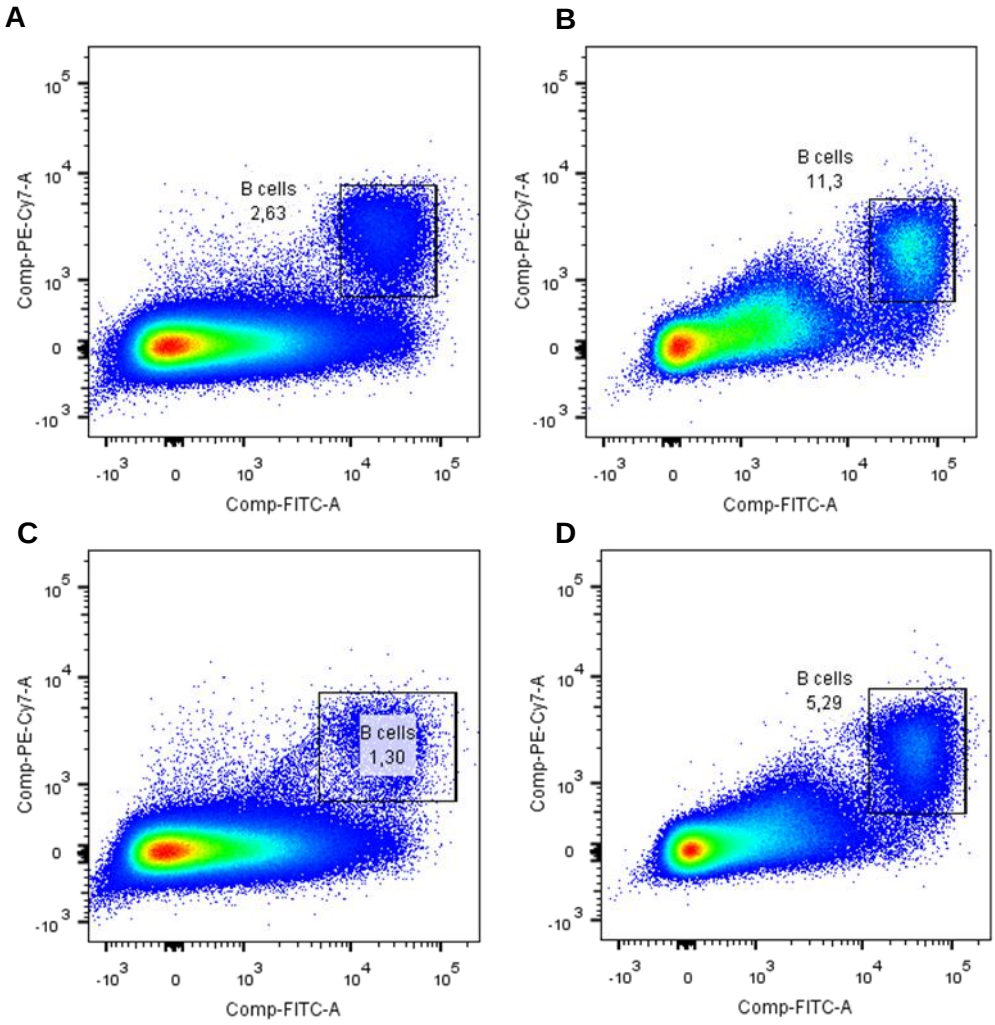
Supplementary Figure VII-1 *- Immunophenotypic analysis of mice infused with pre-expanded cells – ELGR 359 (A,B) and ELGR 360 (C,D) – peripheral blood analysis at day 60 for T lymphocytes (A,C) and B lymphocytes (B,D)*



Supplementary Figure-VII-2 *- Immunophenotypic analysis of mice infused with expanded cells – ELGR 361 (A,B) and ELGR 362 (C,D) mice – peripheral blood analysis at day 60, for T lymphocytes (A,C) and B lymphocytes (B,D).*



Supplementary Figure VII-3 *- Immunophenotypic analysis of mice infused with expanded cells – ELGR 361 (A,B) and ELGR 363 (C,D) mice – engraftment analysis after 16weeks in Bone Marrow (A,C) and spleen (B,D)*



Supplementary Figure VII-4 *- Immunophenotypic analysis of mice infused with expanded cells – ELGR 361 (A,B) and ELGR 363 (C,D) mice – B lymphocyte analysis after 16weeks in Bone Marrow (A,C) and spleen (B,D).*

CONCLUSIONS AND FUTURE TRENDS

Stem cells hold a great promise in the advanced regenerative medicine field towards the development of innovative and further improved therapies that can be applied in a wide range of inherited and acquired medical conditions.

Blood derived CD34+ stem/progenitor cells, in particular, present several therapeutic advantages due to their intrinsic characteristics. Indeed, this marker is widely used to isolate hematopoietic stem/progenitor cells (HSPCs), as well as to target angiogenic progenitor cells (believed to include blood derived endothelial progenitor cells (EPC)) (Sidney et al., 2014). In addition, hematopoietic cell transplantation is a well and widely established therapy to treat malignant and non-malignant hematopoietic disorders, since the first bone marrow (BM) transplantation in 1956. After transplantation, these cells are able to home and repopulate patient BM and to give rise to all blood cell types. However, hematopoietic cell transplantation with BM cells has some drawbacks as it involves an invasive procedure and it stringently requires a specific HLA matched donor.

Umbilical cord blood (UCB) has arisen as an alternative source for CD34+ stem/progenitor cells, as it presents several advantages namely: i) easy and ready availability (as it is considered as biological waste); ii) non-invasive collection; iii) less HLA restringing (with less probability of GVHD and wider availability); iv) source of more primitive cells. On the other hand, this is accompanied by some step-backs as the cell number that can be retrieved from a single unit is limited and usually enough to treat only a small child. To overcome this, it has been reported the transplantation of 2 partially HLA-matched UCB units to transplant adult patients(Barker et al., 2005). However, the *holy grail* still relies on the *ex vivo* expansion of these cells, not only to achieve clinical meaningful numbers, but also a final product with quality/potency where the maintenance of the progenitor cells is attained. Indeed, an effort has been made in this direction, by optimization of several culture factors as cytokine cocktails (Andrade et al., 2010), initial stem/progenitor cell enrichments (Andrade et al., 2011), the presence/absence of feeder

layers (da Silva et al., 2005a; da Silva et al., 2010a), culture duration (Douay, 2001b) and feeding regime (Csaszar et al., 2012).

On the other hand, as stated previously, Asahara and colleagues stated the isolation of a putative EPC population with angiogenic potential from peripheral blood-derived CD34+ cells (Asahara et al., 1997) and later from BM (Asahara et al., 1999). This population has the potential to be applied for cardiovascular ischemic disease therapy (Kalka et al., 2000; Zhang and Xu, 2014). It is believed that HSPCs and EPCs are originated from a common progenitor throughout embryonic life, the hemangioblast. However, they relation and the distinct pathway is yet to be determined (Ingram et al., 2004). Furthermore, the complete characterization of these cells and mode of action when used in cell therapy has generated controversy amongst the scientific community, with a variety of different isolation and culture methods and assays that hinders an impossible comparison between the works reported and controversy on the clinical benefit.

Alternative suspension culture methods have been developed in order to meet the translational scenario to the clinical setting, with higher quantity and quality (efficient neovascularization in ischemic animal models) of the expanded cells (Bouchentouf et al., 2011; Masuda et al., 2012; Masuda et al., 2014; O et al., 2011). In this context, a successful scale- up of PB-derived CD34+ circulating angiogenic progenitor cells (CAPCs) into GMP compliant cell culture bags combined with defined culture reagents (QQc), suitable for the clinical setting was achieved (*Chapter III*). Our data showed effective expansion of the cultured population (7-fold), while maintaining the stem/progenitor content and increasing the endothelial population with further increased angiogenic potential *in vitro*.

CD34 enrichment using MACS technology is a routinely applied strategy to isolate $CD34^+$ cells as EPC containing cell fraction (Schmidt-Lucke et al., 2010). However, its frequency

in peripheral blood is very low (less than 0.1%), which limits the number of cells that can be obtained (Scheubel et al., 2003). In addition, in the autologous therapy setting, one must take into account that increasing age and disease hinders the cell's angiogenic potential (Numaguchi et al., 2006). Therefore, regarding the aforementioned factors, UCB is an advantageous source for an allogeneic clinical setting. The effects of shear stress and oxygen tension have been extensively described throughout the literature to participate biologically in the angiogenic mechanisms and to promote the angiogenic potential of cells exposed to these (Lee et al., 2013b; Obi et al., 2012; Obi et al., 2009; Papaioannou and Stefanadis, 2005; Yamamoto et al., 2003). Moreover, the chosen medium plays a critical part on culture performance. Based on reports from literature, it was ought to test StemSpan® medium in comparison with the medium used in *Chapter III* (SCGM®) to evaluate its ability on UCB-CD34+ CAPC expansion and *in vitro* angiogenic potential of the final product (*Chapter VI*). It was observed, in agreement with data found in literature (Masuda et al., 2012), a higher performance of StemSpan® medium not only in generating higher cell numbers, but as well as in maintenance/expansion of progenitor and endothelial related lineages, assessed by flow cytometry.

In addition, a scalable dynamic system, in spinner flasks, was tested for the expansion of CAPCs derived from UCB-CD34+ cells to test shear and different oxygen tensions (hypoxia – 5% and normoxia – 21%) on the angiogenic potential of the final product. A successful dynamic scalable process was achieved, with an improvement in terms of cell quantity (total cell number fold increase of 32±0.9 and 46±6.8 at day 7; 84±7.9 and 101±26 at day 10, normoxia and hypoxia respectively) in comparison with unexpanded cells. Moreover, progenitor and endothelial progenitors were shown to be maintained/expanded and it was observed higher angiogenic potential *in vitro* in comparison with pre-expanded cells. Nonetheless, the differences between the systems tested were not significant, although cells cultured under hypoxic culture in the spinner

presented consistently higher immunophenotypic marker expression and tube formation capacity than the other conditions, within the experiments performed. Thus, it is pertinent to further explore this system.

On the other hand, the shear stress that cells were exposed to in this study (0.8 dyn/cm^2) was significantly lower in comparison to physiological shear (2.9 dyn/cm^2 to 65.5 dyn/cm^2). Hence, the next step would be to also to test higher agitation rates (rpm) on the angiogenic profile of cultured cells. Nonetheless, optimization of the feeding regime (cytokines and nutrients), as well as determination of angiogenic factor expression (qPCR) and secretion (ELISA) of the cultured cells, and clonogenic assays (Masuda et al., 2011) should be conducted to render the highest potential of this system and to fully characterize the final product, with ultimately, assessment of *in vivo* potential (Yang et al., 2009).

However, there are several cell types with potential to be applied in ischemic disease therapy (Dragneva et al., 2013; Ouma et al., 2013; Wu et al., 2018). Specifically, mesenchymal stem/stromal cells (MSCs) present attractive advantages since after systemic administration, MSCs preferentially home to site of injury and/or inflammation, whereupon they set a regenerative microenvironment through mechanisms which involve both the secretion of bioactive molecules and cell-to-cell interaction, which regulate and/or modulate local innate, and promote tissue-specific cell proliferation and repair (Caplan and Correa, 2011a; Prockop and Oh, 2012). Importantly, these cells present immunomodulatory properties (Meirelles et al., 2009), rendering them as potential allogeneic *off the shelf* therapy upon their GMP compliant *ex vivo* expansion (Dos Santos et al., 2014). Moreover, MSCs have been extensively investigated in cardiac regenerative therapy trials during the past decade due to their angiogenic properties (Kanelidis et al., 2017; Squillaro et al., 2016; Watt et al., 2013).

Benefits of MSC therapy for myocardial infraction are still a matter of debate, that may be due to methodological differences, mainly cell dose and administration route. Nonetheless, intracoronary MSC administration allow for the deliver of high cell number into the myocardial region, and therefore being widely studied. (Dib et al., 2011). However, MSC IC delivery may increase the risk of vessel obstruction due to their bigger size (10-20 µm) in comparison with heart capillaries (5-10 µm), prevailng a matter of debate s(Sanganalmath and Bolli, 2013). In this context, and in collaboration with António Fiarresga, MD, Hospital Santa Marta, PhD work, it was aimed to assess if the index of microcirculatory resistance (IMR) was able to detect changes on microcirculation in a pig model upon MSC IC administration and ultimately to compare microcirculatory obstruction and regenerative potential of MSC expanded under two platforms (conventional – 2D with FBS 'large'; innovative – dynamic microcarrier based in xenofree conditions, 'small') known to produce different sized cells, in an acute myocardial infarction (AMI) pig model (*Chapter IV*). It was observed that IC MSC delivery caused microcirculatory obstruction assessed in real time by IMR. Moreover, IMR was not able to detect significant differences between the different sized cells and, ultimately, no regenerative function was seen upon cell administration. Of notice, successful dynamic cell expansion was attained for different sized spinner flasks (100mL and 250mL). Although no difference of the different sized cells was not detected by IMR, this technique can be considered as a microcirculation disruption subclinical marker, whose real-time assessment may be useful during intracoronary BM MSC delivery and could help to define safety thresholds.

The discrepancies encountered in the regenerative potential upon MSC administration (Makkar et al., 2005; Shake et al., 2002), may be derived to methodological issues, namely expansion method, handling, administration method and effect measurement and thus, the evaluation of other variables of ventricular function as well as regenerative

potential (e.g. secretion of paracrine factors) could add further insights to the study and are a matter of further research.

Some efforts have been made towards the use of more readily available MSC sources, as AT and UCM, with clinical trials reports of safety and, in some cases, clinical improvement (Bartolucci et al., 2017; Gao et al., 2015; Musialek et al., 2015; Perin et al., 2014). Nonetheless, it has been reported that MSCs from different sources also may present different characteristics, such as immunological properties, as well as proliferative and differentiation abilities (Dmitrieva et al., 2012; Hass et al., 2011a; Pill et al., 2015; Puissant et al., 2005),. Thus, further research in order to assess the potential of these cells in an AMI setting should be pursued.

In parallel, MSCs have shown to have an important role in the hematopoietic BM niche and, to mimic physiological conditions, and they have been applied to HSPC-MSC co-culture systems, which have shown that the stromal layer plays an important role on the *ex vivo* expansion and maintenance of HSPCs (da Silva et al., 2005a; da Silva et al., 2010a). In addition, our lab has established an optimized cytokine cocktail for HSPC expansion co-cultured with BM-MSC derived feeder layer (Andrade et al., 2010), which has been used routinely. However, as mentioned previously, other MSC sources are more attractive due to their availability but may present different characteristics. Based on this, it was aimed to perform a systematic and comprehensive comparison of different MSCs sources (BM (including cells isolated based on Stro-1 expression by MACS vs. plastic adherence), AT, UCM, fetal liver (FL)) on the HSPC *ex vivo* expansion supportive capacity (*Chapter V*), which was not available to date. All feeder layers were able to support the expansion of CD34+-enriched cells, with AT yielding the highest fold increase in total cells (32.7±5.5) and FL and the *No Stroma* control with the lowest and similar values. Maintenance/ expansion of progenitors (CD34+, CD34+/CD133+ and CD34+/CD90+), differentiative potential, CFUs and CAFCs formation ability, was roughly the highest and similar between BM-MSCs (plastic adherence), Stro-1 BM-MSCs and

AT. Taking all together, although all three best feeder layers could be considered, AT-MSCs seems to be a highly candidate for to replace BM feeder cells in clinical trials attesting the administration of UCB cells expanded in co-culture systems, as led to similar amount of total progenitor cells and presents a more available MSC source. Nonetheless, further studies to assess UCM-MSC HSPC expansion support need to be completed due to methodological problems on the establishment of this feeder layer establishment.

On the other hand, in order to overcome possible contaminants from compounds of animal origin such as fetal bovine serum (FBS), efforts towards the use of human origin, clinical-grade GMP-compliant MSCs need to be addressed, Thus, the next step would rely on the repetition of this study with MSCs expanded under xeno-free conditions in order to establish a clinical grade co-culture system.

Furthermore, it has been previously stated the importance of culture medium on the performance of cell expansion and final product quality, as well as in the financial manufacturing viability (*Cost of Goods* – CoGs). As partners for Alpha testing of Company X new clinical grade defined xenofree medium, SP, it was ought to test its performance on HSPC expansion and progenitor maintenance with and without stromal layer (*Chapter VII*). SP medium have shown to be able to expand cells, in both static and dynamic cultures, in a higher extent while maintaining higher progenitor cell content in comparison with the medium used routinely in our lab (QBSF). Although SP medium presents an attractive alternative, comparison with other commercial available mediums should be conducted, as well as a cost analysis to assess the best manufacturing choice with attained cell product quality. In addition, in collaboration with Instituto de Medicina Molecular (IMM), a preliminary study of SP+Str expanded cells engraftment potential in an *in vivo* NSG mouse model was conducted. However, results indicate a poor long-term engraftment and hence the results are inconclusive and further studies are needed.

Overall, the work presented in this thesis give important insights for stem cell based therapies, where the potential of different cell types (CD34+ and MSCs) and end applications (Ischemic diseases and hematological disorders) were addressed and correlated. Bioengineering strategies and critical component factors (such as medium) were explored in the different systems towards the optimization of final cell product quantity and quality clinical grade manufacture. It is predicted a field contribution to a further fully controlled systems, which will possibly render higher productivity, and concomitant cost reduction moving towards a financially viable advanced cell therapy. Importantly, exchange of knowledge and translation between different fields were attained, by the collaborative projects that were performed.

Finally, considering the recent encouraging findings of cell based therapy in preclinical and clinical trials, further research needs to be pursued in order to depict effective therapeutic potential, as to determine best cell population, doses and administration route for a specific clinical need. In addition, it has been difficult to compare therapeutic potential with contradictory reports in the field. This would be achieved by a standardization on isolation methods as well as culture methodologies. Future advances will probably rely on the systematic exploitation of scalable and clinical grade expanded cells therapeutic potential, as conventional methods not only present a cost-effective process to achieve the high number demanded by the clinical need, are also usually associated with the use of animal components, which is critically seen by regulatory agencies.

REFERENCES

Andrade, P.Z., da Silva, C.L., dos Santos, F., Almeida-Porada, G., and Cabral, J.M. (2011). Initial CD34+ cell-enrichment of cord blood determines hematopoietic stem/progenitor cell yield upon ex vivo expansion. Journal of cellular biochemistry *112*, 1822-1831.

Andrade, P.Z., dos Santos, F., Almeida-Porada, G., da Silva, C.L., and JM, S.C. (2010). Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells. Molecular bioSystems *6*, 1207-1215.

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M., and Isner, J.M. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circulation research *85*, 221-228.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964-967.

Barker, J.N., Weisdorf, D.J., DeFor, T.E., Blazar, B.R., McGlave, P.B., Miller, J.S., Verfaillie, C.M., and Wagner, J.E. (2005). Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. Blood *105*, 1343-1347.

Bartolucci, J., Verdugo, F.J., Gonzalez, P.L., Larrea, R.E., Abarzua, E., Goset, C., Rojo, P., Palma, I., Lamich, R., Pedreros, P.A*., et al.* (2017). Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial (RIMECARD Trial [Randomized Clinical Trial of Intravenous Infusion Umbilical Cord Mesenchymal Stem Cells on Cardiopathy]). Circulation research *121*, 1192-1204.

Bouchentouf, M., Forner, K., Cuerquis, J., Boulassel, M.R., Routy, J.P., Waller, E.K., Quyyumi, A.A., Paradis, P., Schiffrin, E.L., and Galipeau, J. (2011). A novel and simplified method of culture of human blood-derived early endothelial progenitor cells for the treatment of ischemic vascular disease. Cell transplantation *20*, 1431-1443.

Cabrita, G.J., Ferreira, B.S., da Silva, C.L., Goncalves, R., Almeida-Porada, G., and Cabral, J.M. (2003). Hematopoietic stem cells: from the bone to the bioreactor. Trends Biotechnol *21*, 233-240.

Caplan, A.I., and Correa, D. (2011). The MSC: An Injury Drugstore. Cell stem cell *9*, 11-15.

Corre, J., Barreau, C., Cousin, B., Chavoin, J.P., Caton, D., Fournial, G., Penicaud, L., Casteilla, L., and Laharrague, P. (2006). Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors. Journal of cellular physiology *208*, 282-288.

Csaszar, E., Kirouac, D.C., Yu, M., Wang, W., Qiao, W., Cooke, M.P., Boitano, A.E., Ito, C., and Zandstra, P.W. (2012). Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell stem cell *10*, 218-229.

da Silva, C.L., Gonçalves, R., Crapnell, K.B., Cabral, J.M., Zanjani, E.D., and Almeida-Porada, G. (2005). A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Experimental hematology *33*, 828-835.

da Silva, C.L., Goncalves, R., dos Santos, F., Andrade, P.Z., Almeida-Porada, G., and Cabral, J.M. (2010). Dynamic cell-cell interactions between cord blood

haematopoietic progenitors and the cellular niche are essential for the expansion of CD34+, CD34+CD38- and early lymphoid CD7+ cells. Journal of tissue engineering and regenerative medicine *4*, 149-158.

Dib, N., Khawaja, H., Varner, S., McCarthy, M., and Campbell, A. (2011). Cell therapy for cardiovascular disease: a comparison of methods of delivery. Journal of cardiovascular translational research *4*, 177-181.

Dmitrieva, R.I., Minullina, I.R., Bilibina, A.A., Tarasova, O.V., Anisimov, S.V., and Zaritskey, A.Y. (2012). Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells: Differences and similarities. Cell Cycle *11*, 377-383.

dos Santos, F., Andrade, P.Z., Eibes, G., da Silva, C.L., and Cabral, J.M. (2011). Ex Vivo Expansion of Human Mesenchymal Stem Cells on Microcarriers. Methods Mol Biol *698*, 189-198.

Dos Santos, F., Campbell, A., Fernandes-Platzgummer, A., Andrade, P.Z., Gimble, J.M., Wen, Y., Boucher, S., Vemuri, M.C., da Silva, C.L., and Cabral, J.M. (2014). A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. Biotechnology and bioengineering *111*, 1116-1127.

Douay, L. (2001). Experimental culture conditions are critical for ex vivo expansion of hematopoietic cells. J Hematother Stem Cell Res *10*, 341-346.

Dragneva, G., Korpisalo, P., and Yla-Herttuala, S. (2013). Promoting blood vessel growth in ischemic diseases: challenges in translating preclinical potential into clinical success. Disease models & mechanisms *6*, 312-322.

Gao, L.R., Chen, Y., Zhang, N.K., Yang, X.L., Liu, H.L., Wang, Z.G., Yan, X.Y., Wang, Y., Zhu, Z.M., Li, T.C.*, et al.* (2015). Intracoronary infusion of Wharton's jelly-derived mesenchymal stem cells in acute myocardial infarction: double-blind, randomized controlled trial. BMC medicine *13*, 162.

Hass, R., Kasper, C., Bohm, S., and Jacobs, R. (2011). Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. Cell communication and signaling : CCS *9*, 12.

Ingram, D.A., Mead, L.E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M.J., Gilley, D., and Yoder, M.C. (2004). Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood *104*, 2752-2760.

Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W.M., Silver, M., Kearney, M., Li, T., Isner, J.M., and Asahara, T. (2000). Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proceedings of the National Academy of Sciences of the United States of America *97*, 3422-3427.

Kanelidis, A.J., Premer, C., Lopez, J., Balkan, W., and Hare, J.M. (2017). Route of Delivery Modulates the Efficacy of Mesenchymal Stem Cell Therapy for Myocardial Infarction: A Meta-Analysis of Preclinical Studies and Clinical Trials. Circulation research *120*, 1139-1150.

Lee, S.H., Lee, J.H., Yoo, S.Y., Hur, J., Kim, H.-S., and Kwon, S.M. (2013). Hypoxia inhibits cellular senescence to restore the therapeutic potential of old human endothelial progenitor cells via the hypoxia-inducible factor-1α-TWIST-p21 axis. Arteriosclerosis, thrombosis, and vascular biology *33*.

Makkar, R.R., Price, M.J., Lill, M., Frantzen, M., Takizawa, K., Kleisli, T., Zheng, J., Kar, S., McClelan, R., Miyamota, T.*, et al.* (2005). Intramyocardial injection of allogeneic bone marrow-derived mesenchymal stem cells without immunosuppression preserves cardiac function in a porcine model of myocardial infarction. Journal of cardiovascular pharmacology and therapeutics *10*, 225-233.

Masuda, H., Alev, C., Akimaru, H., Ito, R., Shizuno, T., Kobori, M., Horii, M., Ishihara, T., Isobe, K., Isozaki, M.*, et al.* (2011). Methodological development of a clonogenic assay to determine endothelial progenitor cell potential. Circulation research *109*, 20-37.

Masuda, H., Iwasaki, H., Kawamoto, A., Akimaru, H., Ishikawa, M., Ii, M., Shizuno, T., Sato, A., Ito, R., Horii, M.*, et al.* (2012). Development of serum-free quality and quantity control culture of colony-forming endothelial progenitor cell for vasculogenesis. Stem cells translational medicine *1*, 160-171.

Masuda, H., Tanaka, R., Fujimura, S., Ishikawa, M., Akimaru, H., Shizuno, T., Sato, A., Okada, Y., Iida, Y., Itoh, J.*, et al.* (2014). Vasculogenic conditioning of peripheral blood mononuclear cells promotes endothelial progenitor cell expansion and phenotype transition of anti-inflammatory macrophage and T lymphocyte to cells with regenerative potential. Journal of the American Heart Association *3*, e000743.

Meirelles , L.S., Fontes, A.M., Covas, D.T., and Caplan, A.I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. Cytokine & growth factor reviews *20*, 419-427.

Musialek, P., Mazurek, A., Jarocha, D., Tekieli, L., Szot, W., Kostkiewicz, M., Banys, R.P., Urbanczyk, M., Kadzielski, A., Trystula, M.*, et al.* (2015). Myocardial regeneration strategy using Wharton's jelly mesenchymal stem cells as an off-the-shelf 'unlimited' therapeutic agent: results from the Acute Myocardial Infarction First-in-Man Study. Postepy w kardiologii interwencyjnej = Advances in interventional cardiology *11*, 100-107.

Nishiwaki, S., Nakayama, T., Saito, S., Mizuno, H., Ozaki, T., Takahashi, Y., Maruyama, S., Nishida, T., Murata, M., Kojima, S.*, et al.* (2012). Efficacy and safety of human adipose tissue-derived mesenchymal stem cells for supporting hematopoiesis. International journal of hematology *96*, 295-300.

Numaguchi, Y., Sone, T., Okumura, K., Ishii, M., Morita, Y., Kubota, R., Yokouchi, K., Imai, H., Harada, M., Osanai, H.*, et al.* (2006). The impact of the capability of circulating progenitor cell to differentiate on myocardial salvage in patients with primary acute myocardial infarction. Circulation *114*, I114-119.

O, E., Lee, B.H., Ahn, H.Y., Shin, J.C., Kim, H.K., Kim, M., Park, I.Y., Park, Y.G., and Joe, Y.A. (2011). Efficient nonadhesive ex vivo expansion of early endothelial progenitor cells derived from CD34+ human cord blood fraction for effective therapeutic vascularization. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *25*, 159-169.

Obi, S., Masuda, H., Shizuno, T., Sato, A., Yamamoto, K., Ando, J., Abe, Y., and Asahara, T. (2012). Fluid shear stress induces differentiation of circulating phenotype endothelial progenitor cells. J Appl Physiol *303*, 595-606.

Obi, S., Yamamoto, K., Shimizu, N., Kumagaya, S., Masumura, T., Sokabe, T., Asahara, T., and Ando, J. (2009). Fluid shear stress induces arterial differentiation of endothelial progenitor cells. J Appl Physiol *106*, 203-211.

Ouma, G.O., Zafrir, B., Mohler, E.R., 3rd, and Flugelman, M.Y. (2013). Therapeutic angiogenesis in critical limb ischemia. Angiology *64*, 466-480.

Papaioannou, T.G., and Stefanadis, C. (2005). Vascular Wall Shear Stress: Basic Principles and Methods. Hellenic Journal of Cardiology *46*, 9-15.

Perin, E.C., Sanz-Ruiz, R., Sanchez, P.L., Lasso, J., Perez-Cano, R., Alonso-Farto, J.C., Perez-David, E., Fernandez-Santos, M.E., Serruys, P.W., Duckers, H.J.*, et al.* (2014). Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial. American heart journal *168*, 88-95 e82.

Pill, K., Hofmann, S., Redl, H., and Holnthoner, W. (2015). Vascularization mediated by mesenchymal stem cells from bone marrow and adipose tissue: a comparison. Cell regeneration *4*, 8.

Prockop, D.J., and Oh, J.Y. (2012). Medical therapies with adult stem/progenitor cells (MSCs): A backward journey from dramatic results in vivo to the cellular and molecular explanations. Journal of cellular biochemistry *113*, 1460-1469.

Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P.*, et al.* (2005). Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. British Journal of Haematology *129*, 118-129.

Sanganalmath, S.K., and Bolli, R. (2013). Cell therapy for heart failure: a comprehensive overview of experimental and clinical studies, current challenges, and future directions. Circulation research *113*, 810-834.

Scheubel, R.J., Zorn, H., Silber, R.-E., Kuss, O., Morawietz, H., Holtz, J., and Simm, A. (2003). Age-dependent depression in circulating endothelial progenitor cells inpatients undergoing coronary artery bypass grafting. Journal of the American College of Cardiology *42*, 2073–2080.

Schmidt-Lucke, C., Fichtlscherer, S., Aicher, A., Tschope, C., Schultheiss, H.P., Zeiher, A.M., and Dimmeler, S. (2010). Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol. PloS one *5*, e13790.

Shake, J.G., Gruber, P.J., Baumgartner, W.A., Senechal, G., Meyers, J., Redmond, J.M., Pittenger, M.F., and Martin, B.J. (2002). Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg *73*, 1919-1925.

Sidney, L.E., Branch, M.J., Dunphy, S.E., Dua, H.S., and Hopkinson, A. (2014). Concise Review: Evidence for CD34 as a Common Marker for Diverse Progenitors. Stem cells *32*, 1380-1389.

Squillaro, T., Peluso, G., and Galderisi, U. (2016). Clinical Trials With Mesenchymal Stem Cells: An Update. Cell transplantation *25*, 829-848.

Tipnis, S., and Viswanathan, C. (2010). Umbilical cord matrix derived mesenchymal stem cells can change the cord blood transplant scenario. Int J Stem Cells *3*, 103-118.

Watt, S.M., Gullo, F., van der Garde, M., Markeson, D., Camicia, R., Khoo, C.P., and Zwaginga, J.J. (2013). The angiogenic properties of mesenchymal stem/stromal cells and their therapeutic potential. British medical bulletin *108*, 25-53.

Wu, R., Hu, X., and Wang, J. (2018). Concise Review: Optimized Strategies for Stem Cell-Based Therapy in Myocardial Repair: Clinical Translatability and Potential Limitation. Stem cells.

Yamamoto, K., Takahashi, T., Asahara, T., Ohura, N., Sokabe, T., Kamiya, A., and Ando, J. (2003). Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress. J Appl Physiol *95*, 2081-2088.

Yang, Z., von Ballmoos, M.W., Diehm, N., Baumgartner, I., Kalka, C., and Di Santo, S. (2009). Call for a reference model of chronic hind limb ischemia to investigate therapeutic angiogenesis. Vascular pharmacology *51*, 268-274.

Zhang, L., and Xu, Q. (2014). Stem/Progenitor cells in vascular regeneration. Arteriosclerosis, thrombosis, and vascular biology *34*, 1114-1119.

PUBLICATIONS

Fiarresga A, **Mata MF**, Cunha RMS, Pinto DS, et al. (2016). ***The index of microcirculatory resistance for optimizing the use of intracoronary delivery of mesenchymal stem cells***. Manuscript under preparation.

- **Mata MF**, Lopes JP, Ishikawa M, Alaiti MA, Cabral JM, da Silva CL, Costa MA (2015). ***Scaling up the ex vivo expansion of human circulating CD34(+) progenitor cells with upregulation of angiogenic and anti-inflammatory potential***. Cytotherapy 17(12):1777-84. doi: 10.1016/j.jcyt.2015.09.003 (1 citation).

- Fiarresga A, **Mata MF**, Cavaco-Gonçalves S, Selas M, Simões IN, Oliveira E, et al. (2015). ***Intracoronary Delivery of Human Mesenchymal/Stromal Stem Cells: Insights from Coronary Microcirculation Invasive Assessment in a Swine Model***. PLoS ONE 10(10): e0139870. doi:10.1371/journal.pone.0139870 (0 citations)

ORAL COMMUNICATIONS

-Márcia F. Mata, Cláudia L. da Silva, Joaquim M. S. Cabral: *Bioengineering Approaches for Stem Cell Expansion*. Cardiovascular Research Institute Seminar Series (CRISS), University Hospitals, Case Western Reserve University, Cleveland, Ohio; 01/2014

POSTER COMMUNICATIONS

-Márcia F. Mata, Ana Fernandes-Platzgummer, João Pedro Lopes, Joaquim M. S. Cabral, Marco A. Costa, Cláudia L. da Silva. *''Bioengineering approaches for the ex vivo expansion and angiogenic fortification of human CD34+ progenitor cells*", March 2016, 2nd International Advanced Course on Regenerative Medicine Manufacturing, Hilton Head Island, SC, United States of America.

- **Márcia F. Mata**, João P. Lopes, Joaquim S. Cabral, Cláudia L. da Silva, Marco A. Costa, *''Scaling-up the ex vivo expansion and fortification of circulating human progenitor cells*.'' Heart Without Borders, International Conference, November 2014, Porto, Portugal.

- **Márcia F. Mata**, João Pedro Lopes, Masakazu Ishikawa, Mohamad A Alaiti, Mukesh K. Jain, Daniel I. Simon, Marco A. Costa (2013). "*Safety and stability of CD34+ cell ex-vivo expansion*." 7th Annual CCVC Research Retreat 2013, Cleveland, OH, United States of America.

- João Pedro Lopes, Masakazu Ishikawa, David A. Zidar, Mohamad A Alaiti, **Márcia F. Mata**, Yummei Wang, Mukesh K. Jain, Daniel I. Simon, Carl Orringer, Marco A. Costa (2013). *"Biologic markers and cardiovascular disease in humans*". 7th Annual CCVC Research Retreat 2013, Cleveland, OH, United States of America.